Environmental Science

Shree Nath Singh *Editor*

Microbial Degradation of Xenobiotics

Springer

Environmental Science and Engineering

Environmental Engineering

For further volumes:
http://www.springer.com/series/7487

Shree Nath Singh
Editor

Microbial Degradation of Xenobiotics

Springer

Shree Nath Singh
Environmental Science Division
National Botanical Research Institute
Rana Pratap Marg
226001 Lucknow
India
e-mail: drsn06@gmail.com

ISSN 1863-5520
ISBN 978-3-642-23788-1 e-ISBN 978-3-642-23789-8
DOI 10.1007/978-3-642-23789-8
Springer Heidelberg Dordrecht London New York

Library of Congress Control Number: 2011938665

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

In loving memory of my parents

Preface

Microorganisms are ubiquitous in the environment playing an important role in biogeochemical cycling. However, their ability to metabolize xenobiotic compounds has received much attention in recent years due to their environmental persistence and toxicity. Hence, microbial degradation of xenobiotics is, today, seen as both cost-effective and eco-friendly technology for removing these pollutants by a process known as bioremediation. Earlier researchers have confirmed that microbes are capable of degrading a wide range of organic pollutants. However, process of biodegradation is generally very slow and hence, this process may be accelerated by augmenting pure and mixed cultures of microorganisms in both aerobic and anaerobic conditions. Metabolic intermediates formed in the degradative pathways were also examined for their toxicity assessments using bacteria and higher organisms. Many of degradative genes responsible for xenobiotic metabolism are present on plasmids, transposons or are grouped in clusters on chromosomes. This indicates evolution of degradative pathways and makes the genetic manipulation easier. Development of the transgenic microbial strains highly capable of degrading xenobiotics is now possible through biotechnological approaches. Besides, several catabolic enzymes involved in xenobiotic metabolism have been isolated and characterized. A number of environmental factors, including pH, temperature, bioavailability, nutrient supply and oxygen availability have been shown to affect biodegradation process. These factors have to be optimized to obtain an effective microbial treatment process for the industrial organic wastes at bench and pilot scales. However, in the field scale treatment, all environmental factors cannot be manipulated to enhance the degradation process.

To update the knowledge on bioremediation which is a natural attenuation process, I present before you an edited volume on 'Microbial degradation of xenobiotics' which has focused on different aspects of microbial degradation of xenobiotic compounds, like poly aromatics hydrocarbons, polychlorophenols, polyurethane, dye containing wastewater, water soluble polymers, azo dyes, explosives, chloroorganic pollutants, styrene, trinitrophenol and high molecular weight alkanes. These aspects have been discussed in 17 chapters contributed by the leading scientists drawn from all over the world.

In this endeavor, I am not alone, but assisted by many fellow workers. First of all, I would like to acknowledge all the contributors who responded to my request and very enthusiastically contributed their chapters containing the latest developments on the relevant issues. The services rendered by my own research scholars Mrs. Babita Kumari, Ms. Shweta Mishra, and Mrs. Sadhna Tiwari in this endeavor are remarkable and highly appreciable. Besides, laboratory trainees Ms. Namarata Pandey, Ms. Jyoti, Ms. Rashi Singhal, Ms. Deepika Verma, Ms. Radha Verma, Ms. Shilpi Dupey and Ms. Shilpi Kumari are also duly acknowledged for their multifaceted help and support. Mr. Dilip Chakraborty deserves special appreciation for computer work for preparing the manuscript on the book format.

Lastly, I express my sincere thanks to my family members Mrs. Manorma Singh (wife), Dr. Ragini Singh (daughter), Mr. Pritish Kumar Singh (son) and the little champ Antra for their inspiration, endurance and moral boost up in this endeavor.

S. N. Singh

Contents

Contributors

R. Balasubramanian Department of Civil and Environmental Engineering, National University of Singapore, 1 Engineering Drive 2, Singapore, 117576, Singapore

Anat Bernstein Department of Environmental Hydrology and Microbiology, Zuckerberg Institute for Water Research, Blaustein Institutes for Desert Research, Ben-Gurion University of the Negev, Sede Boqer Campus, 84990 Beer-Sheva, Israel

Tomáš Cajthaml Laboratory of Environmental Biotechnology, Institute of Microbiology, v.v.i., Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague 4, Czech Republic, e-mail: cajthaml@biomed.cas.cz

Pritam Chattopadhyay Plant Biotechnology Laboratory, Department of Botany, Visva-Bharati, Santiniketan, India

Indrani Ghosh Centre for Environmental Science and Engineering, Indian Institute of Technology, Powai, Mumbai 400 076, India

Gary T. Howard Department of Biological Sciences, Southeastern Louisiana University, Hammond, Louisiana 70402, USA, e-mail: ghoward@selu.edu

Leela Iyengar Centre for Post graduate Studies, Jain University, Bangalore, India

Stefan R. Kaschabek Environmental Microbiology, Department of Biological Sciences, Faculty of Chemistry and Physics, TU Bergakademie Freiberg, Leipziger Str. 29, 09599 Freiberg, Germany

Fusako Kawai Center for Nanomaterials and Devices, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan, e-mail: fkawai@kit.ac.jp

Christian Kennes Faculty of Sciences, Campus da Zapateira, University of La Coruña, Rua da Fraga 10, 15008 La Coruña, Spain, e-mail: kennes@udc.es

B. Kumari Environmental Science Division, National Botanical Research Institute, Rana Pratap Marg, Lucknow 226001, India

Toshinari Maeda Department of Biological Functions and Engineering, Graduate School of Life Science and Systems Engineering, Kyushu Institute of Technology, 2-4 Hibikino, Wakamatsu-ku, Kitakyushu 808-0196, Japan, e-mail: toshi.maeda@life.kyutech.ac.jp

Ernest Marco-Urrea Department of Chemical Engineering and Institute of Environmental Science and Technology, Autonomous University of Barcelona, 08193 Bellaterra, Spain

S. Maruthamuthu Corrosion Protection Division, Biocorrosion Group, Central Electrochemical Research Institute, Karaikudi 630 006, India

Shweta Mishra Environmental Science Division, National Botanical Research Institute, Rana Pratap Marg, Lucknow 226001, India

Pijush Kanti Mondal Environmental Research laboratory, Department of Applied Chemistry, Faculty of Engineering and Technology, Aligarh Muslim University, Aligarh, Uttar Pradesh 202 002, India, e-mail: pijushamu@gmail.com

Suparna Mukherji Centre for Environmental Science and Engineering, Indian Institute of Technology, Powai, Mumbai 400 076, India

Hiroaki I. Ogawa Department of Biological Functions and Engineering, Graduate School of Life Science and Systems Engineering, Kyushu Institute of Technology, 2-4 Hibikino, Wakamatsu-ku, Kitakyushu 808-0196, Japan

Anjali Pandey Department of Chemistry, Indian Institute of Technology, Kanpur, India, e-mail: anjali@iitk.ac.in

José A. Perales-Vargas-Machuca Department of Environmental Technologies, Andalusian Centre of Marine Science and Technology (CACYTMAR), University of Cadiz, Puerto Real, 11510, Cadiz, Spain

Pattanathu K. S. M. Rahman Chemical and Bioprocess Engineering Group, School of Science and Engineering, Teesside University, Middlesbrough, Tees Valley, TS13BA, UK, e-mail: p.rahman@tees.ac.uk

A. Rajasekar Department of Civil and Environmental Engineering, National University of Singapore, 1 Engineering Drive 2, Singapore, 117576, Singapore, e-mail: e-mail: esera@nus.edu.sg

Department of Chemical and Biomolecular Engineering, National University of Singapore, 4 Engineering Drive 4, Singapore, 117576, Singapore, e-mail: rajasekar.aruliah@gmail.com

C. A. Reddy Department of Microbiology and Molecular Genetics and The NSF Center for Microbial Ecology, Michigan State University, East Lansing, Michigan 48824-4320, USA, e-mail: reddy@msu.edu

Elisa Rojo-Nieto Department of Environmental Technologies, Andalusian Centre of Marine Science and Technology (CACYTMAR), University of Cadiz, Puerto Real, 11510, Cadiz, Spain, e-mail: elisa.rojo@uca.es

Zeev Ronen Department of Environmental Hydrology and Microbiology, Zuckerberg Institute for Water Research, Blaustein Institutes for Desert Research, Ben-Gurion University of the Negev, Sede Boqer Campus, 84990 Beer-Sheva, Israel, e-mail: zeevrone@bgu.ac.il

Sukanta K. Sen Microbiology Division, Department of Botany, Visva-Bharati, Santiniketan, India, e-mail: sksenvb@rediffmail.com

Antaryami Singh Defence Laboratory, Defence Research and Development Organization, Jodhpur 342 011, India

Poonam Singh Division of Toxicology, Central Drug Research Institute, Lucknow, India

Rita Singh Defence Laboratory, Defence Research and Development Organization, Jodhpur 342 011, India, e-mail: singhritadr@yahoo.com

S. N. Singh Environmental Science Division, National Botanical Research Institute, Rana Pratap Marg, Lucknow 226 001, India, e-mail: drsn06@gmail.com

Shelly Sinha Rabindra Mahavidyalaya, Champadanga, Hooghly, West Bengal, India

Kateřina Svobodová Laboratory of Environmental Biotechnology, Institute of Microbiology, v.v.i., Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague 4, Czech Republic

Y. P. Ting Department of Chemical and Biomolecular Engineering, National University of Singapore, 4 Engineering Drive 4, Singapore, 117576, Singapore

Dirk Tischler Environmental Microbiology, Department of Biological Sciences, Faculty of Chemistry and Physics, TU Bergakademie Freiberg, Leipziger Str. 29, 09599 Freiberg, Germany, e-mail: Dirk-Tischler@email.de

María C. Veiga Faculty of Sciences, Campus da Zapateira, University of La Coruña, Rua da Fraga 10, 15008 La Coruña, Spain

Luying Xun School of Molecular Biosciences, Washington State University, Life Sciences Building, Room 202, Pullman, WA 99164-7520, USA, e-mail: xun@mail.wsu.edu

Chapter 1
Microbial Degradation of Polychlorophenols

Luying Xun

1.1 Introduction

Polychlorophenols are major environmental pollutants, and their degradation by microorganisms has been extensively studied for the purpose of bioremediation. Three different metabolic pathways for aerobic degradation of polychlorophenols have been completely worked out, revealing the metabolic diversity for these structurally similar compounds. Substituted quinols, rather than catechols, are key metabolic intermediates of polychlorophenol biodegradation. Substituted quinols and quinones are also called as *p*-hydroquinones and *p*-benzoquinones, reflecting the reduced and oxidized forms. For example, tetrachloroquinol is the same as tetrachloro-*p*-hydroquinone, and tetrachloroquinone is often referred as tetrachloro-*p*-benzoquinone. Characterization of individual enzymes has led to the discoveries of novel dechlorination mechanisms. The genes coding for the enzymes have been cloned and sequenced, and the gene organization and regulation suggest that recent gene recruitments have occurred for the degradation of some polychlorophenols.

1.1.1 Sources of Polychlorophenols

Trichlorophenols can be naturally produced, but pentachlorophenol (PCP) is anthropogenic in origin. Hoekstra et al. (1999) have reported the production of 2,4,6-trichlorophenol (2,4,6-TCP) and 2,4,5-trichlorophenol (2,4,5-TCP) as well as

L. Xun (✉)
School of Molecular Biosciences, Washington State University,
Life Sciences Building, Room 202, Pullman, WA 99164-7520, USA
e-mail: xun@mail.wsu.edu

S. N. Singh (ed.), *Microbial Degradation of Xenobiotics*,
Environmental Science and Engineering, DOI: 10.1007/978-3-642-23789-8_1,

less chlorinated phenols from spiked $Na^{37}Cl$ in soils of a Douglas fir forest. However, tetrachlorophenols and pentachlorophenol (PCP) are not produced from the spiked ^{37}Cl after one year in situ incubation. There is no evidence of natural production of PCP. PCP is manufactured either by phenol chlorination with chlorine gas or alkaline hydrolysis of hexachlorobenzene, producing a technical grade of PCP that contains other polychlorophenols as impurities (WHO 1987). Merz and Weith first synthesized PCP in 1872 (Merz and Weith 1872). The massive release of PCP into the environment is mainly associated with its use as a wood preservative, a practice starting in the 1930s (Crosby 1981). PCP-treated lumbers are commonly used for outdoor structures, but some have been used to build wine cellars and log houses. The vapors of polychlorophenols released from the building materials can contaminate wine, giving it a corky taste (Suckling et al. 1999), probably due to the formation of chloroanisoles (Coque et al. 2003). People living in PCP-treated log houses have elevated blood levels of PCP over control groups (Cline et al. 1989). Further, polychlorophenol derivatives are often used as herbicides and fungicides. 2,4,5-Trichlorophenoxyacetate (2,4,5-T), a derivative of 2,4,5-TCP, is a potent herbicide and is a major ingredient of "Agent Orange" used for defoliation during the Vietnam War in the 1960s (Firestone 1978). Prochloraz, a derivative of 2,4,6-TCP, is an effective fungicide for plant pathogens (Birchmore and Meneley 1979). Consequently, a wide usage of polychlorophenols and their derivatives have resulted in environmental contamination.

The main sources of polychlorophenol contamination are from their production, application and discharge. The previously uncontrolled disposal has resulted in a widespread contamination of polychlorophenols, e.g. at least 415 locations of former wood preserving facilities are contaminated with polychlorophenols (Middaugh et al. 1994). Their hazardous nature has promoted many countries to regulate their use. In the United States, the release of polychlorophenols requires registration with the Environmental Protection Agency, and the data are published in Toxic Release Inventory: Public Data Release (EPA 2006).

1.1.2 Toxicity of Polychlorophenols

Polychlorophenols are notorious for several reasons. First, they are harmful to all life forms because they disrupt the integrity and function of biological membranes (Cunarro and Weiner 1975; Escher et al. 1996). Second, their metabolites are also toxic. Human uptake of polychlorophenols is rapid via three mechanisms: skin absorption, inhalation, and ingestion (WHO 1986; Proudfoot 2003). High dose leads to hyperthermia, convulsions, and rapid death. The effects of low dose are unclear, resulting in elevated blood chlorophenol levels, which can be metabolized to chloroquinols or conjugated to polychlorophenol glucuronides for renal excretion (Uhl et al. 1986). The oxidation of chloroquinols and reduction of chloroquinones lead to the formation of reactive oxygen species, causing DNA damage (Dahlhaus et al. 1995) and other oxidative stresses (Wang et al. 2001).

Third, technical-grade polyclorophenols contain impurities, e.g. chlorinated dibenzo-*p*-dioxins and dibenzofurans, which are highly carcinogenic (Firestone 1978; Kaiser 2000). They are produced from polychlorophenols during manufacturing processes (Crosby 1981), and they can also be formed via biotransformation in soils (Hoekstra et al. 1999).

1.2 Microbial Degradation of Polychlorophenols

The most efficient and economical approach to the removal of low concentrations of polychlorophenols from contaminated soils and aquifers is bioremediation (Crawford and Mohn 1985; Lamar and Evans 1993; Miethling and Karlson 1996). The position of the chlorine substitution and the number of chlorines influence how the chlorophenols are degraded by microorganisms. Because of the presence of six isomers of trichlorophenols, three isomers of tetrachlorophenols and one pentachlorophenol, various microorganisms have evolved different strategies for the degradation of selected isomers. Bacteria can degrade polychlorophenols under both aerobic and anaerobic conditions, and fungi are able to aerobically metabolize them.

1.2.1 Pentachlorophenol Degradation by Aerobic Bacteria

Chu and Kirsch (1972) reported the first aerobic PCP-degrading bacterium in 1972. Since then, numerous aerobic bacteria that degrade PCP have been isolated from different regions around the globe. The early isolates were originally assigned to various genera, such as *Arthrobacter*, *Pseudomonas*, *Flavobacterium*, *Sphingomonas*, *Rhodococcus*, and *Mycobacterium*. The gram-positive *Rhodococcus* spp. and *Mycobacterium* spp. have been reclassified as *Mycobacterium chlorophenolicum* (Briglia et al. 1994; Haggblom et al. 1994). All the gram-negative, PCP-degrading bacteria, previously known as *Arthrobacter*, *Pseudomonas*, and *Flavobacterium*, were subsequently reclassified as *Sphingomonas chlorophenolica* strains (Crawford and Ederer 1999; Takeuchi et al. 2001), but have been subsequently renamed as *Sphingobium chlorophenolicum* strains (Takeuchi et al. 2001). A PCP-degrading *Sphingomonas* sp. strain UG30A is related to *S. chlorophenolicum* strains, but remains as a *Sphingomonas* sp. (Habash et al. 2009). A related psychrophilic PCP-degrader is *Novosphingobium lentum* MT1 (Tiirola et al. 2005). *S. chlorophenolicum* strains are the most frequently isolated bacteria that degrade PCP; however, other PCP-degrading bacteria have also been reported (Golovleva et al. 1992; Sharma et al. 2009).

1.2.2 2,4,6-Trichlorophenol Degradation by Aerobic Bacteria

Although *S. chlorophenolicum* degrades both PCP and 2,4,6-TCP (Steiert et al. 1987), *Azotobacter* sp. GP1 (Li et al. 1991) and *Ralstonia* (ex. *Pseudomonas*) *pickettii* (Kiyohara et al. 1992) use only 2,4,6-TCP as a sole carbon source. More 2,4,6-TCP degraders have since been identified and isolated: *Cupriavidus necator* (ex. *Ralstonia eutrapha*) JMP134 (Clement et al. 1995), *Sphingopyxis chilensis* (ex. *Pseudomonas paucimobilis*) S37 (Aranda et al. 1999), *Aureobacterium* sp. C964 (Bock et al. 1996), *Rhodococcus percolatus* MBS1T (Briglia et al. 1996), *Sphingobium subarctica* (Puhakka et al. 1995; Nohynek et al. 1996), *Pseudomonas* sp., *Agrobacterium* sp. (Wang et al. 2000), *Nocardioides* sp. (Mannisto et al. 1999), *Flavobacterium* sp. and *Caulobacter* sp. (Mannisto et al. 1999). It appears that the 2,4,6-TCP degrading ability is widespread among the soil bacteria.

1.2.3 2,4,5-Trichlorophenol Degradation by Aerobic Bacteria

Several bacteria are known to degrade 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). *Burkholderia* (ex *Pseudomonas*) *cepacia* AC1100, isolated from an enrichment culture, is a gram-negative bacterium that uses 2,4,5-T as a sole carbon source for the growth (Kilbane et al. 1982). The bacterium degrades 2,4,5-T with 2,4,5-TCP as the first metabolic intermediate (Karns et al. 1983). Two other *Burkholderia* spp. that degrade 2,4,5-T have recently been reported (Lü et al. 2003; Rice et al. 2005). A different 2,4,5-T degrader is *Nocardioides simplex* 3E that is a gram-positive actinomycete, able to grow on 2,4,5-T as a sole carbon source (Golovleva et al. 1990). This microorganism may have two pathways for 2,4,5-T degradation: one with 2,4,5-TCP as the first metabolic intermediate, and the other with dichlorohydroxyphenoxyacetate as the first metabolic intermediate. Since 2,4,5-TCP degradation is an integral part of 2,4,5-T degradation, the characterized pathway for 2,4,5-T degradation is reviewed here.

1.2.4 Anaerobic Degradation of Polychlorophenols

Microorganisms also degrade polychlorophenols under anaerobic conditions. Reductive dechlorination of PCP to tetrachlorophenols, trichlorophenols, dichlorophenols, and monochlorophenols was first observed in anaerobic paddy soils in the 1970s (Ide et al. 1972). The degradation has been confirmed by studies with enrichment cultures and bacterial isolates. An anaerobic bacterial consortium completely dechlorinates PCP to phenol and then mineralizes the produced phenol (Mikesell and Boyd 1986). *Desulfitobacterium frappieri* converts PCP by sequential reductive dehalogenation to 3-chlorophenol (Bouchard et al. 1996). These anaerobic bacteria use polychlorophenols as terminal electron acceptors for anaerobic

respiration to produce less substituted chlorophenols and phenol (Crawford and Mohn 1985), and these phenols are further degraded by other organisms in enrichment cultures or in the environment (Mikesell and Boyd 1986). Progress has been made towards understanding the biochemistry and genetics of reductive dechlorination of polychlorophenols (Boyer et al. 2003; Bisaillon et al. 2010).

1.2.5 Fungal Degradation of Polychlorophenols

Fungal degradation of PCP was reported as early as 1960s (Duncan and Deverall 1964), and the non-specific breakdown of PCP by fungal laccase, tyrosinase, and peroxidase was implied (Lyr 1963). Research on fungal degradation of polychlorophenols has progressed rapidly since then, especially with white-rot fungi (Reddy et al. 1998; Reddy and Gold 2000). The metabolic pathways of 2,4,6-TCP and PCP degradation have been studied with cell extracts of white-rot fungus *Phanerochaete chrysosporium* (Reddy et al. 1998; Reddy and Gold 2000), and a glutathione conjugate reductase involved in PCP degradation has been purified and characterized (Reddy and Gold 2001). *Phanerochaete* spp. have been used for the removal of PCP from contaminated soils (Lamar and Dietrich 1990; Lamar and Evans 1993) and for the disposal of PCP-treated woods (Lamar and Dietrich 1992).

1.3 Biochemistry of Polychlorophenol Degradation

The aerobic breakdown of aromatic compounds starts with monooxygenases or dioxygenases that introduce hydroxyl groups into the aromatic rings. Many aromatic compounds, including phenol, benzene and anthranilate, are converted to catechol or substituted catechols. Then intradiol or extradiol catechol dioxygenases break the aromatic rings to produce aliphatic compounds, which are further channelized into the tricarboxylic acid cycle for the complete mineralization (Harwood and Parales 1996). However, polychlorinated phenols are converted to substituted quinols before ring-cleavage: *S. chlorophenolicum* L-1 (ex. *S. chlorophenolicum* ATCC 39723) metabolizes PCP to 2,6-dichloroquinol (Cai and Xun 2002), *C. necator* JMP134 converts 2,4,6-TCP to 6-chlorohydroxyquinol (Louie et al. 2002), and *B. cepacia* AC1100 channels 2,4,5-TCP to hydroxyquinol (Zaborina et al. 1998).

1.3.1 Pentachlorophenol Metabolic Pathway of S. Chlorophenolicum L-1

PCP degradation pathways have been thoroughly investigated in *S. chlorophenolicum* L-1 and partially studied in *Mycobacterium chlorophenolicum*. Studies with cell

extracts of *M. chlorophenolicum* have shown that PCP is converted to tetrachloroquinol and then to hydroxyquinol before ring-cleavage (Apajalahti and Salkinoja-Salonen 1987; Uotila et al. 1995). The details of this pathway are unknown, as the enzymes and genes have not been identified. For PCP degradation by *S. chlorophenolicum* L-1, the complete degradation pathway has been determined, which is different from that of *M. chlorophenolicum* (Cai and Xun 2002). *S. chlorophenolicum* L-1 is isolated from a PCP-contaminated soil and it is able to completely mineralize PCP to CO_2 and chlorine (Saber and Crawford 1985). Genetic approaches were initially used to study the metabolic pathway in *S. chlorophenolicum* L-1; however, transposon mutagenesis has been unsuccessful (Orser and Lange 1994) and chemical mutagenesis has produced a single mutant (Steiert and Crawford 1986). Since a plasmid vector that replicates in *S. chlorophenolicum* L-1 has not been obtained, the mutated gene cannot be identified by complementation.

1.3.1.1 Pentachlorophenol 4-Monooxygenase (PcpB)

The metabolic pathway has been elucidated from biochemical studies and confirmed by gene inactivation (Fig. 1.1). In order to characterize PCP metabolizing enzyme, a convenient assay has to be developed. Detection of chlorine released from PCP metabolism would be a logical choice, but most buffers have relatively high background levels of chlorine that interferes with the assay. Since PCP-induced *S. chlorophenolicum* L-1 cells also degrade 2,4,6-triiodophenol, an enzyme that release iodide from 2,4,6-triiodophenol is identified in the cell extracts of PCP-induced *S. chlorophenolicum* L-1 (Xun and Orser 1991c) and purified (Xun and Orser 1991a). The protein (PcpB) is a flavin protein, containing a single flavin adenine dinucleotide (FAD). PcpB converts not only 2,4,6-triiodophenol to 2,6-diiodoquinone but also PCP to tetrachloroquinone with the consumption of NADH and O_2. 2,6-diiodoquinone and tetrachloroquinone are rapidly reduced to 2,6-diiodoquinol and tetrachloroquinol by NADH or ascorbic acid added in the reaction mixture (Xun et al. 1992c; Dai et al. 2003; Belchik and Xun 2008). The gene (*pcpB*) is cloned by using a probe of degenerated oligonucleotides designed according to the N-terminal sequence of PcpB (Orser et al. 1993a). When *pcpB* is inactivated by homologous recombination, the *pcpB* mutant is unable to degrade PCP (Lange et al. 1996; Cai and Xun 2002). PcpB has a calculated molecular weight of 59,932, and it is homologous to other FAD-dependent monooxygenases that hydroxylate aromatic rings. PcpB has a broad substrate range, hydroxylating the *para* position of several substituted phenols, including PCP, 2,3,5,6-tetrachlorophenol, 2,4,6-triiodophenol, 2,4,6-tribromophenol, 2,4,6-TCP, and 2,6-dichlorophenol (Xun et al. 1992c). As long as the 2,6-positions are occupied by chlorine, bromine, or iodine, PcpB can hydroxylate the *para*-position of the substituted phenols. When the *para*-position is substituted, the substituted group will be removed after PcpB catalysis. The reaction consumes one NADH when the substituted group is an electron-donating group, such as a

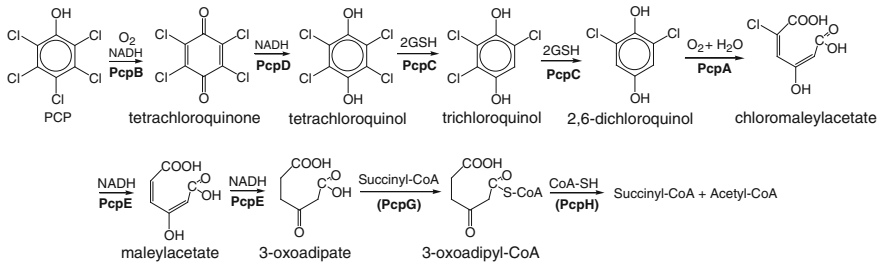


Fig. 1.1 PCP degradation pathway of *S. chlorophenolicum* L-1. PcpB, PCP 4-monooxygenase; PcpD, tetrachloroquinone reductase; PcpC, tetrachloroquinol reductive dehalogenase; PcpA, 2,6-dichloroquinol 1,2-dioxygenase; PcpE, chloromaleylacetate reductase; PcpG, 3-oxoadipate: succinyl-CoA transferase; and PcpH, 3-oxoadipyl-CoA thiolase. PcpG and PcpH are hypothetical, but they are common enzymes in biodegradation of aromatic compounds

hydrogen or an amino group. On the other hand, the overall reaction consumes two NADH when the substituted group is an electron-withdrawing group, i.e. halogen, nitro group, or cyano group.

1.3.1.2 Tetrachloroquinone Reductase (PcpD)

The consumption of two NADH for the dehalogenation of substituted phenols is carefully examined with *p*-hydroxybenzoate 3-monooxygenase, a related FAD-dependent monooxygenase (Husain et al. 1980). For fluoride elimination from tetraflouro-*p*-hydroxybenzoate, the direct product is a quinone, which is chemically reduced by NADH to produce triflouro-3,4-dihydroxybenzoate, and the overall reaction consumes two NADH. *p*-Hydroxybenzoate 3-monooxygenase has been extensively studied as a model system for flavin containing monooxygenases. The normal hydroxylation reaction produces a non-aromatic compound as a transient reaction intermediate, which rearranges back to an aromatic compound (Entsch et al. 1976). For halogen removal, a more oxidized non-aromatic intermediate is generated, and it rearranges to produce a quinone (Husain et al. 1980). The authors have suggested that halide and nitrite elimination catalyzed by similar monooxygenases should also produce quinones. This prediction has been validated and expanded. First, Haigler et al. (1996) have reported that a *Burkholderia* DNT4-methyl-5-nitrocatechol 5-monooxygenase converts 4-methyl-5-nitrocatechol to 2-hydroxy-5-methylquinone with nitrite elimination, and a quinone reductase reduces the quinone to 2-hydroxy-5-methylquinol. Second, the gene immediately after *pcpB* is *pcpD*, whose translated product is homologous to the reductase component of several oxygenases (Orser and Lange 1994). The function of PcpD had remained elusive for several years, until Dai et al. (2003) reported that PcpD reduces tetrachloroquinone with NADH as the reductant (Fig. 1.1). PcpD is not required for PCP 4-monooxygenase to convert PCP to tetrachloroquinone, which can be reduced either

enzymatically by PcpD or chemically by reducing agents. However, PcpD enhances PCP 4-monooxygenase activity under in vitro conditions. A *pcpD* mutant can degrade PCP at a reduced rate, and the mutant is more sensitive to high concentrations of PCP. Thus, PcpD is named as tetrachloroquinone reductase.

1.3.1.3 Tetrachloroquinol Reductive Dehalogenase (PcpC)

The third and fourth steps of PCP degradation (Fig. 1.1) are catalyzed by PcpC, tetrachloroquinol reductive dehalogenase, with glutathione (GSH) as the reducing agent. Tetrachloroquinol is expected to be converted to 2,6-dichloroquinol because a mutant obtained by chemical mutagenesis accumulates the latter (Steiert and Crawford 1986), and the reaction is believed to be reductive dechlorination. The reductive conversion of tetrachloroquinol to 2,6-dichloroquinol is observed with the cell extracts under anaerobic conditions, which are essential for the initial observation, as the reaction is slow and tetrachloroquinol is unstable in the presence of oxygen. When several known cellular reducing agents are tested as the reducing power, only GSH significantly stimulates the reaction rate (Xun et al. 1992b). Subsequently, tetrachloroquinol reductive dehalogenase (PcpC), a glutathione transferase, is purified from the cell extracts of *S. chlorophenolicum* L-1 (Xun et al. 1992a). The enzyme converts tetrachloroquinol to trichloroquinol and then 2,6-dichloroquinol with the concomitant oxidation of GSH. The corresponding gene (*pcpC*) has been cloned by using probes designed from the N-terminal sequence of the purified protein, and the sequence determined (Orser et al. 1993b). PcpC is necessary for *S. chlorophenolicum* L-1 to completely degrade PCP, as a *pcpC* mutant accumulates tetrachloroquinol during PCP degradation. Tetrachloroquinol is unstable in the presence of oxygen and is further transformed into a colored compound and accumulated in the culture medium (Cai and Xun 2002). PcpC is slightly homologous to the Zeta class of glutathione transferases that catalyze maleylacetoacetate isomerization, a reaction in tyrosine metabolism. Despite low sequence similarity (19.4% sequence identity with human GSTZ1), PcpC has maleylacetoacetate isomerase activity and is characterized as a Zeta-class glutathione transferase (Anandarajah et al. 2000). However, a comprehensive phylogenetic analysis groups PcpC with LinD, another chloroquinol reductive dehalogenase and the analysis separate them from the Zeta class of glutathione transferases (Marco et al. 2004).

1.3.1.4 2,6-Dichloroquinol 1,2-Dioxygenase (PcpA)

The third essential enzyme in the PCP degradation pathway is PcpA, which is the first identified protein involved in PCP degradation. When *S. chlorophenolicum* L-1 cells are treated with EDTA, a chelating agent, the cells lose their ability to degrade PCP and several major proteins are released to the solution (Xun and Orser 1991b). One of the EDTA released proteins is present in the PCP-induced

cells, but absent from the uninduced cells. The protein is purified, and the corresponding gene is cloned; however, the gene product had no homology to any entries in the GenBank in 1991. Thus, PcpA was reported as a PCP-induced protein with unknown function in 1991 (Xun and Orser 1991b). In 1999, three groups reported PcpA as a ring-cleavage dioxygenase (Ohtsubo et al. 1999; Xu et al. 1999; Xun et al. 1999). PcpA is 2,6-dichloroquinol 1,2-dioxygenase that oxidizes 2,6-dichloroquinol to 2-chloromaleylacetate chloride (Fig. 1.1), which is spontaneously hydrolyzed to 2-chloromaleylacetate (Fig. 1.1) (Ohtsubo et al. 1999; Xun et al. 1999). PcpA has a molecular weight of 36,513 and requires free Fe^{2+} as a cofactor. PcpA was originally thought to be a periplasmic protein because of its release from EDTA treatment of *S. chlorophenolicum* L-1 cells (Xun and Orser 1991b). Given the requirement of free Fe^{2+} as a cofactor (K_m value of 5 μM Fe^{2+}) (Xun et al. 1999), which is available only in the cytoplasm of aerobic bacteria (Raymond et al. 2003), PcpA should be a cytoplasmic protein, the same as other enzymes of the PCP degradation pathway, all of which require cofactors available in the cytoplasm. PcpA is required for PCP metabolism because a *pcpA* inactivation mutant accumulates 2,6-dichloroquinol (Chanama and Crawford 1997). PcpA has 51.6% of amino acid sequence identity to LinE, which is a 2-chloroquinol 1,2-dioxygenase (Ohtsubo et al. 1999), essentially catalyzing the same reaction as PcpA. LinE also oxidizes quinol to gamma-hydroxymuconic semialdehyde. Other hypothetical proteins, e.g. YdfO (PIR:E69781), YodE (PIR:B69903) and YkcA (PIR:C69855) of *Bacillus subtilis*, similar to PcpA and LinE, are likely quinol ring-cleavage dioxygenases. A recent BLAST search of microbial genomes with PcpA (October 5, 2010) identified many PcpA homologs (more than 30% sequence identity), and they are quite common in Halobacteria of Archaea, Gram-positive bacteria (*Bacillus* and *Lactorbacillus*) and Gram-negative bacteria (alpha Proteobacteria). *S. chlorophenolicum* is an alpha Proteobacterium.

1.3.1.5 2-Chloromaleylacetate Reductase (PcpE)

2-Chloromaleylacetate is a relatively common metabolic intermediate for the degradation of chloroaromatic compounds, and it is reduced by (chloro)maleylacetate reductase to maleylacetate and then 3-oxoadipate with the consumption of NADH (Kaschabek et al. 2002). In *S. chlorophenolicum* L-1, a (chloro) maleylacetate reductase reduces 2-chloromaleylacetate to 3-oxoadipate (Cai and Xun 2002) (Fig. 1.1). The reductase gene (*pcpE*) is identified by sequencing DNA around *pcpA*. PcpE is involved in PCP degradation in *S. chlorophenolicum* L-1 because a *pcpE* inactivation mutant transitorily accumulates 2-chloromaleylacetate during PCP degradation. The result also suggests that *S. chlorophenolicum* L-1 has additional maleylacetate reductase that is produced when 2-chloromaleylacetate is accumulated in the mutant culture. A BLAST search of the recently released *S. chlorophenolicum* L-1 genome identified two additional genes coding for maleylacetate reductases.

1.3.1.6 3-Oxoadipate:succinyl-CoA Transferase and 3-Oxoadipyl-CoA Thiolase

3-Oxoadipate is a common metabolic intermediate of many aromatic compounds, and two additional enzymes are required to channel it into the tricarboxylic acid cycle for complete mineralization (Harwood and Parales 1996). The enzymes have been characterized in other bacteria, but not in *S. chlorophenolicum* (Kaschabek et al. 2002). The first enzyme is 3-oxoadipate:succinyl-CoA transferase that catalyzes the CoA exchange between 3-oxoadipate and succinyl-CoA to generate 3-oxoadipyl-CoA and succinate. The enzyme consists of subunits A and B. The *S. chlorophenolicum* L-1 genome has two sets of genes potentially coding for oxoadipate:succinyl-CoA transferases. One set of the genes is located next to a gene encoding 3-oxoadipyl-CoA thiolase that catalyzes the conversion of 3-oxoadipyl-CoA and CoA-SH to succinyl-CoA and acetyl-CoA. Both succinyl-CoA and acetyl-CoA are completely mineralized via the tricarboxylic acid cycle.

1.3.1.7 Glutathionyl-(chloro)quinol Reductase (PcpF)

Glutathionyl-(chloro)quinol reductase (PcpF) plays a maintenance role of the PCP metabolic pathway. PcpC catalyzes the GSH-dependent conversion of tetrachloroquinol to trichloroquinol and then to 2,6-dichloroquinol (Xun et al. 1992a; Orser et al. 1993b). The Cys-14 residue of PcpC is required for the reaction (Warner et al. 2005). When the Cys-14 residue is oxidized (or mutated), the oxidatively-damaged PcpC (PcpC-ox) transforms tetrachloroquinol to glutathionyl-trichloroquinol (GS-trichloroquinol) and trichloroquinol to GS-dichloroquinol (Fig. 1.2). PcpC and PcpC-ox cannot further transform GS-trichloroquinol and GS-dichloroquinol. Another enzyme, PcpF, catalyzes GSH-dependent reduction of GS-trichloroquinol and GS-dichloroquinol to trichloroquinol and dichloroquinol, respectively, which re-enter the PCP degradation pathway (Fig. 1.2) (Huang et al. 2008). PcpF plays a maintenance role but not essential for PCP degradation by *S. chlorophenolicum* L-1. When the *pcpF* gene is inactivated, the mutant degrades PCP more slowly and becomes more sensitive to PCP toxicity than the wild type.

PcpF is discovered due to its gene location next to the *pcpC* gene on the chromosome of *S. chlorophenolicum* L-1. Both PcpC and PcpF are glutathione transferases. The two proteins share 17.7% sequence identity. PcpC does not share high sequence identity with any glutathione transferase in sequenced bacterial genomes. The closest relative with 27.3% sequence identity is a hypothetical glutathione transferase (GenBank: AAZ25344) of *Colwellia psychrerythraea* 34H. PcpF is completely different. It has homologs (more than 30% sequence identity) in halobacteria (Archaea), bacteria (Bacteria), fungi (Eukarya), and plants (Eukarya). *S. cerevisiae* has three PcpF homologs (YGR154c, ECM4, YMR251w) with ECM4 (extra cellular mutant 4) being the most similar to PcpF. Although they were initially characterized as Omega-class glutathione transferases for their ability to use the substrates of the Omega-class glutathione transferases (Garcera

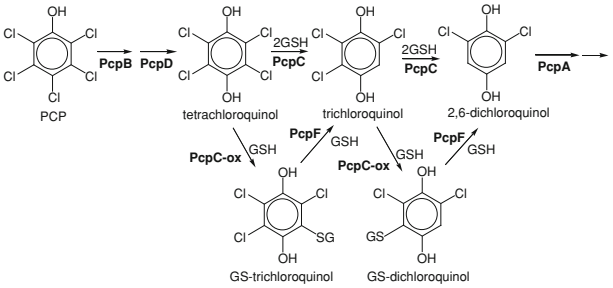


Fig. 1.2 The maintenance role of PcpF in PCP degradation by *S. chlorophenolicum* L-1. PcpC-ox oxidatively damaged PcpC; PcpF, GS-(chloro)quinol reductase

et al. 2006), they share less than 20% sequence identity with the Omega-class glutathione transferases. *A. thaliana* has four PcpF homologs, listed as ECM4-like proteins (NP_199315, NP_001031671, NP_199312, NP_568632).

1.3.1.8 PcpF Homologs are GS-(chloro)quinol Reductases

Phylogenetic analsysis grouped PcpF homologs into a distinct group, separated from any glutathione transferase class. Several bacterial PcpF homolog and yeast ECM4 all actively reduced GS-trichloroquinol. Thus, they are characterized as GS-(chloro)quinol reductases, a new class of glutathione transferases (Xun et al. 2010). GS-(chloro)quinol reductases can also reduce GS-quinol to quinol at the expense of GSH (Lam and Xun, unpublished data). They are related to the newly discovered Omega class, Lambda class and dehydroascorbate (DHA) reductases of glutathione transferases. Unlike other glutathione transferases, the new classes have little or no activity for transferring GSH to electrophilic compounds; however, they catalyze GSH-dependent reductions. The Lambda-class glutathione transferases perform only thiol transfer, and DHA reductases catalyze both thiol transfer and the reduction of DHA to ascorbate (Dixon et al. 2002). The Omega-class glutathione transferases catalyze GSH-dependent reductions of disulfide bonds (thiol transfer), DHA, and dimethylarsinate (Whitbread et al. 2005). Glutathionyl-(chloro)hydroquinone reductases not only catalyze the activities of the Omega-class glutathione transferases, but also reduce GS-trichloroquinol to trichloroquinol (Xun et al. 2010).

S. chlorophenolicum L-1 uses the PCP-metabolizing enzymes for the degradation of other polychlorophenols. *S. chlorophenolicum* L-1 is also able to mineralize a broad spectrum of chlorinated phenols, as long as the 2,6-positions are substituted with chlorines (Steiert et al. 1987). The roles of PcpB, PcpC, and PcpA in degrading these compounds are summarized in Fig. 1.3. It now becomes clear why the 2 and 6-substitutions are required for the cells to completely degrade the chlorinated phenols, as 2,6-dichloroquinol is the necessary metabolic intermediate before PcpA can break the aromatic ring. Besides chlorinated phenols, bromophenols and iodophenols are degraded by the same enzymes (Xun and

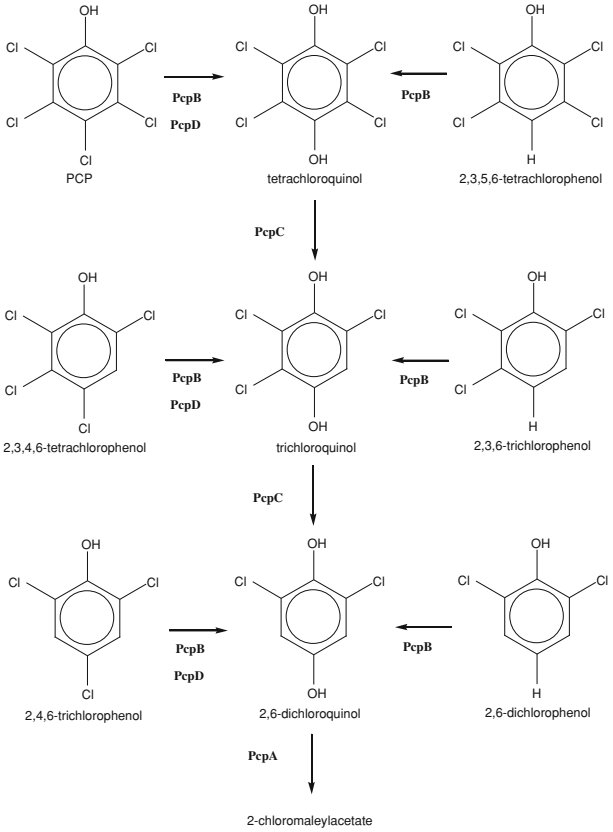


Fig. 1.3 The versatility of the PCP-degrading enzymes for the degradation of polychlorophenols. *S. chlorophenolicum* L-1 degrades several polychlorophenols as long as the 2,6-positions are occupied with chlorines. The combined actions of PcpB, PcpD, PcpC, and PcpA are responsible for the degradation. When PcpB dechlorinates, a quinone is formed. PcpD can reduce the quinone to the corresponding quinol

Orser 1991c; Xun et al. 1992c). Further, most of the halogenated phenols can induce the expression of the PCP degrading genes (Cai and Xun 2002). The *pcpB*, *pcpC*, and *pcpA* homologues have been identified from other PCP degrading *S. chlorophenolicum* strains and *Novosphingobium* spp. (Ederer et al. 1997; Tiirola et al. 2002). Thus, it is reasonable to conclude that the sphingomonads may use homologous enzymes for polychlorophenol degradation.

1.3.2 2,4,6-Trichlorophenol Metabolic Pathway of C. Necator JMP134

C. necator JMP134, best known for its ability to degrade 2,4-dichlorophenoxyacetate, also degrades 2,4,6-TCP (Clement et al. 1995). The initial proposed pathway for

2,4,6-TCP degradation includes 2,6-dichloroquinol as a metabolic intermediate (Padilla et al. 2000). The enzymes responsible for 2,4,6-TCP degradation in *C. necator* JMP134 have been identified, purified, and characterized by our group (Louie et al. 2002). Further characterization of 2,4,6-TCP 4-monooygenase has shown that 2,6-dichloroquinol is a by-product of the enzyme and not a metabolic intermediate in *C. necator* JMP134 (Xun and Webster 2004).

1.3.2.1 The *tcp* Gene Cluster

When *C. necator* JMP134 grows on sodium glutamate, it does not degrade 2,4,6-TCP. After glutamate is used up, JMP134 rapidly consumes 2,4,6-TCP, indicating the expression of 2,4,6-TCP degrading genes is subject to catabolic repression (Louie et al. 2002). 2,4,6-TCP 4-monooxygenase (TcpA) is partially purified from JMP134 cell extracts and characterized as a reduced flavin adenine dinucleotide ($FADH_2$)-dependent monooxygenase. The corresponding gene is identified by PCR using primers designed from conserved regions of $FADH_2$-dependent monooxygenases, and a gene cluster (*tcpABC*) has been determined. *tcpA* encodes the $FADH_2$-dependent 2,4,6-TCP 4-monooxygenase, and *tcpC* codes for 6-chlorohydroxyquinol 1,2-dioxygenase, respectively. The three genes have been individually inactivated in *C. necator* JMP134. The *tcpA* mutant fails to degrade 2,4,6-TCP; the *tcpB* mutant is not essential for 2,4,6-TCP degradation; and the *tcpC* mutant accumulated 6-chlorohydroxyquinol from 2,4,6-TCP degradation (Louie et al. 2002). After the genome of *C. necator* JMP134 is sequenced, a complete gene cluster *tcpRXABCYD* involved in TCP degradation is identified (Matus et al. 2003). The *tcpR* gene codes for a gene regulator that is required for the expression of the other *tcp* genes (Sánchez and González 2007). The functions of these *tcp* gene products have been characterized with recombinant proteins produced in *Escherichia coli*.

1.3.2.2 2,4,6-Trichlorophenol 4-Monooxygenase (TcpA)

Recombinant TcpA, purified from *E. coli*, transforms 2,4,6-TCP to 6-chlorohydroxyquinol when $FADH_2$ is supplied by an NADH:FAD oxidoreductase of *E. coli* (Louie et al. 2002). 2,6-dichloroquinol is accumulated to a marginal amount in the reaction mixture, but is hardly consumed by TcpA, questioning whether 2,6-dichloroquinol is a real metabolic intermediate. Further investigation has demonstrated that TcpA converts 2,4,6-TCP to 6-chlorohydroxyquinol according to the scheme presented in Fig. 1.4 (Xun and Webster 2004). Because dechlorination of phenolic compounds by monooxygenases produces quinones (Husain et al. 1980; Haigler et al. 1996; Dai et al. 2003), TcpA oxidizes 2,4,6-TCP to 2,6-dichloroquinone. TcpA then uses the product, while it is still bound to the active site, as its second substrate and removes the second chlorine by hydrolysis to generate 6-chlorohydroxyquinone, which is chemically or enzymatically reduced to

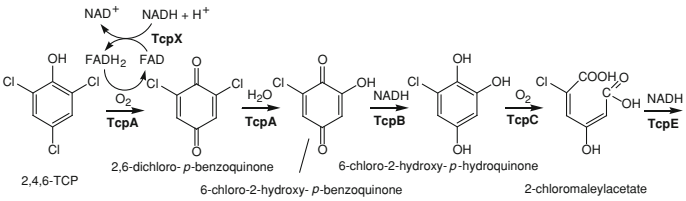


Fig. 1.4 2,4,6-TCP degradation pathway of *C. necator* JMP134. TcpA, 2,4,6-TCP 4-monooxygenase; TcpB, quinone reductase; TcpC, 6-chlorohydroxyquinol 1,2-dioxygenase; and TcpD, chloromaleylacetate reductase

6-chlorohydroxyquinol. When TcpA oxidizes 2,6-dichlorophenol, it simply introduces a hydroxyl group at the *para*-position and produces 2,6-dichloroquinol, which is not further metabolized by TcpA.

TcpA belongs to $FADH_2$-dependent monooxygenases. *E. coli* 4-hydroxyphenylacetate 3-monooxygenase (Galán et al. 2000; Xun and Sandvik 2000), TcpA (Louie et al. 2002), chlorophenol 4-monooxygenase (TftD) (Gisi and Xun 2003), and phenol 2-monooxygenase (Kirchner et al. 2003) are the early reported $FADH_2$-dependent monooxygenases. Sequence analysis suggests that a group of aromatic compound-hydroxylating enzymes are $FADH_2$-dependent monooxygenases, including 2,4,6-TCP 4-hydroxylase of *Ralstonia pickettii* DTP0602 (Takizawa et al. 1995), PhzO for biosynthesis of 2-hydroxylated phenazine antibiotics in *Pseudomonas aureofaciens* 30-84 (Delaney et al. 2001), PvcC involved in siderophore synthesis (Stintzi et al. 1996), and many hypothetical enzymes from GenBank. The characterized $FADH_2$-dependent monooxygenases are involved in biodegradation of aromatic compounds and biosynthesis of antibiotics and siderophores. Since TcpA does not have additional domains in comparison to other $FADH_2$-dependent monooxygenases, it is different from bifunctional enzymes that have two functional domains. Therefore, its ability to catalyze the hydrolytic reaction must be due to catalytic promiscuity, describing the ability of certain enzymes to catalyze unrelated reactions with the same active site.

1.3.2.3 NADH:FAD Oxidoreductase (TcpX)

The *tcpX* gene is immediately upstream *tcpA* in the *tcp* gene cluster, *tcpRXABCYD*. Since TcpX is highly homologous to the characterized flavin reductases (TftC and HpaC) that supply $FADH_2$ to $FADH_2$-dependent monooxygenases, TcpX is expected to be the flavin reductase that provides TcpA with $FADH_2$ (Matus et al. 2003). The recombinant TcpX produced in *E. coli* actively reduces FAD or FMN with NADH as the reducing power (Belchik and Xun 2008). Because $FADH_2$ is unstable in the presence of O_2, it is generated by TcpX in the reaction mixture and used by TcpA to oxidize 2,4,6-TCP (Fig. 1.4). TcpA can also

use $FADH_2$ generated by other flavin reductases. In fact, TcpA is characterized with a general *E. coli* flavin reductase (Fre) that provides $FADH_2$ for TcpA (Louie et al. 2002; Xun and Webster 2004).

1.3.2.4 Quinone Reductase (TcpB)

TcpB belongs to the nitroreductase family (pfam00881) (Bateman et al. 2002) of flavin reductases. The enzymes in this family catalyze the reduction of nitroaromatics, quinones, and flavins. Although some family members, e.g. *Vibrio harveyi* NADPH:FMN oxidoreductase and *V. fischeri* NAD(P)H:FMN oxidoreductase, provide bacterial luciferases with $FMNH_2$ (Inouye 1994; Lei et al. 1994), TcpB does not reduce FAD. TcpB is a quinone reductase with menadione as the best substrate among several tested substrates, including FAD, FMN, and Ferricyanide (Belchik and Xun 2008). The reduction of 6-chlorohydroxyquinone to 6-chlorohydroxyquinol by TcpB is indirectly confirmed as TcpB stimulates TcpA activity for 2,4,6-TCP oxidation in vitro. Although 6-chlorohydroxyquinone can be chemically reduced by reducing agents, such as NADH and ascorbic acid, TcpB is more efficient to reduce 6-chlorohydroxyquinone. 6-Chlorohydroxyquinone spontaneously reacts with thiols to form conjugates, and TcpB minimizes the formation of the conjugates. Thus, TcpB effectively reduces 6-chlorohydroxyquinone to 6-chlorohydroxyquinol and protects cellular thiols.

1.3.2.5 6-Chlorohydroxyquinol 1,2-Dioxygenase (TcpC)

TcpC oxidizes 6-chlorohydroxyquinol to 2-chloromaleylacetate (Louie et al. 2002). It is a member of the intradiol ring-cleavage dioxygenases, consisting of all the characterized (chloro)hydroxyquinol dioxygenases together with some catechol 1,2-dioxygenases and protocatechuate 3,4-dioxygenases. Since inactivation of the *tcpC* also causes the mutant to accumulate 6-chlorohydroxyquinol, the data collectively support that JMP134 transforms 2,4,6-TCP to 2-chloromaleylacetate by TcpA and TcpC (Fig. 1.4). In addition, TcpX, a flavin reductase, is required to generate $FADH_2$ for TcpA, and TcpB reduces 6-chlorohydroxyquinone to 6-chlorohydroxyquinol.

1.3.2.6 Chloromaleylacetate Reductase (TcpD)

TcpD and PcpE share 52.9% sequence identity, suggesting that TcpD is a chloromaleylacetate reductase. When the *tcpD* gene is inactivated, the mutant lost the ability to grow with 1.5 mM 2,4,6-TCP as the sole carbon and energy source (Sánchez and González 2007). Although TcpD is not characterized, it is expected to catalyze the same reactions as PcpE (Fig. 1.1) to convert 2-chloromaleylacetate to maleylacetate and then to 3-oxoadipate (Fig. 1.4).

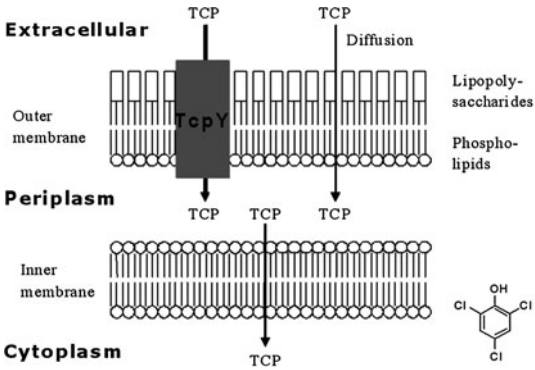


Fig. 1.5 Proposed entry of 2,4,6-TCP via diffusion and through TcpY into *C. necator* JMP134. TCP diffuses through cytoplasmic membranes, but the diffusion may be slowed by lippolysaccharides of outer membrane. TcpY may serve as a TCP channel

1.3.2.7 *β*-Barrel Outer Membrane Protein (TcpY)

TcpY facilitates the uptake of 2,4,6-trichlrophenol into *C. necator* (Belchik et al. 2010). Except *tcpY*, the inactivation of other genes in the *tcpRXABCYD* gene cluster negatively affects 2,4,6-TCP degradation by *C. necator* JMP134. Inactivation of *tcpY* has no apparent effect on 2,4,6-TCP degradation. Sequence analysis associates TcpY with COG4313, a group of hypothetical proteins. Signal peptide, structure and topology analyses indicate that TcpY is a *β*-barrel outer membrane protein of Gram negative bacteria. Structurally similar proteins include *Escherichia coli* outer membrane protein FadL that transports hydrophobic, long-chain fatty acids across the hydrophilic outer leaflet of the outer membrane, consisting of lipopolysaccharides. Several lines of evidence support that TcpY facilitates the uptake of 2,4,6-TCP into *C. necator*. First, constitutive expression of *tcpY* in two *C. necator* strains rendered the cells more sensitive to polychlorophenols, including 2,4,6-TCP. Second, *C. necator* JMP134 expressing cloned *tcpY,* transported more 2,4,6-TCP into the cell than it carrying the cloning vector. 2,4,6-TCP may enter the cell by diffusion or through other *β*-barrel outer membrane proteins (Fig. 1.5). We believe that 2,4,6-TCP uptake is not the limiting step for its degradation by *C. necator* JMP134.

1.3.2.8 The Presence of *tcp* Genes in Other Bacteria

2,4,6-TCP degradation pathways have also been studied in other 2,4,6-TCP degraders that do not mineralize PCP. Chlorohydroxyquinol 1,2-dioxygenases have been identified, purified, and characterized from 2,4,6-TCP-degrading *Azotobacter* sp. GP1 (Zaborina et al. 1995) and *Streptomyces rochei* 303 (Zaborina et al. 1995), and the enzymatic activities have been observed from 2,4,6-TCP degrading *Ralstonia pickettii* (Takizawa et al. 1995). 2,4,6-TCP 4-monooxygenases have been reported from *R. pickettii* (Takizawa et al. 1995) and *Azotobacter* sp. GP1 (Wieser et al. 1997). The two 2,4,6-TCP 4-monooxygenases have high

sequence identity with TcpA (Louie et al. 2002). The *Azotobacter* enzyme has been highly purified and assayed without providing $FADH_2$ as a co-substrate, and the reported enzyme activity (Wieser et al. 1997), which is 47 folds lower than that of TcpA (Louie et al. 2002), is likely due to the insufficient supply of $FADH_2$ in the reaction mixture. Perhaps, due to the low measurable enzyme activity, the main end product 6-chlorohydroxyquinol is not identified from the oxidation of 2,4,6-TCP by the enzyme (Wieser et al. 1997). Thus, the metabolic pathway from earlier studies is incomplete as the link from 2,4,6-TCP to 2-chlorohydroxyquinol is missing, but the studies have laid the foundation for the complete characterization of 2,4,6-TCP degradation pathway in *C. necator* JMP134 (Louie et al. 2002). Since these 2,4,6-TCP degraders all have homologous 2,4,6-TCP 4-monooxygenases and chlorohydroxyquinol 1,2-dioxygenases (Louie et al. 2002; Matus et al. 2003), they should have the same pathway for 2,4,6-TCP degradation (Fig. 1.4). Further, because *S. chlorophenolicum* also mineralizes 2,4,6-TCP (Cai and Xun 2002), some 2,4,6-TCP degraders, that do not carry *pcpC*, may also use PcpB and PcpA homologues for the degradation (Fig. 1.3).

1.3.3 2,4,5-Trichlorophenol Metabolic Pathway of B. Cepacia AC1100

2,4,5-TCP is the first metabolic intermediate of 2,4,5-T degradation by *B. cepacia* AC1100 (Fig. 1.6) (Karns et al. 1983), and the 2,4,5-T degradation pathway has been deciphered by a combination of genetic and biochemical studies. Mutagenesis has produced two types of mutants: one completely loses the ability to degrade 2,4,5-T, and the other degrades it, but accumulates 5-chlorohydroxyquinol (Sangodkar et al. 1988; Haugland et al. 1991). Logically, the first type has a mutation in a gene responsible for the first step of the degradation pathway, and the second type has a mutation in the gene responsible for 5-chlorohydroxyquinol metabolism. Fortunately, several plasmid vectors can replicate in *B. cepacia*. The intact genes of *B. cepacia* AC1100 have been cloned, and transferred into the mutants. The cloned DNA fragments, that complement the mutants to grow on 2,4,5-T, have been mapped to identify the genes responsible for the first degradation step as well as for 5-chlorohydroxyquinol metabolism.

1.3.3.1 2,4,5-Trichlorophenoxyactate Oxygenase (TftAB)

The 2,4,5-T oxygenase has an oxygenase component and a reductase component. It is the first enzyme of the 2,4,5-T degradation pathway of *B. cepacia* AC1100 (Fig. 1.6). The genes encoding the 2,4,5-T oxygenase component (*tftA* and *tftB*) have been cloned for their ability to complement the mutant that does not grow on 2,4,5-T (Hubner et al. 1998). The two genes *tftA* and *tftB* are homologous to the

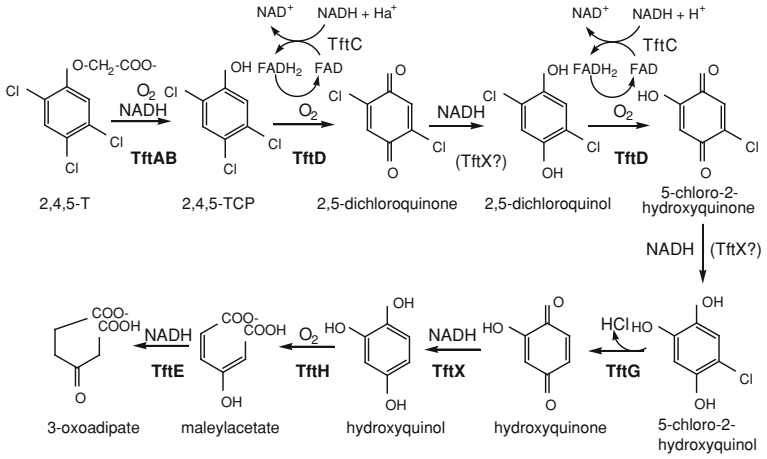


Fig. 1.6 2,4,5-Trichlorophenoxyacetate degradation pathway of *B. cepacia* AC1100. TftAB, 2,4,5-T terminal oxygenase; TftD, 2,4,5-TCP 4-monooxygenase; TftC, NADH:FAD oxidoreductase; TftX, quinone reductase; TftG, 5-chlorohydroxyquinol dehydrochlorinase; TftH, hydroxyquinol 1,2-dioxygenase; and TftE, maleylacetate reductase

α- and β-subunits of the oxygenase component of ring hydroxylating dioxygenases (Hubner et al. 1998), but the gene encoding a reductase component is not next to *tftA* and *tftB*. Theoretically, the oxygenase component requires a reductase component to function. The 2,4,5-T oxygenase has been studied from *B. cepacia* AC1100, and it has an oxygenase component and a reductase component (Xun and Wagon 1995). The 2,4,5-T oxygenase component (TftAB) is a heterotetramer with an $\alpha_2\beta_2$ structure, and each $\alpha\beta$ unit has a Reiske-type [2Fe-2S] center, which gives the protein a reddish color with absorption peaks at 420 (shoulder) and 530 nm. The N-terminal sequences of the $\alpha\beta$ subunits match with those encoded by *tftA* and *tftB*. As expected, the oxygenase component alone has no activity, and it requires a reductase component to oxidize 2,4,5-T to 2,4,5-TCP and glyoxylate. The reaction is a typical monooxygenation reaction, different from the reaction typically catalyzed by most ring hydroxylating dioxygenases; however, it should not be a surprise, as some ring-hydroxylating dioxygenases can catalyze monooxygenation reactions towards certain substrates (Wackett et al. 1988). The reductase component has been only partially purified from *B. cepacia* AC1100 (Xun and Wagon 1995), and the corresponding gene has not been cloned.

1.3.3.2 2,4,5-Trichlorophenol 4-Monooxygenase (TftCD)

Mutations that block 2,4,5-TCP metabolism have not been obtained. The 2,4,5-TCP 4-monooxygenase is identified, purified, and characterized directly from *B. cepacia* AC1100 (Xun 1996). HPLC analysis showed that the cell extract consumes 2,4,5-TCP without any apparent end products. When 2,4,6-TCP is used

as a substrate, the cell extract converts 2,4,6-TCP to 2,6-dichloroquinol at the expense of NADH and O_2. A two-component monooxygenase (TftCD) is purified by assaying the conversion of 2,4,6-TCP to 2,6-dichloroquinol, which is chemically reduced to 2,6-dichloroquinol. The purified enzyme also oxidizes 2,4,5-TCP to 2,5-dichloroquinone, which is chemically reduced to 2,5-dichloroquinol; the monooxygenase then oxidizes the latter to 5-chlorohydroxyquinone, which is chemically reduced to 5-chlorohydroxyquinol (Fig. 1.6). The two steps of quinone reduction are accomplished by NADH and ascorbate in reaction mixtures, but the reaction can be catalyzed by a quinone reductase in vivo. A quinone reductase (TftX) is discussed in a later paragraph. The two genes (*tftCD*) coding for the two enzymes are physically linked and are cloned by using a degenerated oligonucleotide probe designed from the N-terminus of TftC. There are two copies of *tftCD* on two separate replicons in *B. cepacia* AC1100, explaining why transposon mutagenesis is ineffective to obtain a mutant blocked for 2,4,5-TCP metabolism (Hubner et al. 1998).

The two genes are expressed in *E. coli*, and the gene products are characterized as two separate enzymes: an NADH:FAD oxidoreductase (TftC) and an $FADH_2$-dependent 2,4,5-TCP 4-monooxygenase (TftD) (Gisi and Xun 2003). TftC uses NADH, but not NADPH, to preferentially reduce FAD over FMN, and it is assigned as an NADH:FAD oxidoreductase. The function of TftC is to supply TftD with $FADH_2$. TftC is highly homologous to TcpX that provides TcpA with $FADH_2$ for 2,4,6-TCP oxidation. TftD is an $FADH_2$-dependent monooxygenase that catalyzes two sequential monooxygenase reactions on 2,4,5-TCP (Fig. 1.6). TftD uses both 2,4,5-TCP and 2,5-dichlorohydroxyquinol as substrates (Fig. 1.6), and kinetic analysis has showed that 2,5-dichlorohydroxyquinol is a better substrate. TftD is highly homologous to TcpA with 61% of amino acid sequence identity. Both TftD and TcpA can use 2,4,6-TCP as a substrate. However, TftD only oxidizes 2,4,6-TCP to 2,6-dichloroquinone, while TcpA converts 2,4,6-TCP to 2,6-dichloroquinone and then to 6-chlorohydroxyquinone (Fig. 1.4).

1.3.3.3 5-Chlorohydroxyquinol Dehydrochlorinase (TftG)

A transposon mutant that accumulates 5-chlorohydroxyquinol from 2,4,5-T degradation was obtained (Sangodkar et al. 1988), and a gene cluster (*tftEFGH*) that complements the mutation was cloned and sequenced (Zaborina et al. 1998). The functions of the corresponding proteins in 2,4,5-T degradation have been demonstrated. TftG, a very small protein of 12 kDa, is 5-chlorohydroxyquinol dehydrochlorinase that converts 5-chlorohydroxyquinol to hydroxyquinone with the elimination of an HCl molecule (Fig. 1.6) (Zaborina et al. 1998). TftG is similar to LinA, a 17-KDa protein, with 19% sequence identity and 46% similarity. LinA is a dechlorinase that removes HCl from hexachlorocyclohexane (Imai et al. 1991). The dechlorination mechanism used by TftG and LinA is HCl elimination from the substrates. The chemistry concerned with the dechlorination by LinA has been studied and the proposed reaction mechanism involves nucleophilic attack by a His

residue on hexachlorocyclohexane, hydrogen abstraction, Cl^- elimination, and end-product release (Trantirek et al. 2001).

1.3.3.4 Quinone Reductase (TftX)

TftF is a glutathione reductase, but it may not be directly involved in 2,4,5-T degradation. It is suspected to reduce hydroxyquinone, but the purified TftF did not reduce the quinone (Zaborina et al. 1998). A quinone reductase (TftX), whose activity is increased when *B. cepacia* AC1100 grows on 2,4,5-T, has been purified and shown to reduce hydroxyquinone (Fig. 1.6). The reductase is a homodimer of a 22-kDa polypeptide. Since the corresponding gene has not been reported, the enzyme has not been assigned to any enzyme family. The purified protein has a typical absorption maximum at 450 nm for flavoproteins. In the presence of free FMN, the purified enzyme uses NADH to reduce hydroxyquinone to hydroxyquinol (Fig. 1.6), which is subject to ring-cleavage by TftH (Zaborina et al. 1998).

1.3.3.5 Hydroxyquinol 1,2-Dioxygenase (TftH)

The gene *tftH* codes for hydroxyquinol 1,2-dioxygenase. TftH is homologous to 6-chlorohydroxyquinol 1,2-dioxygenases of 2,4,6-TCP degrading bacteria, which can use both 6-chlorohydroxyquinol and hydroxyquinol as substrates (Zaborina et al. 1995). However, TftH oxidizes only hydroxyquinol, but not 5-chlorohydroxyquinol nor 6-chlorohydroxyquinol (Zaborina et al. 1998), suggesting that TftH has a high substrate specificity. The high substrate specificity of TftH supports the necessity for the conversion of 5-chlorohydroxyquinol to hydroxyquinol before ring cleavage in the degradation pathway (Fig. 1.6). TftH oxidizes hydroxyquinol to maleylacetate.

1.3.3.6 Maleylacetate Reductase (TftE)

The *tftE* gene codes for a maleylacetate reductase that reduces maleylacetate to 3-oxoadipate. TftE and PcpE share 54.5% sequence identity, and TftE and TcpD have 61.4% sequence identity. Although the initial pathways of the three polychlorophenols are different, the pathways merge at maleylacetate, which is further channeled to the tricarboxylic acid cycle for complete mineralization. The common pathway from 3-oxoadipate to the tricarboxylic acid cycle intermediates has not been investigated in any polychlorophenol degrading bacteria; however, the pathway should be as proposed in Fig. 1.1, which has been demonstrated in other microorganisms (Harwood and Parales 1996; Kaschabek et al. 2002).

1.4 Dechlorination Mechanisms

The biochemistry of polychlorophenol degradation has revealed several dechlorination mechanisms. First, oxygenolytic dechlorination is quite common for the three metabolic pathways. Molecular oxygen is involved in the dechlorination and a chlorine is replaced by a hydroxyl group with the oxygen atom derived from O_2. The dechlorination reactions catalyzed by PcpB (Fig. 1.1) (Xun et al. 1992d), TcpA (Fig. 1.4) (Louie et al. 2002), and TftD (Fig. 1.6) (Xun 1996) are examples of oxygenolytic dechlorination. Second, reductive dechlorination is defined as the substitution of a chlorine by a hydrogen atom. 2-Chloromaleylacetate reduction to maleylacetate catalyzed by PcpE is an example (Fig. 1.1). In addition, some anaerobic microorganisms use reductive dechlorinases as the terminal enzyme of an electron transport chain to remove chlorines from polychlorophenols for anaerobic respiration (Boyer et al. 2003). Third, "thiolytic" dechlorination often involves glutathione, which can be consumed in some reactions. During PCP degradation by *S. chlorophenolicum*, PcpC catalyzes "thiolytic" dechlorination (Fig. 1.1) (Anandarajah et al. 2000). Since glutathione is oxidized and chlorines are replaced by hydrogen atoms in the end product, trichloroquinol (Fig. 1.1), the reaction is also reductive (Xun et al. 1992a). Fourth, dehydrochlorination refers to a reaction that eliminates an HCl molecule from the parent compound. An example of this type of dechlorination is seen in the conversion of 5-chlorohydroxyquinol to hydroxyquinone by TftG (Fig. 1.6) (Zaborina et al. 1998). Fifth, in hydrolytic dechlorination, a chlorine is replaced by a hydroxyl group derived from H_2O. TcpA catalyzes sequential dechlorination of 2,4,6-TCP by an oxidative reaction and a hydrolytic reaction (Fig. 1.4) (Xun and Webster 2004). Dechlorination of polychlorinated aromatic compounds is critical in their biodegradation because partial or complete dechlorination often occurs before ring-cleavage dioxygenases can open aromatic rings (Figs. 1.1, 1.4 and 1.6). Further, chlorines must be removed before any chloro-organic compounds can be channeled into common metabolic pathways for complete mineralization. Research done with the biochemistry of polychlorophenol degradation has enhanced our understanding of enzymatic mechanisms of dechlorination.

1.5 Gene Organization, Regulation and Evolution of the Polychlorophenol Degradation Pathways

The genes (*tcpRXABCYD*) involved in 2,4,6-TCP degradation (Fig. 1.4) are organized in a gene cluster in *C. necator* JMP134 (Fig. 1.7a) (Louie et al. 2002; Matus et al. 2003). TcpR functions as a positive gene regulator, and it is required for the expression of the *tcpXABCYD* operon. The inducer is identified as 2,4,6-TCP (Sánchez and González 2007); however, the induction is subject to catabolic repression by glutamate (Louie et al. 2002). The apparent presence of similar *tcp*

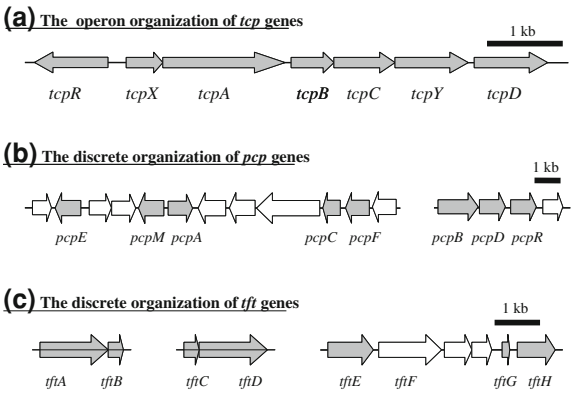


Fig. 1.7 Organization of the genes involved in polychlorophenol degradation. The functions of *tcp* gene products are shown in Fig. 1.4; the functions of the *pcp* gene products are given in Fig. 1.1; the functions of *tft* gene products are presented in Fig. 1.6. **a** The operon organization of *tcp* genes, **b** the discrete organization of *pcp* genes, **c** the discrete organization of *tft* genes

gene clusters in different 2,4,6-TCP degrading microorganisms (Matus et al. 2003) suggests that the genes responsible for 2,4,6-TCP metabolism are well conserved and widespread in different genera of microorganisms. Since 2,4,6-TCP can be naturally produced in forest soils (Hoekstra et al. 1999), it may not be farfetched to conclude that nature had developed the 2,4,6-TCP degradation system prior to the massive release from industrial production and application.

The *pcp* genes are restricted to sphingomonads, primarily *Sphingobium chlorophenolicum*, *Sphingomonas* sp. and *Novosphingobium* spp. Horizontal transfer of *pcpB* genes among sphingomonads has been proposed (Tiirola et al. 2002). Although these bacteria are isolated from different geographical locations, most of them are *S. chlorophenolicum* strains (Crawford and Ederer 1999). The organization of the *pcp* genes in *S. chlorophenolicum* L-1 is unusual because the five PCP-degrading genes, *pcpA*, *pcpB*, *pcp*C, *pcpD*, and *pcpE*, are located at four discrete locations (Figs. 1.1 and 1.7b) (Cai and Xun 2002). Two homologous LysR-type regulator genes, *pcpM* and *pcpR,* are identified. Genes *pcpBDR* are clustered together with *pcpBD* transcribed on the same mRNA, and *pcpR* is transcribed separately. *pcpA* and *pcpM* are next to each other, but transcribed away from each other. PcpM may be involved in *pcpA* expression, but not required, and PcpR is required for the expression of *pcpB*, *pcpA*, and *pcpE*. PCP and other polychlorophenols are co-inducers of PcpR that is the gene activator. The *pcpC* gene is constitutively expressed. The restricted presence of *pcp* genes in sphingomonads, the discrete organization, and lack of regulation of *pcpC* collectively suggests that the *pcp* genes have been recently recruited. Given that PCP is not a natural chemical and that the introduction of PCP on a global scale has occurred only in the last 80 years, the discrete organization and incomplete regulation of *pcp* genes in *S. chlorophenolicum* ACTT 39723 may represent an evolutionary snapshot of a new metabolic pathway.

2,4,5-T is also not found in nature, and its release into the environment has been more recent than PCP contamination (Firestone 1978). The organization and

regulation of the genes involved in 2,4,5-T degradation suggest that the genes have also been recently recruited in *B. cepacia* AC1100 (Fig. 1.7c). The genes are organized in three gene clusters. The first cluster is *tftAB* coding for the oxygenase component of 2,4,5-T oxygenase that catalyzes the first step of 2,4,5-T degradation (Fig. 1.6). The *tftAB* genes are constitutively expressed, using a promoter created by an insertion element (Hubner and Hendrickson 1997). The insertion element has a high frequency of spontaneous deletion, resulting in *B. cepacia* AC1100 mutants that cannot degrade 2,4,5-T (Haugland et al. 1991). The second cluster is *tftCD*, which has two copies in the AC1100 genome (Hubner et al. 1998). The genes are inducible by 2,4,5-TCP (Karns et al. 1983), and the regulation mechanisms have not been studied. The third cluster, containing 6 open reading frames (ORF), is responsible for the conversion of 5-chlorohydroxyquinol to 3-oxoadipate (Fig. 1.6) (Zaborina et al. 1998). Three out of 6 of the genes are involved in the conversion: ORF1 (*tftE*), encoding maleylacetate reductase, ORF5 (*tftG*), coding for 5-chlorohydroxyquinol dehydrochlorinase, and ORF6 (*tftH*), encoding hydroxyquinol 1,2-dioxygenase. ORF2 (*tftF*), ORF3 and ORF4 code for a glutathione reductase and two glutathione S-transferases, and they do not have apparent roles in 2,4,5-T degradation. The genes are expressed when AC1100 grows on 2,4,5-T, but their regulation has not been reported. It seems that the gene clusters are recruited and forced to degrade 2,4,5-T, reflecting the selection pressure applied to enhance the evolution of a 2,4,5-T degrading bacterium in a chemostat, from which AC1100 is isolated (Kellogg et al. 1981).

1.6 Conclusions

Polychlorophenols and their derivatives are widely used as fungicides, pesticides, and herbicides. Despite regulations for their usage and release, they remain a group of major pollutants, highly toxic to humans. The primary action site of polychlorophenols is cell membranes, disrupting the proton motive force and inactivating membrane proteins. Chloroquinones and chloroquinols are metabolic intermediates of aerobic microbial degradation as well as metabolites in fungi and animals, including humans. The formation of chloroquinols is harmful to cells, as the oxidation of chloroquinols and reduction of chloroquinones generate reactive oxygen species, leading to oxidative damage. Thus, chloroquinols are not necessarily the ideal metabolic intermediates for microbial degradation, but their formation is likely determined by the structural properties of polychlorophenols. Due to the formation of chloroquinols, microorganisms have recruited quinol ring-cleavage dioxygenases instead of catechol ring-cleavage dioxygenases for polychlorophenol degradation. Further, GSH spontaneously reacts with chloroquinones to form GS-chloroquinols. When GS-chloroquinols are produced, GS-(chloro)quinol reductases, e.g. PcpF, can release the quinols that re-enter the metabolic pathway. In summarizing the information from literature, phenol and mono- and di-chlorophenols are often degraded to catechol and substituted

catechol before ring-cleavage; whereas, polychlorophenols are usually metabolized to quinol derivatives.

Characterization of polychlorophenol degradation by microorganisms is intended to develop strategies for efficient removal of these pollutants from the environment. The complete metabolic pathways with the enzymes and corresponding genes have only been characterized for three polychlorophenols, PCP, 2,4,6-TCP and 2,4,5-TCP. The results have revealed different metabolic pathways for the degradation of different isomers of polychlorophenols. Conversely, *S. chlorophenolicum* and *C. necator* degrade 2,4,6-TCP by using different pathways. Studies on the biochemistry and molecular biology of polychlorophenol degradation reveal a glimpse of manifold mechanisms and pathways as well as their evolution in microbial degradation of environmental pollutants. Results that support this diverse theme include the discrete organization of *pcp* genes and *tft* genes, the recruitment of two glutathione transferases for tetrachloroquinol metabolism during PCP degradation, and the employment of catalytic promiscuity in 2,4,6-TCP conversion to 6-chlorohydroxyquinol. Research on the biochemistry and molecular biology will continue with immediate progresses possibly in a better understanding of gene regulation and the evolutionary aspects of PCP, 2,4,6-TCP, 2,4,5-TCP degradation, research extension to other isomers of polychlorophenols, and further studies on the biochemistry and genetics of fungal metabolism and anaerobic degradation of polychlorophenols. The studies will provide scientific guidance for bioremediation of polychlorophenols and contribute to the knowledge of dechlorination mechanisms as well as the evolution of pollutant biodegradation.

Acknowledgments I began working on polychlorophenol degradation as a postdoctoral scientist with Dr. Cindy S. Orser at University of Idaho and have continued the research as a faculty member at Washington State University. Pacific Northwest National Laboratory provided me with lab space and equipment from 1992 to 1997. National Science Foundation has supported the research with grants MCB-921873, MCB-9722970, MCB-0323167 and MCB-1021148. My graduate students, postdoctoral scientists, and coworkers have significantly contributed to the progress summarized here. We have collaborated with Dr. A. M. Chakrabarty's group at University of Illinois at Chicago on 2,4,5-T degradation.

References

Anandarajah K, Kiefer PMJ, Donohoe BS, Copley SD (2000) Recruitment of a double bond isomerase to serve as a reductive dehalogenase during biodegradation of pentachlorophenol. Biochemistry 39:5303–5311

Apajalahti JH, Salkinoja-Salonen MS (1987) Complete dechlorination of tetrachlorohydroquinone by cell extracts of pentachlorophenol-induced Rhodococcus chlorophenolicus. J Bacteriol 169:5125–5130

Aranda C, Godoy F, Gonzalez G, Homo J, Martinez M (1999) Effects of glucose and phenylalanine upon 2,4,6-trichlorophenol degradation by *Pseudomonas paucimobilis* S37 cells in a no-growth state. Microbios 100:73–82

Bateman A, Birney E, Cerruti L, Durbin R, Etwiller L, Eddy SR (2002) The Pfam protein families database. Nucleic Acids Res 30:276–280

Belchik SM, Xun L (2008) Functions of flavin reductase and quinone reductase in 2,4,6-trichlorophenol degradation by *Cupriavidus necator* JMP134. J Bacteriol 190:1615–1619

Belchik SM, Schaeffer SM, Hasenoehrl S, Xun L (2010) A beta-barrel outer membrane protein facilitates cellular uptake of polychlorophenols in *Cupriavidus necator*. Biodegradation 21:431–439

Birchmore RJ, Meneley JC (1979) Prochloraz-a new foliar fungicide with potential in a wide range of crops. Phytopathology 69:1022–1026

Bisaillon A, Beaudet R, Lépine F, Déziel E, Villemur R (2010) Identification and characterization of a novel CprA reductive dehalogenase specific to highly chlorinated phenols from *Desulfitobacterium hafniense* strain PCP-1. Appl Environ Microbiol 76(22):7536–7540. doi:10.1128/AEM.01362-10

Bock C, Kroppenstedt RM, Schmidt U, Diekmann H (1996) Degradation of prochloraz and 2,4,6-trichlorophenol by environmental bacterial strains. Appl Microbiol Biotech 45:257–262

Bouchard B, Beaudet R, Villemur R, McSween G, Lepine G, Bisaillon JG (1996) Isolation and characterization of *Desulfitobacterium frappieri* sp. nov., an anaerobic bacterium which reductively dechlorinates pentachlorophenol to 3-chlorophenol. Int J Syst Bacteriol 46:1010–1015

Boyer A, Page-BeLanger R, Saucier M, Villemur R, Lépine F, Juteau P, Beaudet R (2003) Purification, cloning and sequencing of an enzyme mediating the reductive dechlorination of 2,4,6-trichlorophenol from *Desulfitobacterium frappieri* PCP-1. Biochem J 373:297–303

Briglia M, Eggen RI, Van Elsas DJ, De Vos WM (1994) Phylogenetic evidence for transfer of pentachlorophenol-mineralizing *Rhodococcus chlorophenolicus* PCP-I(T) to the genus *Mycobacterium*. Int J Syst Bacteriol 44:494–498

Briglia M, Rainey FA, Stackebrandt E, Schraa G, Salkinoja-Salonen MS (1996) *Rhodococcus percolatus* sp. nov., a bacterium degrading 2,4,6-trichlorophenol. Int J Syst Bacteriol 46:23–30

Cai M, Xun L (2002) Organization and regulation of pentachlorophenol-degrading genes in *Sphingobium chlorophenolicum* ATCC 39723. J Bacteriol 184:4672–4680

Chanama S, Crawford RL (1997) Mutational analysis of pcpA and its role in pentachlorophenol degradation by *Sphingomonas* (*Flavobacterium*) *chlorophenolica* ATCC 39723. Appl Environ Microbiol 63:4833–4838

Chu JP, Kirsch EJ (1972) Metabolism of pentachlorophenol by an axenic bacterial culture. Appl Microbiol 23:1033–1035

Clement P, Matus V, Cardenas L, Gonzalez B (1995) Degradation of trichlorophenols by *Alcaligenes eutrophus* JMP134. FEMS Microbiol Lett 127:51–55

Cline RE, Hill RHJ, Phillips DL, Needham LL (1989) Pentachlorophenol measurements in body fluids of people in log homes and workplaces. Arch Environ Contam Toxicol 18:475–481

Coque JJ, Alvarez-Rodriguez ML, Larriba G (2003) Characterization of an inducible chlorophenol O-methyltransferase from *Trichoderma longibrachiatum* involved in the formation of chloroanisoles and determination of its role in cork taint of wines. Appl Environ Microbiol 69:5089–5095

Crawford RL, Ederer MM (1999) Phylogeny of *Sphingomonas* species that degrade pentachlorophenol. J Ind Microbiol Biotechnol 23:320–325

Crawford RL, Mohn WW (1985) Microbial removal of pentachlorophenol from soil using a *Flavobacterium*. Enzyme Microb Technol 7:617–620

Crosby DG (1981) Environmental chemistry of pentachlorophenol. Pure Appl Chem 53:1052–1080

Cunarro J, Weiner MW (1975) Mechanism of action of agents which uncouple oxidative phosphorylation: direct correlation between proton-carrying and respiratory-releasing properties using rat liver mitochondria. Biochim Biophys Acta 387:234–240

Dahlhaus M, Almstadt E, Henschke P, Luttgert S, Appel KE (1995) Induction of 8-hydroxy-2-deoxyguanosine and single-strand breaks in DNA of V79 cells by tetrachloro-*p*-hydroquinone. Mutat Res 329:29–36

Dai M, Rogers JB, Warner JR, Copley SD (2003) A previously unrecognized step in pentachlorophenol degradation in *Sphingobium chlorophenolicum* is catalyzed by tetrachlorobenzoquinone reductase (PcpD). J Bacteriol 185:302–310

Delaney SM, Mavrodi DV, Bonsall RF, Thomashow LS (2001) phzO, a gene for biosynthesis of 2-hydroxylated phenazine compounds in *Pseudomonas aureofaciens* 30–84. J Bacteriol 183:318–327

Dixon DP, Davis BG, Edwards R (2002) Functional divergence in the glutathione transferase superfamily in plants. Identification of two classes with putative functions in redox homeostasis in *Arabidopsis thaliana*. J Biol Chem 277:30859–30869

Duncan CG, Deverall FJ (1964) Degradation of wood preservatives by fungi. Appl Microbiol 12:57–62

Ederer MM, Crawford RL, Herwig RP, Orser CS (1997) PCP degradation is mediated by closely related strains of the genus *Sphingomonas*. Mol Ecol 6:39–49

Entsch B, Ballou DP, Massey V (1976) Flavin-oxygen derivatives involved in hydroxylation by *p*-hydroxybenzoate hydroxylase. J Biol Chem 251:2550–2563

EPA (2006) Toxic Release Inventory: Public Data Release. http://www.epa.gov/tri/tridata/tri06/

Escher BI, Snozzi M, Schwarzenbach P (1996) Uptake, speciation, and uncoupling activity of substituted phenols in energy transducing membranes. Environ Sci Technol 30:3071–3079

Firestone D (1978) The 2,3,7,8-trtrachlorodibenzo-para-dioxin problem: a review. Stockholm Ecol Bulletin 27:39–52

Galán B, Díaz E, Prieto MA, García JL (2000) Functional analysis of the small component of the 4-hydroxyphenylacetate 3-monnooxygenase of *Escherichia coli* W: a prototype of a new Flavin: NAD(P)H reductase subfamily. J Bacteriol 182:627–636

Garcera A, Barreto L, Piedrafita L, Tamarit J, Herrero E (2006) *Saccharomyces cerevisiae* cells have three Omega class glutathione S-transferases acting as 1-Cys thiol transferases. Biochem J 398:187–196

Gisi MR, Xun L (2003) Characterization of chlorophenol 4-monooxygenase (TftD) and NADH:flavin adenine dinucleotide oxidoreductase (TftC) of *Burkholderia cepacia* AC1100. J Bacteriol 185:2786–2792

Golovleva LA, Pertsova RN, Evtushenko LI, Baskunov BP (1990) Degradation of 2,4,5-trichlorophenoxyacetic acid by a *Nocardioides simplex* culture. Biodegradation 1:263–271

Golovleva LA, Zaborina O, Pertsova R, Baskunov B, Schurukhin Y, Kuzmin S (1992) Degradation of polychlorinated phenols by *Streptomyces rochei* 303. Biodegradation 2:201–208

Habash M, Chu BC, Trevors JT, Lee H (2009) Mutational study of the role of N-terminal amino acid residues in tetrachlorohydroquinone reductive dehalogenase from *Sphingomonas* sp. UG30. Res Microbiol 60:553–559

Haggblom MM, Nohynek LJ, Palleroni NJ, Kronqvist K, Nurmiaho-Lassila EL, Salkinoja-Salonen MS (1994) Transfer of polychlorophenol-degrading *Rhodococcus chlorophenolicus* to the genus *Mycobacterium* as *Mycobacterium chlorophenolicum* comb nov. Int J Syst Bacteriol 44:485–493

Haigler BE, Suen WC, Spain JC (1996) Purification and sequence analysis of 4-methyl-5-nitrocatechol oxygenase from *Burkholderia* sp strain DNT. J Bacteriol 178:6019–6024

Harwood CS, Parales RE (1996) The ß-ketoadipate pathway and the biology of self-identity. Annu Rev Microbiol 50:553–590

Haugland RA, Sangodkar UM, Sferra PR, Chakrabarty AM (1991) Cloning and characterization of a chromosomal DNA region required for growth on 2,4,5-T by *Pseudomonas cepacia* AC1100. Gene 100:65–73

Hoekstra E, de Weerd H, de Leer E, Brinkman U (1999) Natural formation of chlorinated phenols, dibenzo-*p*-dioxins, and dibenzofurans in soil of a douglas fir forest. Environ Sci Technol 33:2543–2549

Huang Y, Xun R, Chen G, Xun L (2008) The maintenance role of a glutathionyl-hydroquinone lyase (PcpF) in pentachlorophenol degradation by *Sphingobium chlorophenolicum* ATCC 39723. J Bacteriol 190:7595–7600

Hubner A, Hendrickson W (1997) A fusion promoter created by a new insertion sequence, IS1490, activates transcription of 2,4,5-trichlorophenoxyacetic acid catabolic genes in *Burkholderia cepacia* AC1100. J Bacteriol 179:2717–2723

Hubner A, Danganan CE, Xun L, Chakrabarty AM, Hendrickson W (1998) Genes for 2,4,5-trichlorophenoxyacetic acid metabolism in *Burkholderia cepacia* AC1100: characterization of the *tftC* and *tftD* genes and locations of the *tft* operons on multiple replicons. Appl Environ Microbiol 64:2086–2093

Husain M, Entsch B, Ballou DP, Massey V, Chapman PJ (1980) Fluoride elimination from substrates in hydroxylation reaction catalyzed by *p*-hydroxybenzoate hydroxylase. J Biol Chem 255:4189–4197

Ide A, Niki Y, Sakamoto F, Watanabe I, Wantanabe H (1972) Decomposition of pentachlorophenol in paddy soil. Agric Biol Chem 36:1937–1944

Imai R, Nagata Y, Fukuda M, Takagi M, Yano K (1991) Molecular cloning of a *Pseudomonas paucimobilis* gene encoding a 17-kilodalton polypeptide that eliminates HCl molecules from gamma-hexachlorocyclohexane. J Bacteriol 173:6811–6819

Inouye S (1994) NAD(P)H-flavin oxidoreductase from the bioluminescent bacterium, *Vibrio fischeri* ATCCis a flavoprotein. FEBS Lett 347:163–168

Kaiser J (2000) Just how bad is dioxin? Science 288:1941–1944

Karns JS, Duttagupta S, Chakrabarty AM (1983) Regulation of 2,4,5-trichlorophenoxyacetic acid and chlorophenol metabolism in *Pseudomonas cepacia* AC1100. Appl Environ Microbiol 46:1182–1186

Kaschabek SR, Kuhn B, Muller D, Schmidt E, Reineke W (2002) Degradation of aromatics and chloroaromatics by *Pseudomonas* sp. strain B13: purification and characterization of 3-oxoadipate:succinyl-coenzyme A (CoA) transferase and 3-oxoadipyl-CoA thiolase. J Bacteriol 184:207–215

Kellogg ST, Chatterjee DK, Chakrabarty AM (1981) Plasmid-assisted molecular breeding: new technique for enhanced biodegradation of persistent toxic chemicals. Science 214:1133–1135

Kilbane JJ, Chatterjee DK, Karns JS, Kellogg ST, Chakrabarty AM (1982) Biodegradation of 2,4,5-trichlorophenoxyacetic acid by a pure culture of *Pseudomonas cepacia*. Appl Environ Microbiol 44:72–78

Kirchner U, Westphal AH, Muller R, van Berkel WJ (2003) Phenol hydroxylase from *Bacillus thermoglucosidasius* A7, a two-protein component monooxygenase with a dual role for FAD. J Biol Chem 278:47545–47553

Kiyohara H, Hatta T, Ogawa Y, Kakuda T, Yokoyama H, Takizawa N (1992) Isolation of *Pseudomonas pickettii* strains that degrade 2,4,6-trichlorophenol and their dechlorination of chlorophenols. Appl Environ Microbiol 58:1276–1283

Lamar RT, Dietrich DM (1990) In situ depletion of pentachlorophenol from contaminated soil by *Phanerochaete* spp. Appl Environ Microbiol 56:3093–3100

Lamar RT, Dietrich DM (1992) Use of lignin-degrading fungi in the disposal of pentachlorophenol-treated wood. J Ind Microbiol 9:181–191

Lamar RT, Evans JW (1993) Solid-phase treatment of a pentachlorophenol-contaminated soil using lignin-degradting fungi. Environ Sci Technol 27:2566–2571

Lange CC, Schneider BJ, Orser CS (1996) Verification of the role of PCP 4-monooxygenase in chlorine elimination from pentachlorophenol by *Flavobacterium* sp. strain ATCC 39723. Biochem Biophys Res Commun 219:146–149

Lei B, Liu M, Huang S, Tu SC (1994) *Vibrio harveyi* NADPH-flavin oxidoreductase: cloning, sequencing and overexpression of the gene and purification and characterization of the cloned enzyme. J Bacteriol 176:3552–3558

Li DY, Eberspacher J, Wagner B, Kuntzer J, Lingens F (1991) Degradation of 2,4,6-trichlorophenol by *Azotobacter* sp. strain GP1. Appl Environ Microbiol 57:1920–1928

Louie TM, Webster CM, Xun L (2002) Genetic and biochemical characterization of a novel 2,4,6-trichlorophenol degradation pathway in *Ralstonia eutropha* JMP134. J Bacteriol 184:3492–3500

Lü Z, Min H, Wu S, Ruan A (2003) Phylogenetic and degradation characterization of *Burkholderia cepacia* WZ1 degrading herbicide quinclorac. J Environ Sci Health B 38: 771–782

Lyr H (1963) Enzymatische detoxifikation chlorierter phenole. Phytopath Zeitschr 47:73–83

Mannisto MK, Tiirola MA, Salkinoja-Salonen MS, Kulomaa MS, Puhakka JA (1999) Diversity of chlorophenol-degrading bacteria isolated from contaminated boreal groundwater. Arch Microbiol 171:189–197

Marco A, Cuesta A, Pedrola L, Palau F, Marín I (2004) Evolutionary and structural analyses of GDAP1, involved in Charcot-Marie-Tooth disease, characterize a novel class of glutathione transferase-related genes. Mol Biol Evol 21:176–187

Matus V, Sanchez MA, Martinez M, Gonzalez B (2003) Efficient degradation of 2,4,6-Trichlorophenol requires a set of catabolic genes related to tcp genes from *Ralstonia eutropha* JMP134(pJP4). Appl Environ Microbiol 69:7108–7115

Merz V, Weith W (1872) On the characteristics of pentachlorophenol (in German). Ber Dtsch Chem Ges 5:458–463

Middaugh DP, Thomas RL, Lantz SE, Heard CS, Mueller JG (1994) Field-scale testing of a hyperfiltration unit for removal of creosote and pentachlorophenol from ground water: chemical and biological assessment. Arch Environ Contam Toxicol 26:309–319

Miethling R, Karlson U (1996) Accelerated mineralization of pentachlorophenol in soil upon inoculation with *Mycobacterium chlorophenolicum* PCP1 and *Sphingomonas chlorophenolica* RA2. Appl Environ Microbiol 62:4361–4366

Mikesell MD, Boyd SA (1986) Complete reductive dechlorination and mineralization of pentachlorophenol by anaerobic microorganisms. Appl Environ Microbiol 52:861–865

Nohynek LJ, Nurmiaho-Lassila EL, Suhonen EL, Busse HJ, Mohammadi M, Hantula J (1996) Description of chlorophenol-degrading *Pseudomonas* sp. strains KF1T, KF3, and NKF1 as a new species of the genus *Sphingomonas*, *Sphingomonas subarctica* sp. nov. Int J Syst Bacteriol 46:1042–1055

Ohtsubo Y, Miyauchi K, Kanda K, Hatta T, Kiyohara H, Senda T (1999) PcpA, which is involved in the degradation of pentachlorophenol in *Sphingomonas chlorophenolica* ATCC39723, is a novel type of ring-cleavage dioxygenase. FEBS Lett 459:395–598

Orser CS, Lange CC (1994) Molecular analysis of pentachlorophenol degradation. Biodegradation 5:277–288

Orser CS, Dutton J, Lange C, Jablonski P, Xun L, Hargis M (1993a) Characterization of a *Flavobacterium* glutathione S-transferase gene involved in reductive dechlorination. J Bacteriol 175:2640–2644

Orser CS, Lange CC, Xun L, Zahrt TC, Schneider BJ (1993b) Cloning, sequence analysis, and expression of the *Flavobacterium* pentachlorophenol-4-monooxygenase gene in *Escherichia coli*. J Bacteriol 175:411–416

Padilla L, Matus V, Zenteno P, Gonzalez B (2000) Degradation of 2,4,6-trichlorophenol via chlorohydroxyquinol in *Ralstonia eutropha* JMP134 and JMP222. J Basic Microbiol 40: 243–249

Proudfoot AT (2003) Pentachlorophenol poisoning. Toxicol Rev 22:3–11

Puhakka JA, Herwig RP, Koro PM, Wolfe GV, Ferguson JF (1995) Biodegradation of chlorophenols by mixed and pure cultures from a fluidized-bed reactor. Appl Microbiol Biotechnol 42:951–957

Raymond KN, Dertz EA, Kim SS (2003) Enterobactin: an archetype for microbial iron transport. Proc Natl Acad Sci USA 100:3584–3588

Reddy GV, Gold MH (2000) Degradation of pentachlorophenol by *Phanerochaete chrysosporium*: intermediates and reactions involved. Microbiology 146:405–413

Reddy GV, Gold MH (2001) Purification and characterization of glutathione conjugate reductase: a component of the tetrachlorohydroquinone reductive dehalogenase system from *Phanerochaete chrysosporium*. Arch Biochem Biophys 391:271–277

Reddy GV, Gelpke MD, Gold MH (1998) Degradation of 2,4,6-trichlorophenol by *Phanerochaete chrysosporium*: involvement of reductive dechlorination. J Bacteriol 180:5159–5164

Rice JF, Menn FM, Hay AG, Sanseverino J, Sayler GS (2005) Natural selection for 2,4,5-trichlorophenoxyacetic acid mineralizing bacteria in agent orange contaminated soil. Biodegradation 16:501–512

Saber DL, Crawford RL (1985) Isolation and characterization of *Flavobacterium* strains that degrade pentachlorophenol. Appl Environ Microbiol 50:1512–1518

Sánchez MA, González B (2007) Genetic characterization of 2, 4, 6-trichlorophenol degradation in *Cupriavidus necator* JMP134. Appl Environ Microbiol 73:2769–2776

Sangodkar UM, Chapman PJ, Chakrabarty AM (1988) Cloning, physical mapping and expression of chromosomal genes specifying degradation of the herbicide 2,4,5-T by *Pseudomonas cepacia* AC1100. Gene 71:267–277

Sharma A, Thakur IS, Dureja P (2009) Enrichment, isolation and characterization of pentachlorophenol degrading bacterium *Acinetobacter* sp. ISTPCP-3 from effluent discharge site. Biodegradation 5:643–650

Steiert JG, Crawford RL (1986) Catabolism of pentachlorophenol by a *Flavobacterium* sp. Biochem Biophys Res Commun 141:825–830

Steiert JG, Pignatello JJ, Crawford RL (1987) Degradation of chlorinated phenols by a pentachlorophenol-degrading bacterium. Appl Environ Microbiol 53:907–910

Stintzi A, Cornelis P, Hohnadel D, Meyer JM, Dean C, Poole K (1996) Novel pyoverdine biosynthesis gene(s) of *Pseudomonas aeruginosa* PAO. Microbiology 142:1181–1190

Suckling J, Mansson P-H, Mann J, Morgan J (1999) Corky wines linked to cellars in France. In: Wine Spectator. In February 28, 1999 Issue

Takeuchi M, Hamana K, Hiraishi A (2001) Proposal of the genus *Sphingomonas* sensu stricto and three new genera, *Sphingobium, Novosphingobium* and *Sphingopyxis*, on the basis of phylogenetic and chemotaxonomic analyses. Int J Syst Evol Microbiol 51:1405–1417

Takizawa N, Yokoyama H, Yanagihara K, Hatta T, Kiyohara H (1995) A locus of *Pseudomonas pickettii* DTP0602, *had*, that encodes 2,4,6-trichlorophenol-4-dechlorinase with hydroxylase activity, and hydroxylation of various chlorophenols by the enzyme. J Ferment Bioeng 80:318–326

Tiirola MA, Wang H, Paulin L, Kulomaa MS (2002) Evidence for natural horizontal transfer of the *pcpB* gene in the evolution of polychlorophenol-degrading sphingomonads. Appl Environ Microbiol 68:4495–4501

Tiirola MA, Busse HJ, Kämpfer P, Männistö MK (2005) *Novosphingobium lentum* sp nov., a psychrotolerant bacterium from a polychlorophenol bioremediation process. Int J Syst Evol Microbiol 55:583–588

Trantirek L, Hynkova K, Nagata Y, Murzin A, Ansorgová A, Sklenár V, Damborský J (2001) Reaction mechanism and stereochemistry of gamma-hexachlorocyclohexane dehydrochlorinase LinA. J Biol Chem 276:7734–7740

Uhl S, Schmid P, Schlatter C (1986) Pharmacokinetics of pentachlorophenol in man. Arch Toxicol 58:182–186

Uotila JS, Kitunen VH, Coote T, Saastamoinen T, Salkinoja-Salonen MS, Apajalahti JH (1995) Metabolism of halohydroquinones in *Rhodococcus chlorophenolicus* PCP-1. Biodegradation 6:119–126

Wackett LP, Kwart LD, Gibson DT (1988) Benzylic monooxygenation catalyzed by toluene dioxygenase from *Pseudomonas putida*. Biochemistry 27:1360–1367

Wang CC, Lee CM, Lu CJ, Chuang MS, Huang CZ (2000) Biodegradation of 2,4,6-trichlorophenol in the presence of primary substrate by immobilized pure culture bacteria. Chemosphere 41:1873–1879

Wang YJ, Lee CC, Chang WC, Liou HB, Ho YS (2001) Oxidative stress and liver toxicity in rats and human hepatoma cell line induced by pentachlorophenol and its major metabolite tetrachlorohydroquinone. Toxicol Lett 122:157–169

Warner JR, Lawson SL, Copley SD (2005) A mechanistic investigation of the thiol-disulfide exchange step in the reductive dehalogenation catalyzed by tetrachlorohydroquinone dehalogenase. Biochemistry 44:10360–10368

Whitbread AK, Masoumi A, Tetlow N, Schmuck E, Coggan M, Board PG (2005) Characterization of the omega class of glutathione transferases. Methods Enzymol 401:78–99

WHO (1986) Occupational exposures to chlorophenols. IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans vol 41. IARC, Lyon, pp 319–356

WHO (1987) Environmental Health Criteria 71, Pentachlorophenol. World Health Organization, Geneva

Wieser M, Wagner B, Eberspacher J, Lingens F (1997) Purification and characterization of 2,4,6-trichlorophenol-4-monooxygenase, a dehalogenating enzyme from *Azotobacter* sp. strain GP1. J Bacteriol 179:202–208

Xu L, Resing K, Lawson SL, Babbitt PC, Copley SD (1999) Evidence that pcpA encodes 2,6-dichlorohydroquinone dioxygenase, the ring cleavage enzyme required for pentachlorophenol degradation in *Sphingomonas chlorophenolica* strain ATCC 39723. Biochemistry 38:7659–7669

Xun L (1996) Purification and characterization of chlorophenol 4-monooxygenase from *Burkholderia cepacia* AC1100. J Bacteriol 178:2645–2649

Xun L, Orser CS (1991a) Purification, properties of pentachlorophenol hydroxylase, a flavoprotein from *Flavobacterium* sp. strain ATCC 39723. J Bacteriol 173:4447–4453

Xun L, Orser CS (1991b) Purification of a *Flavobacterium* pentachlorophenol-induced periplasmic protein (PcpA) and nucleotide sequence of the corresponding gene (pcpA). J Bacteriol 173:2920–2926

Xun L, Orser CS (1991c) Biodegradation of triiodophenol by cell-free extracts of a pentachlorophenol-degrading *Flavobacterium* sp. Biochem Biophys Res Commun 174:43–48

Xun L, Sandvik ER (2000) Characterization of 4-hydroxyphenylacetate 3-hydroxylase (HpaB) of *Escherichia coli* as a reduced flavin adenine dinucleotide-utilizing monooxygenase. Appl Environ Microbiol 66:481–486

Xun L, Wagon K (1995) Purification and properties of 2,4,5-trichlorophenoxyacetate oxygenase from *Pseudomonas cepacia* AC1100. Appl Environ Microbiol 61:3499–3502

Xun L, Webster CM (2004) A monooxygenase catalyzes sequential dechlorinations of 2,4,6-trichlorophenol by oxidative and hydrolytic reactions. J Biol Chem 279:6696–6700

Xun L, Topp E, Orser CS (1992a) Purification and characterization of a tetrachloro-*p*-hydroquinone reductive dehalogenase from a *Flavobacterium* sp. J Bacteriol 174:8003–8007

Xun L, Topp E, Orser CS (1992b) Glutathione is the reducing agent for the reductive dehalogenation of tetrachloro-p-hydroquinone by extracts from a *Flavobacterium* sp. Biochem Biophy Res Comm 182:361–366

Xun L, Topp E, Orser CS (1992c) Diverse substrate range of a *Flavobacterium* pentachlorophenol hydroxylase and reaction stoichiometries. J Bacteriol 174:2898–2902

Xun L, Topp E, Orser CS (1992d) Confirmation of oxidative dehalogenation of pentachlorophenol by a *Flavobacterium* pentochlorophenol hydroxylase. J Bacteriol 174:5745–5747

Xun L, Bohuslavek J, Cai M (1999) Characterization of 2,6-dichloro-p-hydroquinone 1,2-dioxygenase (PcpA) of *Sphingomonas chlorophenolica* ATCC 39723. Biochem Biophy Res Comm 266:322–325

Xun L, Belchik SM, Xun R, Huang Y, Zhou H, Sanchez E (2010) S-Glutathionyl-(chloro)hydroquinone reductases: a novel class of glutathione transferases. Biochem J 428:419–427

Zaborina O, Latus M, Eberspacher J, Golovleva LA, Lingens F (1995) Purification and characterization of 6-chlorohydroxyquinol 1,2-dioxygenase from *Streptomyces rochei* 303: comparison with an analogous enzyme from *Azotobacter* sp. strain GP1. J Bacteriol 177:229–234

Zaborina O, Daubaras DL, Zago A, Xun L, Saido K, Klem T (1998) Novel pathway for conversion of chlorohydroxyquinol to maleylacetate in *Burkholderia cepacia* AC1100. J Bacteriol 180:4667–4675

Chapter 2
Degradation of Chloro-organic Pollutants by White Rot Fungi

Ernest Marco-Urrea and C. A. Reddy

2.1 Introduction

Ligno-cellulosic plant biomass is the most abundant renewable organic resource on earth and contains cellulose, hemicellulose, and lignin polymers as its key components. Lignin, the most abundant aromatic polymer in the biosphere, is a highly complex, three dimensional, branched, recalcitrant polymer. Because of its recalcitrance, biodegradation of lignin is the rate-limiting step in the degradation of lignocellulosic biomass and thus lignin plays a pivotal role in global carbon cycling. Bacteria and most fungi are unable to mineralize lignin, but white rot fungi, a group of basidiomycetes that cause white rot decay of wood materials, are considered the most efficient organisms in mineralizing lignin in nature (Buswell and Odier 1987).

Three families of extracellular lignin modifying enzymes (LME) consisting of lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase are key components of the lignin-degrading enzyme system of white rot fungi. These enzymes are relatively non-specific and provide white rot fungi the unique ability to degrade a broad array of environmental pollutants such as dioxins, polychlorinated biphenyls (PCBs), petroleum hydrocarbons, munitions wastes (such as trinitrotoluene), industrial dye effluents, herbicides and pesticides (Aust 1990; Reddy 1995; Pointing 2001; Reddy and Mathew 2001). Of these, chloro-organic pollutants are some of the

E. Marco-Urrea
Department of Chemical Engineering and Institute of Environmental Science and Technology, Autonomous University of Barcelona, 08193 Barcelona, Spain

C. A. Reddy (✉)
Department of Microbiology and Molecular Genetics and The NSF Center for Microbial Ecology, Michigan State University, East Lansing, MI 48824-4320, USA
e-mail: reddy@msu.edu

S. N. Singh (ed.), *Microbial Degradation of Xenobiotics*, Environmental Science and Engineering, DOI: 10.1007/978-3-642-23789-8_2,

most common and most toxic pollutants encountered in the environment. Similar to ligninolysis, a feature of the secondary metabolism of white rot fungi, degradation of a number of environmental pollutants (but not all) by these organisms is triggered by limitation for nutrients, such as N and C and is also temporally correlated to lignin mineralization (Bumpus et al. 1985; Baldrian 2008). Moreover, many white rot fungi do not utilize environmental pollutants as sources of carbon and energy; instead, they utilize other available sources of energy in their environment, such as sugars and polysaccharides and gratuitously breakdown various pollutant chemicals, which are usually present in minute amounts (ppm or ppb) (Aust 1990; Reddy and Mathew 2001). Furthermore, although wood is their natural substrate, some white rot fungi have the ability to survive in soil and effectively compete with soil microflora (Baldrian 2008).

What are the characteristics of white rot fungi that make them attractive candidates for use in bioremediation applications? There are several reasons as mentioned by various workers (Aust 1990; Reddy 1995; Pointing 2001; Reddy and Mathew 2001; Baldrian 2008). 1. They are widely distributed in nature worldwide, particularly in forest soils where woody materials are undergoing decay, and thus are readily available for isolation and utilization in bioremediation studies. 2. White rot fungi are extremely versatile as they are able to degrade a long list of commonly used chloro-organic pollutants individually or in mixtures. 3. Constitutive nature of key biodegradation enzymes in white rot fungi eliminates or reduces the need for adaptation to the pollutant of interest. 4. White rot fungi can oxidatively breakdown pollutant substrates with low solubility because peroxidases and laccases, the key enzymes involved in lignin degradation as well as in pollutant degradation, are extracellular obviating the need for internalizing the substrates. 5. White rot fungi do not use organo-pollutants as sources of carbon and energy to any significant extent; therefore, relatively inexpensive lignocellulose sources, such as saw dust, peanut hulls, corn cobs, straw and other such materials can be provided for effective fungal colonization and biomass production at the contaminated sites. 6. White rot fungi (and filamentous fungi in general) grow by hyphal extension through the soil and have an advantage in gaining better access to some of the pollutant chemicals that accumulate in tiny pores in soil (Baldrian 2008; Pinedo-Rilla et al. 2009).

A number of reviews dealing with degradation of environmental pollutants by white rot fungi have been published (Bumpus et al. 1985; Reddy 1995; Raghukumar 2000; Pointing 2001; Reddy and Mathew 2001; Wesenberg et al. 2003; Chang 2008; Pinedo-Rilla et al. 2009; Majeau et al. 2010). In this review, we focused on degradation of selected classes of chloro-organic pollutants by white rot fungi with emphasis on work done in the last decade. Also, we focused on enzyme mechanisms and pathways including identification of metabolic intermediates involved in degradation of chloro-organics. *Phanerochaete chrysosporium* is the most intensively studied model white rot fungus in investigations on enzymology, molecular biology, and genetics of lignin degradation as well as on biodegradation of chloro-organics (Gold and Alic 1993; Reddy and D'Souza 1994; Hofrichter et al. 2010; Lundell et al. 2010) and hence received greater focus in this review. *P. chrysosporium* is also the

first white rot fungus for which whole genome sequence is available (Martinez et al. 2004; Vanden Wymelenberg et al. 2006, 2009).

2.2 Enzymology

2.2.1 Extracellular Peroxidases and Laccases

The unique ability of white rot fungi to degrade lignin is largely attributable to the non-specific free radical mediated oxidizing reactions carried out by their extracellular LMEs, peroxidases and laccases (Dosoretz and Reddy 2007; Wong 2009; Hofrichter et al. 2010; Lundell et al. 2010; Majeau et al. 2010). These enzymes are believed to have evolved to give white rot fungi (and a few other groups of fungi) the ability to breakdown lignin in plant biomass and obtain a better access to the cellulose and hemicellulose, which are not efficiently accessed by bacteria. LMEs cleave the C–C and C–O bonds of lignin regardless of the chiral conformations of the lignin molecule (Dosoretz and Reddy 2007). This manner of bond fission is partially contributed by the free radical mechanism employed by white rot fungi in lignin degradation (Reddy and D'Souza 1994; Dosoretz and Reddy 2007; Hofrichter et al. 2010). In addition, free radical species generated during the degradation process (of either lignin or organo-pollutants) may serve as secondary oxidants that may, in turn, mediate oxidation of other compounds away from the active sites of the enzymes.

Extracellular peroxidases of white rot fungi include lignin peroxidase (LiP; EC.1.11.1.14), manganese peroxidase (MnP; EC 1.11.1.13), and versatile peroxidase (VP; EC 1.11.1.16). These enzymes belong to Class II fungal heme peroxidases (Lundell et al. 2010). Both LiPs and MnPs belong to a family of multiple isozymes coded by multiple genes and are produced during idiophasic growth (Gold and Alic 1993; Reddy and D'Souza 1994; Lundell et al. 2010).

LiP, MnP, and VP have similar catalytic cycles based on two electron oxidation of the enzyme by using H_2O_2 as primary oxidant to yield Compound I (Fig. 2.1; Table 2.1). Two consecutive one electron reduction steps of Compound I via Compound II by electron donor substrates return the enzyme to the initial ferric oxidation state. The H_2O_2 required by ligninolytic peroxidases is mainly generated by direct reduction of O_2 to H_2O_2 catalyzed by extracellular enzymes of the fungi, such as glyoxal oxidase, pyranose oxidase and aryl alcohol oxidase (Dosoretz and Reddy 2007).

LiP (Class II heme peroxidases, group A.1) possesses a higher redox potential and lower pH optimum than most other isolated peroxidases and oxidases and is able to oxidize non-phenolic aromatic substrates, typically not oxidized by MnPs (Table 2.1). It attacks nonphenolic phenyl propanoid units of the lignin polymer and the stable cation-centered radicals formed during theses oxidations may serve as redox mediators for LiP-catalyzed reactions effectively extending the substrate

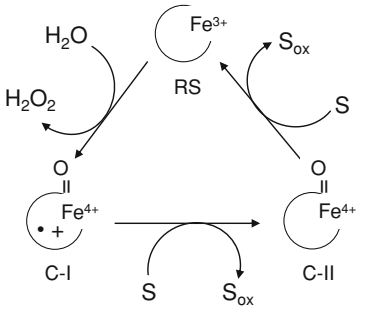


Fig. 2.1 Catalytic cycle of ligninolytic peroxidases (LiP, MnP, and VP). The enzyme resting state (RS, containing Fe^{3+}) undergoes oxidation by hydrogen peroxide to yield Compound I (C–I, containing Fe^{4+}-oxo and porphyrin cation radical). Reduction of C–I in two one electron steps results in the formation of Compound II (C-II, containing $Fe^{4+} = O$ after porphyrin reduction) which is then converted to RS. During the catalytic cycle, two substrate (S) molecules are oxidized which could be low redox potential phenols or Mn^{2+} in the cases of MnP and VP

range (Barr et al. 1992; Dosoretz and Reddy 2007; Hofrichter et al. 2010; Lundell et al. 2010). Similar to other peroxidases, LiP is also capable of oxidizing most phenolic compounds through the generation of phenoxy radicals. Besides the direct catalytic action of the enzyme, *P. chrysosoporium* produces veratryl alcohol (VA), that temporally parallels LiP production, and VA is suspected to have the role of a redox mediator in the oxidation of lignin. Veratryl alcohol is oxidized by LiP to veratryl alcohol radical cation intermediate and the latter can initiate free radical reactions oxidizing oxalate, a natural substrate present in wood, but also produced by white rot fungi, to carboxylate anion radical ($CO_2^{\bullet -}$). This anion radical possesses a reduction potential (−1.9 V vs. NHE) that was shown sufficient to reductively dechlorinate some chloro-organics (Shah et al. 1993; Khindaria et al. 1995). Reactions catalyzed by LiP include ring opening and side chain cleavage reactions, benzyl alcohol oxidations, oxidative dechlorination reactions, and methoxylations.

MnP, unlike LiP, belongs to Class II heme peroxidases, group B (Lundell et al. 2010). MnPs of different fungal species have strikingly similar sequence homology. MnP, unlike LiP, oxidizes Mn(II) via Compound I and Compound II to yield Mn(III) and it is the later that is responsible for the oxidation of organic compounds catalyzed by MnP (Fig. 2.1 and Table 2.1) (Dosoretz and Reddy 2007; Hofrichter et al. 2010). Mn(III) is a diffusible oxidant that is able to penetrate the small molecular pores between cellulose microfibrils, which preclude the action of LiP because of steric hindrances. Organic acids, such as oxalate, malate, and fumarate, which are produced in cultures of white rot fungi, chelate Mn(III) and these stable complexes are involved in oxidation of the substrate. Although MnP does not normally oxidize non-phenolic lignin substructures, the latter have been shown to be slowly co-oxidized when MnP peroxidatively oxidizes unsaturated

Table 2.1 Comparison of the main lignin modifying enzymes produced by white rot fungi

Enzyme	Production	Mechanism and specificity	Fungus producing	References[a]
Laccase	Extracellular, most basidiomycetes	One electron oxidation of organic substrates coupled to 4-electron reduction of molecular oxygen to water. Broad specificity. Oxidation of phenolic compounds. Oxidation of non-phenolic compounds in the presence of mediators	*T. versicolor, B. adusta, G. lucidum, C. maxima, P. ostreatus, T. pubescens*	Cañas and Camarero (2010), Thurston (1994), Hildén et al. (1994)
Lignin peroxidase	Extracellular, most basidiomycetes	Two electron oxidation of the enzyme by H_2O_2 to yield Compound I which undergoes two consecutive one electron reduction steps by oxidizing organic substrates. Broad specificity. Oxidation of phenolic and nonphenolic compounds	*P. chrysosporium, P. sordida, P. radiata, P. tremellosa, T. versicolor, B. adusta*	Hofrichter et al. (2010), ten Have et al. (1998), Sugiura et al. (2009)
Manganese peroxidase	Extracellular, most basidiomycetes	Two electron oxidation of the enzyme by H_2O_2 to yield Compound I which undergoes two consecutive one electron reduction steps by oxidizing Mn^{2+} into Mn^{3+} that in turn oxidizes phenolic compounds	*P. chrysosporium, P. sordida, C. subvermispora., P. radiata, D. squalens, P. rivulosus*	Gold et al. (2000), Hakala et al. (2006)
Versatile peroxidase	Extracellular, most basidiomycetes	VP oxidizes Mn^{2+} into Mn^{3+} as MnP does, and also high redox potential aromatic compounds, as LiP does; has broad specificity and oxidizes nonphenolic, phenolic and dye substrates	P. eryngii, P. ostreatus, B. adusta, Trametes spp.	Ruiz-Dueñas et al. (2009), Moreira et al. (2005)

[a] Includes mainly more recent reviews when available

fatty acids. MnP mediates oxidation of phenols, dyes, chlorophenols and other organopollutants (Pointing 2001; Reddy and Mathew 2001).

Versatile peroxidase (VP) belongs to fungal class II heme peroxidases, group A.3. It has been reported from genera *Pleurotus*, *Bjerkandera*, and *Trametes* and shares catalytic properties of both LiP and MnP (Dosoretz and Reddy 2007; Hofrichter et al. 2010). Similar to MnP, it exhibits high affinity for Mn(II) and catalyzes oxidation of Mn(II) to Mn(III), and also oxidizes both phenolic and non-phenolic substrates that is typical for LiP in the absence of Mn(II) (Table 2.1). VP oxidizes dimethoxybenzene, lignin dimers, phenols, amines, dyes, and aromatic alcohols (Dosoretz and Reddy 2007; Hofrichter et al. 2010). In the absence of Mn(II), VP oxidizes phenolic and non-phenolic substrates similar to LiP.

Another enzyme designated hybrid manganese peroxidase (hMnP) belongs to fungal class II heme peroxidases, group A.2. It has been isolated from *Phlebia radiata*, *Trametes versicolor* and other species and shares catalytic characteristics of VP and MnP (Hofrichter et al. 2010).

Laccases (EC1.10.3.2) are blue multicopper oxidases that catalyze the four-electron reduction of O_2 to water coupled with the oxidation of various organic substrates (Table 2.1). These are perhaps the most widely distributed oxidases in white rot fungi. These four-copper metalloenzymes catalyze O_2-dependent oxidation of a variety of phenolic compounds and do not require H_2O_2 or Mn(II) for activity. Similar to peroxidases, laccases catalyze subtraction of one electron from phenolic hydroxyl groups of phenolic compounds to form phenoxy radicals as intermediates. They also oxidize non-phenolic substrates in the presence of mediators which are oxidized to reactive radical or cation substrates by laccase and undergo further oxidation of non-phenolic targets (Dosoretz and Reddy 2007; Hofrichter et al. 2010; Rodgers et al. 2010).

With regard to the oxidation potential of LMEs, LiP possess the highest redox potential ($E'_0 \sim 1.2$ V, pH 3), followed by MnP ($E'_0 \sim 0.8$ V, pH 4.5) and laccase ($E'_0 \sim 0.79$ V, pH 5.5) enzymes (Wong 2009). A comparison of LiP, MnP and laccase in the oxidation of a homologous series of various methoxybenzenes (ranged from 0.81 to 1.76 V at pH 3) showed a correlation between the redox potential of the enzymes and the compound substrate (Kersten et al. 1990; Popp and Kirk 1991). However, it should be noted that the affinity of LMEs to degrade chloro-organic pollutants varies from one compound to the other and should be tested on a case by case basis.

Another extracellular fungal class II heme peroxidase (EC 1:11:1:7; CiP), originally described from *Coprinopsis cinerius*, has low redox potential and is unable to oxidize veratryl alcohol. It lacks the Mn(II) binding site of MnP and VP (Hofrichter et al. 2010). It is commercially available from Novozyme (Baylase®) and has been used to clean up phenolics from waste water streams. Yet another peroxidase, designated dye-decolorizing peroxidase (EC 1:1:1:x) from *Agaricus* type fungi, was reported to catalyze the oxidation of dyes and phenolic compounds (Hofrichter et al. 2010).

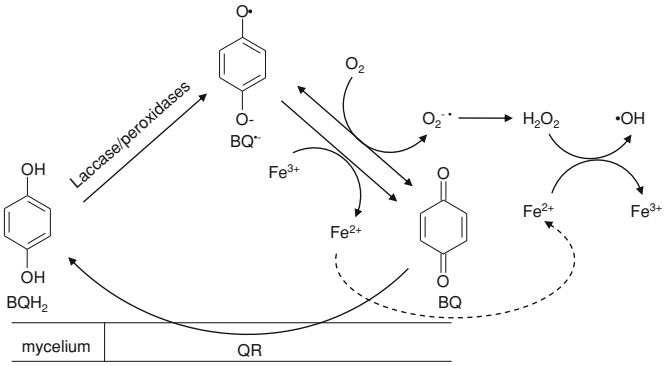


Fig. 2.2 Reaction scheme involved in the production of hydroxyl radical by white rot fungi via quinone redox cycling (Gomez-Toribio et al. 2009a, b). 1,4-benzoquinone (*BQ*) is reduced by quinone reductase (*QR*) producing hydroquinone (BQH_2), which is oxidized by any of the lignin modifying enzymes to semiquinones ($BQ^{\cdot-}$). The production of superoxide anion radicals ($O_2^{\cdot-}$) by $BQ^{\cdot-}$ autooxidation is mainly catalyzed by Fe^{3+}, that is reduced to Fe^{2+}. Fenton's reagent formation is accomplished by $O_2^{\cdot-}$ dismutation to H_2O_2

2.2.2 Induction of Hydroxyl Radicals

Hydroxyl radicals are highly reactive, non-specific, and potent oxidants described in cultures of white rot fungi during degradation of pollutants. Redox potential of hydroxyl radicals is estimated to be 2.8 V (Lawton and Robertson 1999) which allows degradation of some environmental pollutants that are not susceptible to peroxidases and laccases.

A simple strategy for the induction of extracellular hydroxyl radicals in white rot fungi, via Fenton's reaction employing the quinone redox cycling, has been described (Gomez-Toribio et al. 2009a, b). Quinone redox cycling involves cell-bound reduction of quinones to hydroquinones. Hydroquinones are known substrates of all LMEs, and undergo further extracellular oxidation to semiquinones. In the presence of O_2, semiquinones are autooxidized to regenerate the quinone, while O_2 is reduced to superoxide anion radical ($O_2^{\bullet-}$) (Guillen et al. 1997). In this strategy, the incubation of the mycelium with quinones and ferric ion (Fe^{3+}) generates the required Fenton's reagent (Fe^{2+} and H_2O_2) to produce hydroxyl radical via reduction of Fe^{3+} to Fe^{2+} by semiquinone radical and spontaneous dismutation of $O_2^{\bullet-}$ to H_2O_2 (Fig. 2.2). H_2O_2 and Fe^{2+} react to produce $^{\bullet}OH$ and Fe^{3+}. Involvement of hydroxyl radicals in the degradation of chloro-organics, petroleum hydrocarbons, pharmaceuticals, and dyes has recently been reported (Gomez-Toribio et al. 2009a; Marco-Urrea et al. 2009a, 2010; Aranda et al. 2010).

White rot fungi produce cellobiose dehydrogenase (CDH) when grown on cellulose as the carbon source. CDH catalyzes the two electron oxidation of cellobiose to cellobionolactone coupled to the reduction of quinones, phenolic intermediates, O_2 or Fe^{3+} and catalyzes the formation of Fenton's reagent (Kremer

and Wood 1992; Mason et al. 2003). It has been reported that hydroxyl radicals produced by CDH oxidize oxalate to carboxylate anion radical ($CO_2^{\bullet -}$), which was shown to dechlorinate chloro-organic compounds, similar to that described above for LiP, when incubated with veratryl alcohol and $CO_2^{\bullet -}$ (Cameron and Aust 1999). Also, LiP has been shown to act as an indirect source of hydroxyl radicals in a cascade of reactions mediated by veratryl alcohol, ferric ion and oxalate, although this mechanism is apparently not employed for degradation of chloroaromatic pollutants (Barr et al. 1992).

2.2.3 Cytochrome P450 System

Originally, the ability of ligninolytic (nitrogen-limited) cultures of *P. chrysosporium* to degrade pollutants was attributed to the action of LMEs, particularly to LiP and MnP. Nevertheless, several chloro-organic pollutants were found to be degraded and even mineralized by non-ligninolytic (nitrogen-rich) cultures of *P. chrysosporium* that were not expressing LiP and MnP (Yadav and Reddy 1992, 1993). This indicated that enzyme system(s), other than peroxidases were involved in the degradation of the pollutants studied by these investigators. Recent studies indicate that cytochrome P450 system is important in catalyzing the detoxification of several organic pollutants, including chloro-organics, by white rot fungi. The evidence for this is based on marked decrease in degradation of chloro-organics in the presence of cytochrome P450 inhibitors as well as the induction pattern of cytochrome P450-encoding genes in response to several of the chloro-organics (Doddapaneni and Yadav 2004). Furthermore, the whole genome sequence of *P. chrysosporium* revealed an estimated number of 148 P450 monooxygenase genes, the highest number known until that time among the fungal genomes (Martinez et al. 2004; Yadav et al. 2006). The main reactions catalyzed by these intracellular cytochrome P450 enzymes include epoxidation of C=C double bonds and hydroxylation of aromatic compounds. It has been suggested that cytochrome P450 enzyme system in nature plays an important role in the mineralization of lignin metabolites resulting from peroxidase-depolymerized lignin polymer (Subramanian and Yadav 2008).

2.2.4 Phase II Conjugation Reactions

Biotransformation of xenobiotics by higher animals is usually carried out in two steps known as phase I and phase II reactions. Phase I reactions are generally reactions which modify the chemical by adding a functional group. These reactions typically involve oxidation by the cytochrome P450 system leading to a new intermediate that contains a reactive chemical group (such as the addition of hydroxyl, carboxyl, or an amine group). Besides oxidations, reductions and hydrolyses are also common phase I reactions. Phase II reactions are conjugation

reactions, in which the phase I metabolite is conjugated to form sulfates, glucuronides, glucosides, or glutathione conjugates. In general, a conjugated metabolite is more water-soluble, less toxic, and more easily excreted from the body than the original xenobiotic or phase I metabolite. The whole genome sequence of the white rot fungus *P. chrysosporium* revealed a high diversity of glutathione *S*-transferases, a class of phase II detoxifying enzymes found mainly in cytosol. Some phase II enzymes have peroxidase activities with a probable role in protecting cells against H_2O_2–induced cell death. It has been hypothesized that phase II enzymes are related to a large number of cytochrome P450 sequences identified in the whole genome sequence of *P. chrysosporium* (Morel et al. 2009). However, little definitive data are available on the likely role of phase II enzymes of white rot fungi in detoxification processes. Some phase II enzymes, such as glucosidases and xylosidases of white rot fungi have been reported to catalyze the conversion of some chlorinated organic compounds to the corresponding conjugated derivatives, that showed markedly lower cytotoxicity than the parent compound (Reddy et al. 1997; Hundt et al. 2000).

2.3 Biodegradation of Chloro-organic Compounds by White Rot Fungi

2.3.1 Chlorinated Alkanes and Alkenes

Contamination of soils and aquifers by the aliphatic halocarbons trichloroethylene (TCE) and perchloroethylene (PCE), widely used as degreasing solvents, is a serious environmental pollution problem. TCE and PCE are among the most frequently detected chemicals at hazardous waste sites and are on the EPA's list of priority pollutants. Khindaria et al. (1995) reported that TCE is mineralized by *P. chrysosporium* cultures grown aerobically. These investigators proposed that TCE is subject to in vitro reductive dehalogenation catalyzed by LiP of *P. chrysosporium* in the presence of veratryl alcohol, H_2O_2, and EDTA (or oxalate) leading to the production of the corresponding reduced chlorinated radicals (Fig. 2.3a). Later research by Yadav et al. (2000) showed TCE mineralization by *P. chrysosporium* in malt extract medium and in a nitrogen-rich defined medium, in which the LiP and MnP production by the fungus is known to be suppressed, as well as in nitrogen-limited medium in which normal levels of Lip and MnP are known to be produced. Comparison of values for total degradation (46.2%) and mineralization (38.5%) using [^{14}C] TCE as the substrates showed that most of the TCE was transformed to $^{14}CO_2$. Therefore, contrary to the results of Khindaria et al. (1995), an alternate enzyme system that does not involve LiP or MnP appeared to be important in TCE degradation by *P. chrysosporium*. More recently, Marco-Urrea et al. (2008a) showed that three other white rot fungi including *T. versicolor*, *Irpex lacteus* and *Ganoderma lucidum* degraded TCE. Their results

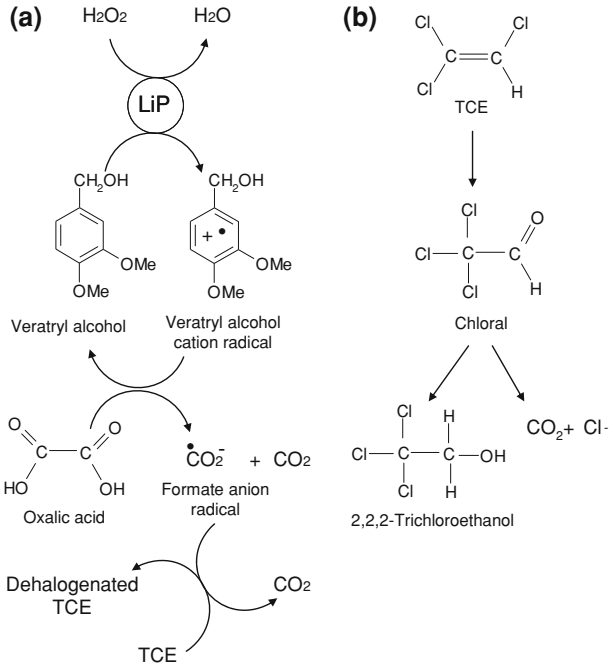


Fig. 2.3 Mechanisms of trichloroethylene (TCE) degradation by white rot fungi. **a** Proposed reductive dechlorination of TCE by LiP of *P. chrysosporium* using oxalate as the electron donor (Khindaria et al. 1995). **b** Oxidation of TCE catalyzed by cytochrome P450 system in *T. versicolor* (Marco-Urrea et al. 2008b)

further indicated that TCE degradation by *T. versicolor* involves cytochrome P450 system, as suggested by inhibition of TCE degradation in the presence of 1-aminobenzotriazole (a known inhibitor of cyt. P450). Also, *T. versicolor* was shown to mineralize 53% of the added [^{13}C] TCE, while the rest accumulated in the form of [^{13}C] 2,2,2-trichloroethanol, a common intermediate in [^{13}C]TCE degradation mediated by mammalian cytochrome P450 (Fig. 2.3b).

For many years, PCE was considered to be non-biodegradable under aerobic conditions, attributable to the high oxidation state of the molecule. However, Marco-Urrea et al. (2006) reported for the first time aerobic PCE degradation by *T. versicolor*. Their results indicated that PCE is first transformed to trichloroacetyl chloride, which was rapidly hydrolyzed in water (abiotically) to trichloroacetic acid. The PCE conversion to trichloroacetic acid appears to be catalyzed by cytochrome P450 system, as evidenced by marked inhibition of this conversion in the presence of cytochrome P450 inhibitor, 1-aminobenzotriazole.

Extracellular hydroxyl radicals produced by *T. versicolor*, via quinone redox cycling, were also shown to catalyze degradation of PCE and TCE (Marco-Urrea et al. 2009a). The advantage of using this strategy lies in the higher dechlorination

ratio obtained, in comparison to the cytochrome P450 mediated system. Using isotopic labeled [^{13}C] TCE, these investigators showed that mineralization of TCE by hydroxyl radicals produced by *T. versicolor* does not involve 2,2,2-trichloroethanol as a product. CDH produced by *P. chrysosporium* was also able to catalyze dechlorination of bromotrichloromethane to trichloromethyl radical after the production of carboxylate anion radical (Cameron and Aust 1999). Also, *P. chrysosporium* was shown to use the gaseous natural product chloromethane as methyl donor for veratryl alcohol biosynthesis (Harper et al. 1990).

2.3.2 Polychlorinated Biphenyls

Polychlorinated Biphenyls (PCBs) is a generic name for a family of compounds with multiple chlorines (usually 2–8) per biphenyl molecule. Because of their environmental persistence and toxicity, they have been banned now, but in the past, they had a wide range of industrial applications, such as heat transfer, dielectric, and hydraulic fluids, solvent extenders, flame retardants, and plasticizers. Commercial PCBs were manufactured as mixtures under the trade names Aroclor, Clophen, and Declor and consist of a mixture of congeners which differ in the number and positions of chlorines on the biphenyl nucleus (Table 2.2).

About 150 congeners of PCBs have been found in the environment. *P. chrysosporium* was the first white rot fungus that was shown to degrade a wide range of PCB congeners (Bumpus et al. 1985; Yadav et al. 1995a, b). In general, the extent of degradation/mineralization of PCBs decreases with increase in the number of chlorines on the biphenyl nucleus. For instance, negligible levels of mineralization for individual hexa- and tetrachlorobiphenyls were reported for several of the white rot fungi studied (Bumpus et al. 1985; Thomas et al. 1992; Vyas et al. 1994), but the level of degradation dramatically increased when PCBs with three or less chlorine substitutions were used (Dietrich et al. 1995; Beaudette et al. 1998). Mineralization of commercial PCB mixtures by *P. chrysosporium* also appears to follow the trend expected on the basis of PCB chlorination levels. Thus, mineralization of [^{14}C]Aroclor 1242 (42% chlorine by weight) was about 20%, while that of [^{14}C]Aroclor 1254 (54% chlorine by weight) ranged from 10 to 14% (Eaton 1985; Bumpus and Aust 1987; Yadav et al. 1995b). Furthermore, Yadav et al. (1995b) showed that PCB degradation decreased with an increase in chlorine content (Aroclor 1242>Aroclor 1254>Aroclor 1260). Also, commercial PCB mixtures with varying number of ortho, meta, and para substitutions on the biphenyl ring were degraded extensively (Yadav et al. 1995b). In more recent studies, Kamei et al. (2006b) reported undetectable levels of degradation when a chlorine was changed from an *ortho* position to *meta* position in 2,3′,4,4′,5,5′-hexachlorobiphenyl and 3,3,4,4,5,5′-hexachlorobiphenyl in cultures of *P. brevispora*. Other investigators showed lack of mineralization of 3,3′,4,4′-tetrachlorobiphenyl as compared to 10% mineralization observed with 2,2′,4,4′-tetrachloro biphenyl in *P. chrysosporium* cultures (Thomas et al. 1992; Dietrich et al. 1995).

Table 2.2 Degradation of individual chlorinated biphenyl (CBP) congeners by white rot fungi

Compound	Fungus/enzyme	Comments on mineralization and metabolic intermediates	Reference
2-CBP	*P. chrysosporium*	Mineralization of [^{14}C]2-CBP.	Thomas et al. (1992)
2,5-diCBP	*T. versicolor*	Identification of dichlorobenzenes, chlorophenols and alkylated benzenes as intermediates.	Koller et al. (2000)
4,4′-diCBP	*P. chrysosporium* *Phanerochaete* sp. *MZ142*	Identification of metabolites 2-OH- and 3-OH-diCBP in *Phanerochaete* sp. MZ142 and 3-OH- and 4-OH-diCBP in *P. chrysosporium*; and 3-methoxy-4,4′-diCBP, 4-CBZ, 4-CBZH, and 4-CBZOH in both.	Kamei et al. (2006a)
	P. chrysosporium	Mineralization of [^{14}C]4,4′-diCBP. Identification of CBZ and CBZOH as metabolites.	Dietrich et al. (1995)
2,4′,5-triCBP	*P. chrysosporium, B. adusta* *T. versicolor, P. ostreatus.*	Mineralization of [^{14}C]2,4′,5-triCBP: *T. versicolor* > *B. adusta* > *P. ostreatus* > *P. chrysosporium*. No correlation established between degradation, mineralization, and peroxidase production.	Beaudette et al. (1998)
	T. versicolor	Mineralization of [^{14}C]2,4,5-triCBP was stimulated by the surfactant Triton X-100.	Beaudette et al. (2000)
2,2′,4, 4′-tetraCBP	*P. chrysosporium*	Mineralization of [^{14}C]2,2′,4, 4′-tetraCBP.	Thomas et al. (1992)
3,3′,4, 4′-tetraCBP	*P. brevispora*	Identification of 5-methoxy-3,3′,4, 4′-tetraCBP as metabolite.	Kamei et al. (2006b)
	P. chrysosporium	Low mineralization of [^{14}C]3,3′,4, 4′-tetraCBP (0.8%).	Dietrich et al. (1995)
	P. chrysosporium	Low mineralization of [^{14}C]3,3′,4, 4′-tetraCBP (1.1%).	Bumpus et al. (1985)
	P. chrysosporium	Low mineralization of [^{14}C]3,3′,4, 4′-tetraCBP (1.4%).	Vyas et al. (1994)

(continued)

Table 2.2 (continued)

Compound	Fungus/enzyme	Comments on mineralization and metabolic intermediates	Reference
2,3′,4,4′,5-pentaCBP	*P. brevispora*	3-methoxy, 5′-methoxy-pentaCBP, 4-methoxy-2,3′,4′,5-tetraCBP, and as intermediates	Kamei et al. (2006b)
2,3,3′,4,4′-pentaCBP	*P. brevispora*	Three metabolites identified: 5-methoxy-, 5′-methoxy-pentaCBP and 4-methoxy-tetraCBP.	Kamei et al. (2006b)
3,3′,4,4′,5-pentaCBP	*P. brevispora*	Identification of 5′-methoxy-pentaCBP as metabolite.	Kamei et al. (2006b)
3,3′,4,4′,5,5′-hexaCBP	*P. brevispora*	Identification of 5′-methoxy-pentaCBP.	Kamei et al. (2006b)
2,2′,4,4′,5,5′-hexaCBP	*P. chrysosporium*	Low mineralization of [^{14}C]2,2′,4,4′,5,5′-hexaCBP (0.9%).	Bumpus et al. (1985)

CBP chlorinated biphenyl, *OH* hydroxyl group, *CBZ* chlorobenzoic acid, *CBZH* chlorobenzaldehyde, *CBZOH* chlorobenzyl alcohol

P. ostreatus strains showed selective PCB degradation with preference for congeners with chlorine atoms in *ortho* > *meta* > *para* position in the commercial mixture Declor 103 (Kubatova et al. 2001). On the contrary, *P. chrysosoporium* did not show any noticeable specificity for the position of chlorine substitutions in Aroclor 1242, 1242 and 1260 (Yadav et al. 1995b). However, additional studies are required to better understand the apparent inconsistencies in the observed results.

The effect of addition of surfactants was studied to see if the hydrophobic PCBs would become more bioavailable leading to increased rates of PCB degradation (Ruiz-Aguilar et al. 2002). Addition of Triton X-100 and Dowfax 8390 at low concentration did not affect levels of total biodegradation of PCB mixtures, but mineralization of 2,4′,5-trichlorobiphenyl increased by 12%, when Triton X-100 was used (Beaudette et al. 2000).

In further studies on the nature of degradation of PCBs by white rot fungi, it was reported that hexa-, penta- and tetrachloro-PCB congeners were methoxylated, leading to some dechlorination. Transformation products derived from ring fission are only reported for dichlorobiphenyls. *P. brevispora* transformed 3,3,4,4′-tetra-, 2,3,3′,4,4′-penta-, 2,3′,4,4′,5-penta-, 3,3′,4,4′,5-penta-chlorobiphenyl, and 2,3′,4,4′,5,5-hexachlorobiphenyl (HCB) to *meta*-monomethoxylated PCBs (Kamei et al. 2006b). Dechlorination was detected in the degradation of 2,3,3′,4,4′-penta-; 2,3′,4,4′,5-penta-; and 2,3′,4,4′,5,5′-hexachlorobiphenyls and occurred exclusively at the *para* position (Kamei et al. 2006b). Therefore, it appears that orthochlorines in monochlorobiphenyls are more likely to undergo dechlorination and that lack of chlorine in the meta position favors ring fission. Kamei et al. (2006a) demonstrated that 4,4′- dichlorobiphenyl (DCB) was hydroxylated by *Phanerochaete* sp. MZ142 and *P. chrysosporium* at different positions. In the case of strain MZ142, 4, 4′-DCB oxidation produced 2-hydroxyl-4,4′-DCB and 3-hydroxyl-4,4′-DCB. The metabolic pathway for 3-hydroxyl-4,4′-DCB was branched to produce the corresponding methoxylated product and to form 4-chlorobenzoic acid, 4-chlorobenzaldehyde, and 4-chlorobenzyl alcohol (Fig. 2.4). Also, 2-hydroxyl-4,4′-DCB was not methoxylated. These results are in agreement with the previous results reporting the formation of 4-chlorobenzoic acid and 4-chlorobenzyl alcohol from 4,4′-DCB by *P. chrysosporium* (Dietrich et al. 1995). Transformation of hydroxylated products produced from 4,4′-DCB into 4-chlorobenzoic acid, 4-chlorobenzaldehyde, and 4-chlorobenzyl alcohol by *Phanerochaete* cultures appears to be favored in low-nitrogen medium, but not in nitrogen-rich medium, in which the production of ligninolytic peroxidases is suppressed, indicating that ligninolytic enzymes could play a role in this conversion (Kamei et al. 2006a). It was hypothesized that a nitrate reductase enzyme was involved in dechlorination of hexachlorobiphenyl (HCB) by *P. chrysosporium*, but the specific enzyme involved in dechlorination of HCB remains yet to be identified (De et al. 2006).

The capacity of white rot fungi to degrade hydroxylated PCBs (OH-PCBs) was also studied, since these compounds enter the environment in high concentrations

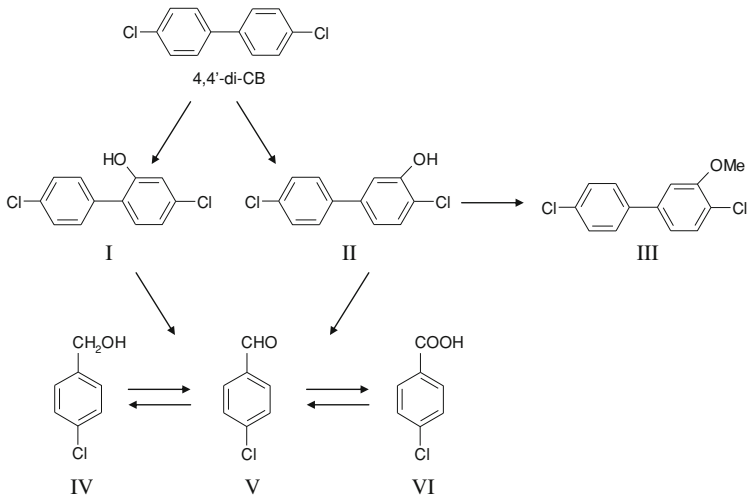


Fig. 2.4 Pathway for 4,4′-dichlorobiphenyl (4,4′-diCBP) degradation by *Phanerochaete* sp. MZ142 (Kamei et al. 2006a). Symbols: I: 2-hydroxy-4,4′-dichlorobiphenyl, II: 3-hydroxy-4,4′-dichlorobiphenyl, III: 3-methoxy-4,4′-dichlorobiphenyl, IV: 4-chlorobenzyl alcohol, V: 4-chlorobenzoaldehyde, VI: 4-chlorobenzoic acid

from PCB-contaminated sites. Unlike PCBs, hydroxylated PCBs are transformed by laccase. As a general rule, degradation rate constants of hydroxylated PCBs by purified laccases decreased with increase in the degree of chlorination, which is positively correlated with the ionization potential of the molecules (Keum and Li 2004; Fujihiro et al. 2009). Mono-, di-, and trichloro-OH-PCBs are readily oxidized by purified laccases from diverse white rot fungi, but higher chlorinated OH-PCBs could require the presence of laccase mediators to achieve this transformation (Keum and Li 2004). Transformation reactions of OH-PCBs by laccases include oxidative dechlorination of the molecule and/or dimer formation from the coupling of two OH-PCBs. Dimers are formed either by C–C or C–O–C bonds and the resulting oligomer is not necessarily dechlorinated (Schultz et al. 2001; Fujihiro et al. 2009; Kordon et al. 2010). For instance, 2-OH-5-monoCB is reported to be dechlorinated to 2-phenyl-p-benzoquinone and non-dechlorinated coupling products by purified laccases from *Pycnoporus cinnabarinus* and *Myceliophthora thermophila* (Kordon et al. 2010). In contrast, Schultz et al. (2001) carried out in vitro assays with laccase produced by *Pycnoporus cinnabarinus* and described the formation of dechlorinated dimer 5,5′-di-(2-hydroxybiphenyl) and two different non-dechlorinated dimers from 2-OH-5-CB. Laccase also oxidizes di- and trichloro-OH-PCBs to dechlorinated quinoid and hydroquinoid derivatives (Kordon et al. 2010), but oxidation of pentachlorinated-OH-PCBs produced two non-dechlorinated dimmers (Fujihiro et al. 2009).

2.3.3 Phenoxyalkanoic Herbicides

This group includes 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). 2,4-D is perhaps the most commonly used broad leaf herbicide around the world; 2,4,5-T is a component of Agent Orange that was widely used as a defoliant. 2,4-D is quite susceptible to bacterial degradation and generally does not persist for long in the environment except under adverse conditions, such as low soil pH and low temperature which increase its longevity in soil. 2,4,5-T is relatively more resistant to microbial degradation and tends to persist in the environment. It has been blamed for serious illnesses in many veterans of Vietnam war, where they got exposed to Agent Orange that was used as a defoliant. 2,4-D and 2,4,5-T were also reported to be mutagenic agents. Furthermore, during the manufacture of 2,4,5-T, it gets contaminated with low levels of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) which is very toxic to humans.

Ryan and Bumpus (1989) showed 2,4,5-T degradation by *P. chrysosporium* both in liquid culture as well as in soil. Extensive mineralization of ^{14}C-labeled 2,4-D and 2,4,5-T by *P. chrysosporium* has been demonstrated in liquid media (Pathway for 4,4′-dichlorobiphenyl Yadav and Reddy 1992, 1993). These investigators, by using a peroxidase-negative mutant of *P. chrysosporium*, demonstrated that LiP and MnP of this organism are not involved in the degradation of 2,4-D and 2,4,5-T by *P. chrysosporium* and further observed faster 2,4-D and 2,4,5-T mineralization when the wild type was grown in nutrient rich (non-ligninolytic) media. Yadav and Reddy (1993) also observed that ring-labeled 2,4-D is mineralized faster in nutrient rich (non-ligninolytic) media and that 2,4,5-T and 2,4-D were simultaneously mineralized at a higher rate when presented as a mixture. Subsequently, Reddy et al. (1997) reconfirmed that ligninolytic peroxidases were not involved in the initial cleavage reaction of 2,4-D and 2,4,5-T nor in the subsequent transfomation of the side chain of 2,4,5-T and 2,4-D. However, they showed that ligninolytic peroxidases of *P. chrysosporium* and *Dichomitus squalens* were involved in the degradation of chlorinated phenolic intermediates of 2,4-D and 2,4,5-T. These results were based on the increased degradation of ring-labeled and side chain-labeled 2,4,5-T and 2,4-D by *D. squalens* on addition of Mn^{2+} (a known inducer of MnP) to the medium and on increased degradation by *P. chrysosporium* in nitrogen-limited medium (in which production of both LiP and MnP is induced). Degradation of 2,4-D by *D. squalens* appears to involve an initial ether cleavage resulting in the formation of 2,4-dichlorophenol and acetate, under conditions of nitrogen depletion when peroxidase system is induced. The chlorophenol intermediate underwent subsequent oxidative dechlorination to a benzoquinone intermediate, followed by mineralization to CO_2 (Fig. 2.5) (Reddy et al. 1997). In vitro assays with purified laccase showed no activity when 2,4-D was used, but degraded up to 60% of 2,4-dichlorophenol (Sanino et al. 1999).

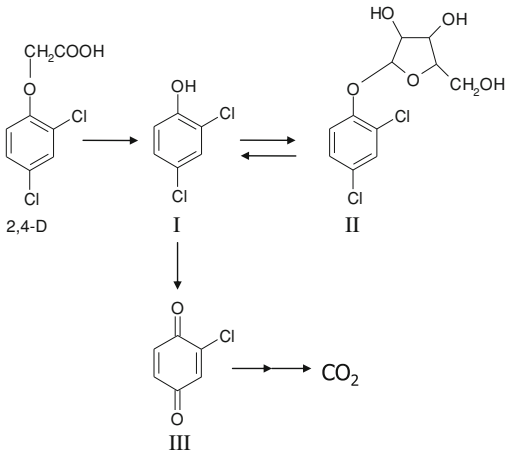


Fig. 2.5 Initial steps in the proposed pathway for degradation of 2,4-dichlorophenoxyacetic acid (2,4-D) by *Dichomitus squalens* (Reddy et al. 1997). Symbols: I: 2,4-dichlorophenol, II: 2, 4-dichlorophenol xyloside, III: 2-chloro-p-benzoquinone *Note*: 2,4,5-T apparently undergoes similar transformations during its degradation

2.3.4 Triazine Herbicides

Atrazine is a commonly used triazine herbicide and is degraded by a number of white rot fungi (Masaphy et al. 1993; Mougin et al. 1994; Entry et al. 1996; Bending et al. 2002; Nwachukwu and Osuji 2007). Degradation of atrazine by *Pleurotus pulmonarius* and *P. chrysosporium* leads to the accumulation of the N-dealkylated products deethylatrazine, deisopropylatrazine, deethyl-deisopropylatrazine and hydroxyisopropylatrazine (the latter produced only by *P. pulmonarius*). Atrazine oxidation by these two appears to involve cytochrome P450 system as evidenced by inhibition of oxidation upon addition of cytochrome P450 inhibitors piperonyl butoxide and 1-aminobenzotriazole (Masaphy et al. 1996a; Mougin et al. 1997b). Transformation of atrazine did not occur in vitro using purified LiP and MnP (Hickey et al. 1994; Mougin et al. 1997b). The ability of white rot fungi to degrade atrazine when added to pasteurized and unpasteurized lignocellulosic substrates, cotton + wheat straw (CWS) was evaluated (Hickey et al. 1994; Masaphy et al. 1996b; Bastos and Magan 2009). Masaphy et al. (1996b) showed loss of nearly 70% of the total radioactivity added as ^{14}C-ring-labeled atrazine two weeks after colonization with *Pleurotus* in pasteurized CWS, while only about 30% of atrazine loss was noted in non-inoculated CWS. No mineralization of the triazine ring was found after six weeks of incubation, but chlorinated and dechlorinated degradation products of atrazine were found. *T. versicolor* was able to grow and actively degrade atrazine in non-sterile soil with low organic matter and low water availability conditions (-0.7–2.8MPa) that limited the metabolic activity of autochthonous microbial community (Bastos and Magan 2009). These results suggested that *T. versicolor* is potentially useful for bioremediation of semi-arid soils contaminated with triazine herbicides.

P. chrysosporium was also shown to effect *N*-dealkylation of triazine herbicides simazine, propazine and terbuthylazine (Mougin et al. 1997b). *T. versicolor* and

P. chrysosporium degraded about 80% of simazine in a soil extract broth regardless of osmotic potential used and the range of concentrations tested (0–30 mg l^{-1}) (Fragoeiro and Magan 2005). The inoculation of the above-mentioned fungi on wood chips in soil microcosms containing simazine enhanced the degradation of the herbicide and increased the extracellular enzymes in soil (Fragoeiro and Magan 2008).

Using radiolabelled atrazine, Donnelly et al. (1993) showed that ericoid mycorrhizal fungus, *Hymenoscyphus ericae* 1318, degraded atrazine and relatively high levels of atrazine carbon was incorporated into its tissue. In general, as the nitrogen concentration increased, the extent of atrazine degradation increased.

2.3.5 Chlorinated Dioxins

Polychlorinated dibenzodioxins (PCDDs) are a group of highly toxic environmental pollutants that are confirmed human carcinogens and tend to bioaccumulate in humans and animals due to their lipophilic properties. Polychlorinated dibenzo-*p*-dioxins (PCDD) and polychlorinated dibenzofurans (PCDF) have been shown to be degraded by several species of white rot fungi (Table 2.3). 2,7-dichlorodibenzo-*p*-dioxin (2,7-diCDD) was used as the model in most of these studies. *P. chrysosporium* degraded 50% of the added 2,7-diCDD under ligninolytic conditions while only 10% was degraded under nonligninolytic conditions, suggesting the possible involvement of LiP and MnP (Valli et al. 1992). The pathway for 2,7-diCDD degradation involved oxidative cleavage of 2,7-diCDD by LiP resulting in the production of 4-chloro-1,2-benzoquinone and 2-hydroxy-1,4-benzoquinone (Fig. 2.6), followed by cycles of oxidation involving LiP and/or MnP leading to production of hydroquinones or catechols and subsequent methylation reactions generating methoxybenzenes (Fig. 2.6). It is of interest that the white rot fungus *Panellus stypticus*, which (unlike *P. chrysosporium*) does not produce either LiP or MnP, metabolizes 2,7-diCDD and produces 4-chlorocatechol as an intermediate suggesting that its degradation system for 2,7-diCDD is different from that of *P. chrysosporium* (Sato et al. 2002). Inhibition of 2,7-diCDD degradation upon addition of the cyt. P450 inhibitor, piperonyl butoxide, suggested the possible involvement of cyt. P450 enzyme in dioxin degradation by *P. stypticus*. Mori and Kondo (2002a) reported that *Phlebia lindtneri*, *Phlebia* sp. MG-60 and an unidentified white rot fungus mineralized [^{14}C]-2,7-diCDD to a maximum extent of 6.5%. Several of the *Phlebia* species produced one hydroxylated and two methoxylated intermediates as degradation products of 2,7-diCDD, which are different from those seen in the LiP-catalyzed reaction of *P. chrysosporium* (Mori and Kondo 2002a, b; Kamei and Kondo 2005).

Kamei et al. (2005) showed that *Phlebia* species are able to degrade higher chlorinated dioxins, such as 2,3,7-triCDD (18.4–27%), 1,2,8,9-tetraCDD (11.9–21.1%), and 1,2,6,7-tetraCDD (14.2–21.5%). Higher degradation rate of 1,2,6,7-tetraCDD compared to that of 2,3,7-triCDD by *P. lindtneri*, suggested that

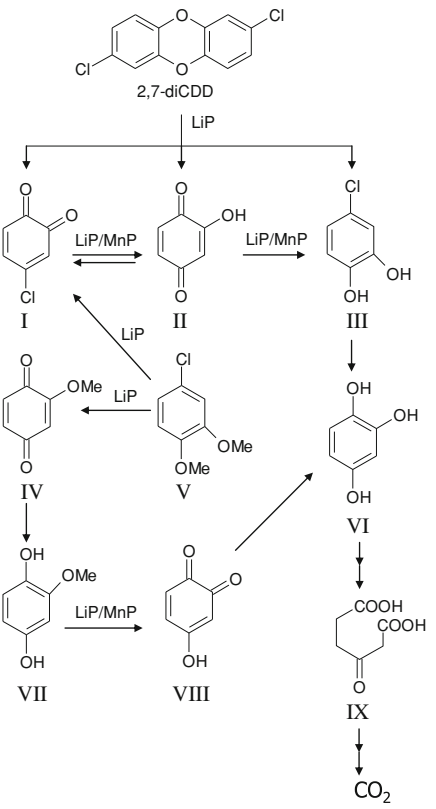


Fig. 2.6 Pathway for degradation of 2,7-dichlorodibenzo-p-dioxin (2,7-diCDD) by *P. chrysosporium* (Valli et al. 1992). Symbols: I: 4-chloro-1,2-benzoquinone, II: 2-hydroxy-1,4-benzoquinone, III: 4-chlorocatechol, IV: 2-methoxy-1,4-benzoquinone, V: 4-chloroveratrole, VI: 1,2,4-trihydroxybenzene, VII: 2-methoxyhydroquinone, VIII: 4-hydoxy-1,2-benzoquinone, IX: *β*-ketoadipic acid; LiP: lignin peroxidase, MnP: manganese peroxidase

chlorination pattern rather than the number of chlorine substitutions affected the rate of degradation of chlorinated dioxins by this fungus (Kamei and Kondo 2005). Degradation intermediates of 2,3,7-triCDD, 1,2,8,9-tetraCDD, and 1,2,6,7-tetraCDD by *P. lindtneri* included hydroxylated and methoxylated products in accordance with the pattern observed with 2,7-diCDD degradation by this organism (Fig. 2.7) (Kamei and Kondo 2005).

Phlebia brevispora, unlike several other *Phlebia* strains examined, was able to degrade 1,3,6,8-tetraCDD (28% in 28 d) and produce hydroxylated-tetraCDD, methoxy-tetraCDD, dimethoxy-tetraCDD, dimethoxy-triCDD and 3,5-dichlorocatechol as intemediates (Kamei et al. 2005). Hydroxylation of 1,3,6,8-tetraCDD was hypothesized to be the initial reaction catalyzed by cytochrome-P450 enzyme (Kamei et al. 2005). Two *Bjerkandera* strains (MS325 and MS1167), that produced

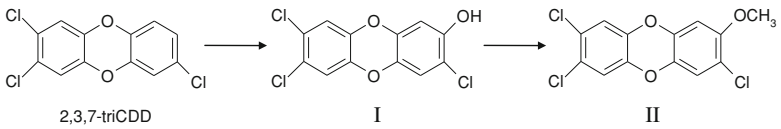


Fig. 2.7 Degradation pathway of 2,3,7-trichlorodibezo-*p*-dioxin (2,3,7-triCDD) by *Phlebia lindtneri* (Kamei and Kondo, 2005). Symbols: I: monohydroxy-triCDD, II: monomethoxy-triCDD

MnP and LiP, showed 16–21% degradation of 1,3,6,8-tetraCDD in 7 d(Manji and Ishihara 2004). An additional *Bjerkandera* strain, that did not produce LiP, was unable to degrade 1,3,6,8-tetraCDD, although this does not necessarily mean that LiP is indispensable for 1,3,6,8-tetraCDD degradation in all cases.

The degradation of 2,3,7,8-tetraCDD is of high environmental interest, because it is the most hazardous dioxin. *P. chrysosporium* mineralizes 2,3,7,8-tetraCDD (Bumpus et al. 1985). Takada et al. (1996) studied degradation of 10 kinds of 2,3,7,8-substituted tetra- to octaCDDs and tetra- to octaCDFs by *Phanerochaete sordida* YK-624 and also included *P. chrysosporium* for comparison. Degradation products of 2,3,7,8-tetraCDD and octa-CDD by *P. sordida* included 4,5-dichlorocatechol and tetrachlorocatechol, respectively. Formation of these products suggested the involvement of LiP-catalyzed reaction similar to that described for 2,7-diCDD (Valli et al. 1992). However, *P. sordida* produced MnP, but no LiP and crude MnP showed degradation of the dioxins, indicating that this fungus used an alternative enzymatic system different from LiP for this purpose.

There have been a few studies to date on PCDF degradation by white rot fungi. Degradation of 2,8-diCDF by *P. lindtneri* produced hydroxyl-diCDF as an intermediate, but the enzymes involved were not elucidated (Mori and Kondo 2002b). All the PCDD and PCDF congeners containing 2,3,7,8-substitutions were partially degraded by *P. sordida*, but the highest level of degradation was seen with 2,3,7,8-hexaCDD (Takada et al. 1996).

Studies on the application of white rot fungi for the cleanup of chlorinated dioxins-contaminated soils and fly ash have been sparse. Designed PCR primers for the specific amplification of *Ceriporia* sp. (MZ-340) DNA in soils have been successfully used to monitor this strain during bioremediation of contaminated fly ash *on site* (Suhara et al. 2003). The total concentration of chlorinated dioxins and furans in fly ash decreased $\geq$ 50% by *Ceriporia* sp. in 12 weeks when compared with the control (Suhara et al. 2003). Kamei et al. (2009) studied the influence of soil properties on the growth of *P. brevispora* and its dioxin degradation ability using 1,3,6,8-tetraCDD and 2,7-diCDD as models. Slurry-state condition was found to be more suitable for fungal soil treatment than the solid state condition. When the fungus was applied to a historically contaminated paddy soil, 1,3,6,8-tetraCDD was degraded approximately 50% over 90 d of incubation. Also, the use of organic-rich soil decreased the biodegradation activity of the fungus (Kamei et al. 2009) (Table 2.3).

Table 2.3 Relevant details on the degradation of various chlorinated dioxins by white rot fungi[a]

Compound	Fungus/enzyme	Comments	Reference
1-monoCDD	Cyt P450	Yeast clones expressing individual cyt P450s of the fungus show metabolism towards 1-monoCDD.	Kasai et al. (2010)
	Cyt P450	Yeast clones expressing individual cyt P450s of the fungus show metabolism towards 1-monoCDD.	Kasai et al. (2010)
2-monoCDD	LiP	LiP transformed 2-monoCDD to the corresponding cation radical as immediate product.	Hammel et al. (1986)
	P. chrysosporium	Identification of metabolites and elucidation of enzymatic mechanisms shown in Fig. 2.6.	Valli et al. (1992)
2,7-diCDD	*P. lindtneri*	Identification of one hydroxylated metabolite.	Mori and Kondo (2002b)
	P. lindtneri,Phlebia sp. MG-60; Unidentified -MZ-227	Mineralization of [^{14}C]2,7-diCDD. Nine other white rot fungi showed lower ability to mineralize.	Mori and Kondo (2002a)
	P. stypticus	Identification of metabolite III (Fig. 6); cyt P450 involvement in the initial oxidation of 2,7-diCDD.	Sato et al. (2002)
	P. lindtneri	Identification of hydroxylated, methoxylated and dimethoxylated metabolites.	Kamei and Kondo (2005)
	Phlebia spp.	Identification of hydroxylated and methoxylated metabolites.	Kamei et al. (2005)
2,3-diCDD	CytP450	Yeast clones expressing individual cyt P450s of the fungus show activity towards 2,3-diCDD.	Kasai et al. (2010)
2,3,7-triCDD	*P. lindtneri*	Identification of hydroxylated and methoxylated metabolites.	Kamei and Kondo (2005)
	Phlebia spp.	Degradation of substrate.	Kamei et al. (2005)
	P. chrysosporium	Low mineralization of [^{14}C]2,3,7,8-tetraCDD (2%).	Bumpus et al. (1985)
	P. sordida	Identification of 4,5-chlorocatechols as metabolite in *P. sordida*. This strain does not produce LiP.	Takada et al. (1996)

(continued)

Table 2.3 (continued)

Compound	Fungus/enzyme	Comments	Reference
2,3,7,8-tetraCDD	*P. chrysosporium*	Crude enzymes of MnP did not oxidize CDDs.	Takada et al. (1996)
	P. brevispora	Identification of monohydroxylated, monomethoxylated, dimethoxylated metabolites and 3,5-dichlorocatechol. Possible role of cyt P450	Kamei et al. (2005)
1,3,6,8-tetraCDD	*Bjerkandera* spp.	Degradation of substrate.	Manji and Ishihara (2004)
	P. brevispora	Examination of the bioremediation potential in different types of soils.	Kamei et al. (2009)
1,2,6,7-TetraCDD	*P. lindtneri*	Identification of two hydroxylated and one monomethoxylated metabolites.	Kamei and Kondo (2005)
1,2,3,7,8-pentaCDD	*Phlebia* spp.	Based on degradation.	Kamei et al. (2005)
1,2,3,4,7,8-hexaCDD	*P. sordida*	Based on degradation.	Takada et al. (1996)
1,2,3,4,6,7,8-heptaCDD	*P. chrysosporium*		
1,2,3,4,6,7,8,9-octaCDD	*P. sordida*	Identification of tetrachlorocatechol as metabolite in *P. sordida*. This strain does not produce LiP.	Takada et al. (1996)
	P. chrysosporium	Crude MnP from this organism did not oxidize CDDs.	Takada et al. (1996)

[a] *LiP* lignin peroxidase, *MnP* manganese peroxidase, *cytP450* cytochrome P450 monooxygenases. *CDD* chlorinated dibenzo-*p*-dioxins

2.3.6 Chlorobenzenes

Chlorobenzenes are used as high-boiling solvents in many industrial applications and as intermediates in the production of herbicides, pesticides, dyestuffs, and rubber. *P. chrysosporium* extensively degraded and mineralized monochlorobenzene as well as o-, m-, and *p*-dichlorobenzenes in malt extract cultures, in which LiP and MnP are not known to be produced (Yadav et al. 1995b). These results indicated that LiP and MnP were not involved in chlorobenzene degradation by *P. chrysosporium*. Additional evidence for the non-involvement of LiP and MnP was provided by using a *per* mutant that was unable to produce LiP and MnP, and the rates of degradation and mineralization of dichlorobenzene by the mutant were comparable to that of the wild type. Comparison of degradation levels with mineralization values indicated that most of the *p*-dichlorobenzene degraded was mineralized ($\sim$30%) whereas only a smaller portion of monochloro-benzene, *o*-, and *m*-dichlorobenzene was mineralized.

Degradation of 1,2,4-trichlorobenzene and 1,2,3-trichlorobenzene by *T. versicolor* in a defined medium was 91.1 and 79.6%, respectively, after 7 d of incubation (Marco-Urrea et al. 2009b). Addition of purified laccase and four different laccase mediators, such as ABTS (2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) did not lead to increased 1,2,4-trichlorobenzene oxidation. However, addition of cytochrome P450 inhibitors piperonyl butoxide and 1-aminobenzotriazole strongly inhibited both dechlorination and degradation, suggesting the involvement of cytochrome P450 in 1,2,4-trichlorobenzene degradation. These findings support previous observations of the non-involvement of LiP and MnP in monochlorobenzene and dichlorobenzene degradation by *P. chrysosporium* (Yadav et al. 1995b). A degradation pathway for 1,2,4-trichlorobenzene was proposed based on the identification of the intermediates, 5-trichloromuconate, its corresponding carboxymethylene-butenolide, 2- or 5-chloro-4-oxo-2-hexendioic acid and 2- or 5-chloro-5-hydroxy-4-oxo-2-pentenoic acid on the second day of incubation, but were not present after 7 d of incubation (Fig. 2.8). This evidence, together with the near-complete dechlorination of the degraded 1,2,4- and 1,2,3-trichlorobenzene as indicated by chloride release balance, suggested possible mineralization of 1,2,4-trichlorobenzene at the end of the incubation (Marco-Urrea et al. 2009b), but this remains to be confirmed. Under the test conditions, *T. versicolor* was not able to degrade 1,3,5-trichlorobenzene, an isomer that is known to show high resistance to aerobic biotransformation. However, when extracellular hydroxyl radical production was induced via quinone redox cycling in this fungus, over 25% degradation of 1,3,5- trichlorobenzene was observed and the ratio between 1,3,5-trichlorobenzene degradation and chloride release was 1:1.9 (Marco-Urrea et al. 2009a).

Different basidiomycetes were screened for their ability to degrade and mineralize highly chlorinated hexachlorobenzene in contaminated soil (Matheus et al. 2000). Nineteen basidiomycete strains were able to colonize contaminated soils containing 5000–50000 mg of hexachlorobenzene kg^{-1} soil, but only *Psilocybe*

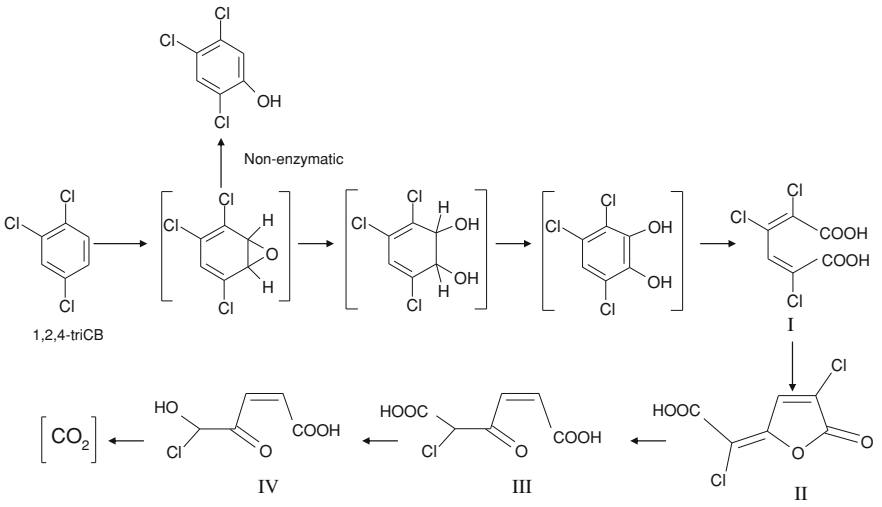


Fig. 2.8 Degradation pathway of 1,2,4-trichlorobenzene (triCB) by *T. versicolor* (Marco-Urrea et al. 2009b). Symbols: I: 2,3,5-trichloromuconate, II: (chloro-) carboxymethylenebutenolide, III: 2- or 5-chloro-4-oxo-2-hexendioic acid, IV: 2- or 5-chloro-5-hydroxy-4-oxo-2-pentenoic acid. Chemicals set into square brackets were not identified in that work

castanella showed a consistent decrease of hexachlorobenzene concentration. The amount of $^{14}CO_2$ from [^{14}C] hexachlorobenzene, however, showed very low levels of mineralization (slightly >1%) in soils inoculated with this fungus.

2.3.7 Chlorinated Insecticides

Lindane (γ isomer of hexachlorocyclohexane) was a widely used pesticide in the past and an estimated 600,000 tons of lindane were produced globally between 1950 and 2000. There is a global ban on the use of lindane now because of its environmental persistence as a pollutant. *P. chrysosporium*, cultured under ligninolytic conditions, was reported to partially mineralize lindane in liquid cultures and in a corncob-amended soils inoculated with *P. chrysosporium* (Bumpus et al. 1985; Kennedy et al. 1990), but lindane degradation was not observed in vitro using purified LiP and MnP from *P. chrysosporium* (Mougin et al. 1996). However, in the presence of the cytochrome P450 inhibitor, 1-aminobenzotriazole, lindane degradation was reduced drastically suggesting the involvement of P450 in lindane degradation by *P. chrysosporium.* When α-, β-, γ-, and δ-hexachlorocyclohexane were tested for their degradation by white rot fungi, γ- and δ-isomers were degraded between 15–71% by six strains, but were found inhibitory to the other white rot fungi tested (Quintero et al. 2008). In non-sterile

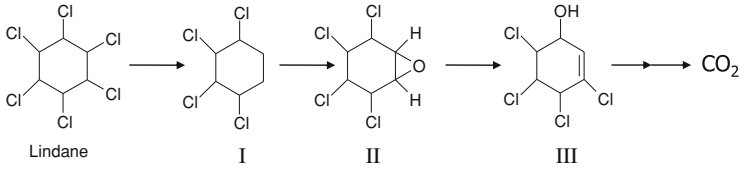


Fig. 2.9 Proposed degradation pathway for lindane by white rot fungi (Mougin et al. 1996; Singh and Kuhad 1999, 2000). Symbols: I. tetrachlorocyclohexane, II: tetrachlorocyclohexane epoxide, III: tetrachlorocyclo-hexenol

soil, all the hexachlorocyclohexane isomers were degraded between 8.2 and 17.5% by *B. adusta* immobilized on corncobs or woodchips. Mineralization of lindane by *P. chrysosporium*, *T. hirsutus* and *P. sordida*, occurred through the production of the intermediates tetrachlorocyclohexene, tetrachlorocyclo-hexene epoxide, and tetrachlorocyclohexenol, whereas only tetrachlorocyclohexenol was produced by *Cyatus bullery* (Fig. 2.9) (Mougin et al. 1996; Singh and Kuhad 1999, 2000). The effect of bioaugmentation on lindane transformation in sterile and non-sterile soils inoculated with *P. chrysosporium* has also been evaluated. In sterile soils, fungal biomass increased rapidly after inoculation with *P. chrysosporium* in the form of spores, but lindane mineralization was not seen (Mougin et al. 1997a). In contrast to this, fungus pre-grown on corncobs and added to sterile soils showed 22.8% mineralization after 60 d incubation (Kennedy et al. 1990). Conversely, either limited fungal growth or antagonist effect of autochthonous microflora on lindane degradation by *P. chrysosporium* and *B. adusta*, inoculated into non-sterile soils, was observed (Mougin et al. 1997a; Quintero et al. 2008).

DDT (1,1,1-trichloro-2,2-bis [4-chlorophenyl] ethane), the first of the chlorinated organic insecticides, was used quite heavily after World War II. High levels of DDT found in agricultural soils are of deep concern, because they present serious threats to food security and human health. The white rot fungi *P.chrysosporium*, *P. ostreatus*, *T. versicolor* and *Phellinus weirii* have been shown to mineralize DDT (Bumpus et al. 1985; Fernando et al. 1987; Purnomo et al. 2010). When glucose was added as the carbon source to nitrogen-deficient cultures of *P. chrysosporium*, 13.2% of DDT was mineralized in the first 21 d and ceased after this point. When either cellulose or starch was used as the growth substrate, substantial rates of mineralization were maintained through 90 d and over 30% mineralization of DDT was observed. *P. chrysosporium* transformed DDT to DDD during the first 3 d of incubation. Upon continued incubation, the amount of DDD decreased and oxidized degradation products 2,2,2-trichloro-l, l-bis(4-chlorophenyl) ethanol (dicofol), 2,2-dichloro-l,l-bis(4-chlorophenyl) ethanol (FW-52), and 4,4′dichlorobenzophenone (DBP) appeared and the fungus started to mineralize DDT. Mineralization of DDT by white rot fungi was reported to occur in nitrogen-deficient media, in which lignin-degrading enzymes such as LiP and MnP are produced, but not in nitrogen-rich media, in which the production of lignin-degrading enzymes is suppressed (Bumpus and Aust 1987). Later investigators,

however, disproved these conclusions by demonstrating DDT disappearance (not mineralization) under nitrogen-rich non-ligninolytic conditions and by demonstrating lack of transformation of DDT when exposed to a concentrated culture broth from ligninolytic cultures containing LiP (Köhler et al. 1988). Purnomo et al. (2008) proposed that cytochrome P450 is involved in DDT conversion to DDD by white rot fungi, but no experimental evidence was provided. On the basis of the reduction potential, dechlorination of dicofol to FW-52 was suggested to be produced via a free radical mechanism by LiP incubated together with oxalate and veratryl alcohol (Khindaria et al. 1995). More recently, a preliminary report appeared on DDT disappearance under different pH and oxygen concentrations in soils containing laccase, but further research is needed to demonstrate the involvement of laccase and other LME from white rot fungi in the mineralization of DDT (Zhao and Yi 2010).

It was of great interest that brown rot fungi *Gloeophyllum trabeum*, *Fomitopsis pinicola* and *Daedalea dickinsii* also showed a high ability to degrade DDT (Purnomo et al. 2008).

Aldrin, a heavily used organochlorine pesticide in the past, has been globally banned since the 1970s, because of its environmental persistence in agricultural soils and is a serious threat to human health and food security. Aldrin is converted by common soil microorganisms to dieldrin, which is the active form of the insecticide. *P. chrysosporium* was also able to carry out the epoxidation of aldrin to dieldrin together with the formation of an unidentified polar intermediate (Kennedy et al. 1990). *P. brevispora* transformed dieldrin to 9-hydroxydieldrin (Kamei et al. 2010); however, dieldrin mineralization by white rot fungi seems to be negligible (Kennedy et al. 1990).

2.3.8 Chlorinated Pharmaceutical and Personal Care Products

An expanding array of substances called 'Endocrine-disrupting compounds' (EDC) include chemicals of natural and anthropogenic origin. EDCs are widely distributed in the environment. They constitute a potential health risk to humans and aquatic life. Only a couple of EDCs are covered here. Triclosan is one such synthetic antimicrobial compound that is present in a wide range of health care products, such as tooth-paste, deodorant sticks and soaps. It has been detected in various environmental matrices due to its low degradation by conventional wastewater treatment processes. The fact, that triclosan shows remarkable toxicity towards bacteria, makes white rot fungi interesting candidates to test for biodegradation of this compound. Triclosan was degraded by seven strains of white rot fungi including *P. chrysosporium* ME 446, *P. magnoliae*, *T. versicolor, I. lacteus, P. ostreatus*, *P. cinnabarinus*, and *D. squalens*, while *B. adusta* was the only organism included in the screening that did not degrade triclosan (Cajthaml et al. 2009). *I. lacteus* and *P. ostreatus* were found to be the most efficient triclosan degraders with a degradation efficiency exceeding 90 and 80%, respectively.

In another study, triclosan was shown to inhibit the growth of *T. versicolor* over the first 3 d of incubation, but the ability of the fungus to transform triclosan to glucoside and xyloside conjugated forms and small amounts of 2,4-dichlorophenol lowered the toxicity due to triclosan and allowed the subsequent normal growth of the fungus (Hundt et al. 2000). The white rot fungus *P. cinnabarinus* also converted triclosan to the glucoside conjugate and additionally produced 2,4,4′-trichloro-2′-methoxydiphenyl (Hundt et al. 2000).

Several studies have focused on the applicability of purified laccases to oxidize and detoxify triclosan. The enzymatic transformation of triclosan by laccase was found to be optimal at pH 5 and 50°C and was negatively affected by the presence of sulfite, sulfide, cyanide, chloride, Fe(III) and Cu (II) ions that may be present in a wastewater matrix (Kim and Nicell 2006; Cabana et al. 2007). The use of polyethylene glycol (PEG) to prevent the inactivation of laccase resulted in an enhancement of triclosan degradation, but influenced negatively the toxicity of the treated solution (Kim and Nicell 2006). Immobilized laccase showed higher ability to degrade triclosan and had greater stability than free laccases (Cabana et al. 2009a, b). Degradation rates of triclosan by laccase were substantially improved through the use of laccase mediators (Murugesan et al. 2010). For example, ~90% triclosan degradation was observed in the presence of mediators, such as 1-hydroxybenzotriazole (HBT) and syringaldehyde (SYD). Other investigators did not see enhanced degradation of triclosan in the presence of mediators (Kim and Nicell 2006; Cabana et al. 2007). Two different mechanisms of triclosan degradation catalyzed by laccase were proposed. In the absence of redox mediators, laccase transformed triclosan to dimers, trimers and tetramers formed by oxidative coupling of the phenoxy radicals of triclosan (Cabana et al. 2007; Murugesan et al. 2010). Although the basic structure of triclosan was unaffected, laccase-mediated detoxification of triclosan was demonstrated using bacterial growth inhibition tests (Murugesan et al. 2010). In the presence of redox mediators HBT and SYD, the ether bond linkage of triclosan was cleaved producing 2,4-dichlorophenol, which was further dechlorinated through oligomerization reaction (in the case of HBT) or oxidative dechlorination to 2-chlorohydroquinone (in the case of SYD) (Murugesan et al. 2010). Recently, direct dechlorination of triclosan resulting in the formation of dechlorinated oligomers was shown in the presence of laccases conjugated with chitosan (Cabana et al. 2010). MnP seems to be a more promising choice than laccase for triclosan degradation, evidenced from the higher degradation rates obtained with MnP than laccase only or laccase + HOBT (Inoue et al. 2010). However, the transformation products were not described. Algal and bacterial growth inhibition tests showed that triclosan treated with MnP results in greatly reduced toxicity in the effluent.

Clofibric acid (CA) is the pharmacologically active derivative of clofibrate and several other fibrates that are used as blood lipid regulators in human medicine. Limited removal of CA, that was observed in municipal wastewater treatment plants, deserves more attention because of the risk of CA passing later barriers in partly closed water cycles. Of the four white rot fungi screened for their ability to degrade CA, only *T. versicolor* was able to degrade nearly 90% of CA (8 mg l^{-1})

in defined liquid medium after 7 d (Marco-Urrea et al. 2009c); but three other fungi, *P. chrysosporium*, *G. lucidum* and *I. lacteus*, were unable to degrade CA under similar conditions. CA was not oxidized by the purified MnP or laccase even in the presence of redox mediators. However, CA degradation by *T. versicolor* cultures was inhibited by more than half in the presence of cytochrome P450 inhibitors piperonyl butoxide or 1-aminobenzotriazole, suggesting that P450 plays a role in CA degradation by this organism (Marco-Urrea et al. 2009c). Degradation of CA in cultures of *T. versicolor* was also attempted by inducing hydroxyl radicals, through the quinone redox cycling mechanism (Marco-Urrea et al. 2010). Based on time-course experiments, using 10 mg l^{-1} of CA, these cultures showed >80% degradations after 6 h of incubation. An intermediate showing hydroxylation of the benzene ring of CA was detected early in the incubation, but it was not detectable in the cultures after 24 h (Marco-Urrea et al. 2010).

2.4 Conclusions

White rot fungi have become increasingly attractive as candidates for designing effective bioremediation strategies, because of the broad substrate specificity of the ligninolytic enzymes which enable these fungi to degrade or mineralize quite a broad spectrum of chloro-organics (and other environmental pollutants). Even though a large majority of the bioremediation studies to date were done with *P. chrysosporium* as the model, a greater number of other genera of white rot fungi have also been studied in recent years and this may help determine the right organism with the right characteristics for carrying out a desired bioremediation application. Basic studies designed to obtain a better understanding of the mechanisms of actions as well as the basic protein and gene structures of the major extracellular ligninolytic enzymes (LiP, MnP, VP, and laccase), that catalyze degradation of chloro-organics through free radical mediated reactions, have been described. Recent studies indicate that intracellular enzymes as exemplified by cytochrome P450 monooxygenases are widespread in white rot fungi and appear to be key players involved in the degradation of a number of the chloro-organic pollutants.

Although most studies on lab or field scale bioremediation were done using bacteria, white rot fungi are beginning to gain prominence in this area, because they offer a number of advantages over bacteria for biodegradation of chloro-organic environmental pollutants. For example, reductive dechlorination of toxic PCE and TCE by bacteria usually leads to accumulation of toxic *cis*-dichloroethylene and vinyl chloride in the environment, whereas white rot fungi not only can degrade PCE and TCE to less toxic intermediates, but also have the ability to mineralize TCE. Also, compared to most degradative enzymes of bacteria, the ligninolytic enzymes of white rot fungi are non-specific and allow these organisms to degrade several classes of organo-pollutants individually or in mixtures.

Most published studies on biodegradation of chloro-organo-pollutants have been carried out in defined or complex laboratory media or in small samples of autoclaved soils and the effect of temperature, pH, moisture, nutrient (mainly C, N and S) and oxygen concentration, and small molecular weight growth/enzyme inducers on the quantitative and qualitative production of degradative enzymes, and biochemical pathways for the degradation of various pollutants have been studied. However, less number of pilot scale or field scale studies have been done to date. Because of the great versatility of white rot fungi in degrading a broad array of environmental pollutants, it is important to study bioremediation applications on a larger scale using large bioreactors and contaminated soils, sediments, and effluent streams, and other pollutant sites. Some of these studies have been reviewed by Baldrian (2008) and he described some of the limitations for the practical applicability of white rot fungi for field scale bioremediations. The specific physico-chemical properties of some chloro-organics can also restrict the application of white rot fungi. For instance, some chloro-organics, such as PCE and TCE are highly volatile, have low solubility, and are denser than water and therefore, they accumulate in the form of pools in groundwater when released into the environment. In these environments, anoxic conditions are predominant and the white rot fungi which are aerobic would require oxygen and this may add to the costs. Successful use of immobilized enzymes of white rot fungi, such as LiP, MnP, and laccases for bioremediation would obviate some of the difficulties associated with the use of whole mycelia cells. In turn, this points to the importance of detailed characterization of the ligninolytic enzymes/isoenzymes produced by different strains or species of white rot fungi that offer the most promise for different bioremediation applications, because fungal strains vary a great deal in their repertoire of enzymes/isoenzymes. Recently, application of laccases immobilized on solid supports has been explored for the treatment of contaminated effluents in adapted bioreactors. In general, immobilization of laccase on solid supports allows enhanced enzyme stability and continuous utilization of the biocatalyst for bioremediation applications. For successful bioremediation, white rot fungi should be able to survive and successfully compete with autochthonous soil flora for carbon, nitrogen, and other nutrient sources. Even though, woody materials are their natural substrates, recent studies indicate that several species of white rot fungi can survive in soil and their soil colonizing ability is comparable to those of soil-inhabiting basidiomycetes utilizing the available lignocellulosic and other substrates in soil. Also, some of the white rot fungi appear to compete well with the complex soil microbial flora.

White rot fungi are extraordinary in their versatility to degrade a large variety of complex and recalcitrant environmental pollutants that contaminate soil and groundwater ecosystems, constituting a potential danger to human and animal health. It is fairly well proven now that white rot (as well as some non-white rot fungi) have the ecological and biochemical capability to degrade important categories of toxic chloro-organic pollutants. There has been much progress in screening and identifying strains/species of white rot fungi isolated from different ecosystems that are increasingly more efficient than previously described

organisms in degrading selected chloro-organic pollutants generated by different industrial processes. There has been also much advancement in our understanding of the basic physiology, biochemistry, and molecular biology of enzymes and enzyme systems involved in the degradation of chloro-organic pollutants by white rot fungi. Current genomics and proteomics tools offer significant opportunities to determine the role and interactions of known genes and for identifying new genes involved in degradation of chloro-organics by white rot fungi. This information would also be of great interest to monitor and better understand the behaviour of white rot fungi in soils and to use them more efficiently as the agents of bioremediation in contaminated sites. More research is needed to obtain better understanding of the ecology, enzymology, genomics, and proteomics of some of the more important species of white rot fungi that appear more desirable from a bioremediation perspective. Despite of great promise of white rot fungi as bioremediation agents, a number of challenges remain to be surmounted in translating the basic knowledge on these organisms into cost-effective practical bioremediation applications.

Acknowledgments The authors wish to acknowledge past students, postdoctoral fellows, and visiting scientists in their labs who contributed to the research results presented in this chapter. C. A. Reddy is also grateful for the funding received from Department of Energy over the years that contributed in part to the research presented here.

References

Aranda E, Marco-Urrea E, Caminal G, Arias ME, Garcia-Romera I, Guillen F (2010) Advanced oxidation of benzene, toluene, ethylbenzene and xylene isomers (BTEX) by *Trametes versicolor*. J Hazard Mater 181:181–186

Aust SD (1990) Degradation of environmental pollutants by *Phanerochaete chrysosporium*. Microb Ecol 20:197–209

Baldrian P (2008) Wood-inhabiting ligninolytic basidiomycetes in soils: ecology and constraints for applicability in bioremediation. Fungal Ecol 1:4–12

Barr DP, Shah MM, Grover TA, Aust SD (1992) Production of hydroxyl radical by lignin peroxidase from *Phanerochaete chrysosporium*. Arch Biochem Biophys 298:480–485

Bastos AC, Magan N (2009) *Trametes versicolor*: potential for atrazine bioremediation in calcareous clay soil, under low water availability conditions. Int Biodeter Biodegrad 63:389–394

Beaudette LA, Davies S, Fedorak PM, Ward OP, Pickard MA (1998) Comparison of gas chromatography and mineralization experiments for measuring loss of selected polychlorinated biphenyl congeners in cultures of white rot fungi. Appl Environ Microbiol 64:2020–2025

Beaudette LA, Ward OP, Pickard MA, Fedorak PM (2000) Low surfactant concentration increases fungal mineralization of a polychlorinated biphenyl congener but has no effect on overall metabolism. Lett Appl Microbiol 30:155–160

Bending GD, Friloux M, Walker A (2002) Degradation of contrasting pesticides by white rot fungi and its relationship with ligninolytic potential. FEMS Microbiol Lett 212:59–63

Bumpus JA, Aust SD (1987) Biodegradation of DDT [1, 1, 1-trichloro-2, 2-bis(4-chlorophenyl)ethane] by the white rot fungus *Phanerochaete chrysosporium*. Appl Environ Microbiol 53:2001–2008

Bumpus JA, Tien M, Wright D, Aust SD (1985) Oxidation of persistent environmental pollutants by a white rot fungus. Science 228:1434–1436

Buswell JA, Odier E (1987) Lignin biodegradation. CRC Crit Rev Microbiol 15:141–168

Cabana H, Jiwan JL, Rozenberg R, Elisashvili V, Penninckx M, Agathos SN, Jones JP (2007) Elimination of endocrine disrupting chemicals nonylphenol and bisphenol A and personal care product ingredient triclosan using enzyme preparation from the white rot fungus *Coriolopsis polyzona*. Chemosphere 67:770–778

Cabana H, Alexandre C, Agathos SN, Jones JP (2009a) Immobilization of laccase from the white rot fungus *Coriolopsis polyzona* and use of the immobilized biocatalyst for the continuous elimination of endocrine disrupting chemicals. Biores Technol 100:3447–3458

Cabana H, Jones JP, Agathos SN (2009b) Utilization of cross-linked laccase aggregates in a perfusion basket reactor for the continuous elimination of endocrine-disrupting chemicals. Biotechnol Bioeng 102:1582–1592

Cabana H, Ahamed A, Leduc R (2010) Conjugation of laccase from the white rot fungus *Trametes versicolor* to chitosan and its utilization for the elimination of triclosan. Biores Technol. doi:10.1016/j.biotech.2010.09.080

Cajthaml T, Kresinova Z, Svobodova K, Moder M (2009) Biodegradation of endocrine-disrupting compounds and suppression of estrogenic activity by ligninolytic fungi. Chemosphere 75:745–750

Cameron MD, Aust SD (1999) Degradation of chemicals by reactive radicals produced by cellobiose dehydrogenase from *Phanerochaete chrysosporium*. Arch Biochem Biophys 367:115–121

Cañas AI, Camarero S (2010) Laccases and their natural mediators: biotechnological tools for sustainable eco-friendly processes. Biotechnol Adv 28:694–705

Chang YS (2008) Recent developments in microbial biotransformation and biodegradation of dioxins. J Mol Microbiol Biotechnol 15:152–171

De S, Perkins M, Dutta SK (2006) Nitrate reductase gene involvement in hexachlorobiphenyl dechlorination by *Phanerochaete chrysosporium*. J Hazard Mater 135:350–354

Dietrich D, Hickey WJ, Lamar R (1995) Degradation of 4,4′-dichlorobiphenyl, 3,3′,4,4′-tetrachlorobiphenyl, and 2,2′,4,4′,5,5′-hexachlorobiphenyl by the white rot fungus *Phanerochaete chrysosporium*. Appl Environ Microbiol 61:3904–3909

Doddapaneni H, Yadav JS (2004) Differential regulation and xenobiotic induction of tandem P450 monooxygenase genes pc-1 (CYP63A1) and pc-2 (CYP63A2) in the white rot fungus *Phanerochaete chrysosporium*. Appl Microbiol Biotechnol 65:559–565

Donnelly PK, Entry JA, Crawford DL (1993) Degradation of atrazine and 2, 4-dichlorophenoxyacetic acid by mycorrhizal fungi at three nitrogen concentrations *in vitro*. Appl Environ Microbiol 59:2642–2647

Dosoretz CG, Reddy CA (2007) Lignin and lignin-modifying enzymes. In: Reddy CA, Beveridge TJ, Breznak JA, Marzluf GA, Schmidt TM, Snyder LJ (eds) Methods for general and molecular microbiology, 3rd edn. American Society for Microbiology, Washington, pp 611–620

Eaton DC (1985) Mineralization of polychlorinated biphenyls by *Phanerochaete chrysosporium*: a ligninolytic fungus. Enzyme Microb Technol 7:194–196

Entry JA, Donnelly PK, Emmingham WH (1996) Mineralization of atrazine and 2, 4-D in soils inoculated with *Phanerochaete chrysosporium* and *Trappea darkeri*. Appl Soil Ecol 3:85–90

Fernando T, Aust SD, Bumpus JA (1987) Effects of culture parameters on DDT [1, 1, 1-trichloro-2, 2-bis(4-chlorophenyl)ethane] biodegradation by *Phanerochaete chrysosporium*. Chemosphere 19:1387–1398

Fragoeiro S, Magan N (2005) Enzymatic activity, osmotic stress and degradation of pesticide mixtures in soil extract liquid broth inoculated with *Phanerochaete chrysosporium* and *Trametes versicolor*. Environ Microb 7:348–355

Fragoeiro S, Magan N (2008) Impact of *Trametes versicolor* and *Phanerochaete chrysosporium* on differential breakdown of pesticide mixtures in soil microcosms at two water potentials and associated respiration and enzyme activity. Int Biodeter Biodegrad 62:376–383

Fujihiro S, Higuchi R, Hisamatsu S, Sonoki S (2009) Metabolism of hydroxylated PCB congeners by cloned laccase isoforms. Appl Microbiol Biotechnol 82:853–860

Gold MH, Alic M (1993) Molecular biology of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Micorbiol Rev 57:605–622

Gold MH, Youngs HL, Sollewijin Gelpke MD (2000) Manganese peroxidase. In: Sigel A, Sigel H (eds) Manganese and its role in biological processes. Marcel Dekker, New York, pp 559–580

Gomez-Toribio V, Garcia-Martin AB, Martinez MJ, Martinez AT, Guillen F (2009a) Enhancing the production of hydroxyl radicals by *Pleurotus eryngii* via quinone redox cycling for pollutant removal. Appl Environ Microbiol 75:3954–3962

Gomez-Toribio V, Garcia-Martin AB, Martinez MJ, Martinez AT, Guillen F (2009b) Induction of extracellular hydroxyl radical production by white rot fungi through quinone redox cycling. Appl Environ Microbiol 75:3944–3953

Guillen F, Martinez MJ, Munoz C, Martinez AT (1997) Quinone redox cycling in the ligninolytic fungus Pleurotus eryngii leading to extracellular production of superoxide anion radical. Arch Biochem Biophys 339:190–199

Hakala TK, Hildén K, Maijala P, Olsson C, Hatakka A (2006) Differential regulation of manganese peroxidases and characterization of two variable MnP encoding genes in the white-rot fungus *Physisporinus rivulosus*. Appl Microbiol Biotechnol 73:839–849

Hammel KE, Kalyanaraman B, Kirk TK (1986) Oxidation of polycyclic aromatic hydrocarbons and dibenzo[p]-dioxins by *Phanerochaete chrysosporium*. J Biol Chem 261:16948–16952

Harper DB, Buswell JA, Kennedy JT, Hamilton JT (1990) Chloromethane, methyl donor in veratryl alcohol biosynthesis in *Phanerochaete chrysosporium* and other lignin-degrading fungi. Appl Environ Microbiol 56:3450–3457

Hickey WJ, Fuster DJ, Lamar RT (1994) Transformation of atrazine in soil by *Phanerochaete chrysosporium*. Soil Biol Biochem 26:1665–1671

Hildén K, Hakala TK, Maijala P, Lundell TK, Hatakka A (2007) Novel thermotolerant laccases produced by the white-rot fungus *Physisporinus rivulosus*. Appl Microbiol Biotechnol 77:301–309

Hofrichter M, Ullrich R, Pecyna MJ, Liers C, Lundell T (2010) New and classic families of secreted fungal heme peroxidases. Appl Microbiol Biotechnol 87:871–897

Hundt K, Martin D, Hammer E, Jonas U, Kindermann MK, Schauer F (2000) Transformation of triclosan by *Trametes versicolor* and *Pycnoporus cinnabarinus*. Appl Environ Microbiol 66:4157–4160

Inoue Y, Hata T, Kawai S, Okamura H, Nishida T (2010) Elimination and detoxification of triclosan by manganese peroxidase from white rot fungus. J Hazard Mater 180:764–767

Kamei I, Kondo R (2005) Biotransformation of dichloro-, trichloro-, and tetrachlorodibenzo-p-dioxin by the white rot fungus *Phlebia lindtneri*. Appl Microbiol Biotechnol 68:560–566

Kamei I, Suhara H, Kondo R (2005) Phylogenetical approach to isolation of white rot fungi capable of degrading polychlorinated dibenzo-p-dioxin. Appl Microbiol Biotechnol 69:358–366

Kamei I, Kogura R, Kondo R (2006a) Metabolism of 4,4′-dichlorobiphenyl by white rot fungi *Phanerochaete chrysosporium* and *Phanerochaete* sp. MZ142. Appl Microbiol Biotechnol 72:566–575

Kamei I, Sonoki S, Haraguchi K, Kondo R (2006b) Fungal bioconversion of toxic polychlorinated biphenyls by white rot fungus, *Phlebia brevispora*. Appl Microbiol Biotechnol 73:932–940

Kamei I, Watanabe M, Harada K, Miyahara T, Suzuki S, Matsufuji Y, Kondo R (2009) Influence of soil properties on the biodegradation of 1, 3, 6, 8-tetrachlorodibenzo-p-dioxin and fungal treatment of contaminated paddy soil by white rot fungus *Phlebia brevispora*. Chemosphere 75:1294–1300

Kamei I, Takagi K, Kondo R (2010) Bioconversion of dieldrin by wood-rotting fungi and metabolite detection. Pest Manag Sci 66:888–891

Kasai N, Ikushiro S, Shinkyo R, Yasuda K, Hirosue S, Arisawa A, Ichinose H, Wariishi H, Sakaki T (2010) Metabolism of mono- and dichloro-dibenzo-p-dioxins by *Phanerochaete chrysosporium* cytochromes P450. Appl Microbiol Biotechnol 86:773–780

Kennedy DW, Aust SD, Bumpus JA (1990) Comparative biodegradation of alkyl halide insecticides by the white rot fungus, *Phanerochaete chrysosporium* (BKM-F-1767). Appl Environ Microbiol 56:2347–2353

Kersten PJ, Kalyanaraman B, Hammel KE, Reinhammar B, Kirk TK (1990) Comparison of lignin peroxidase, horseradish peroxidase and laccase in the oxidation of methoxybenzenes. Biochem J 268:475–480

Keum YS, Li QX (2004) Fungal laccase-catalyzed degradation of hydroxy polychlorinated biphenyls. Chemosphere 56:23–30

Khindaria A, Grover TA, Aust SD (1995) Reductive dehalogenation of aliphatic halocarbons by lignin peroxidase of *Phanerochaete chrysosporium*. Environ Sci Technol 29:719–725

Kim YJ, Nicell JA (2006) Laccase-catalysed oxidation of aqueous triclosan. J Chem Technol Biotechnol 81:1344–1352

Köhler A, Jäager A, Willershausen H, Graf H (1988) Extracellular ligninase of *Phanerochaete chrysosporium* has no role in the degradation of DDT. Appl Microbiol Biotechnol 29:618–620

Koller G, Moder M, Czihal K (2000) Peroxidative degradation of selected PCB: a mechanistic study. Chemosphere 41:1827–1834

Kordon K, Mikolasch A, Schauer F (2010) Oxidative dehalogenation of chlorinated hydroxybiphenyls by laccases of white rot fungi. Int Biodeter Biodegrad 64:203–209

Kremer SM, Wood PM (1992) Cellobiose oxidase from *Phanerochaete chrysosporium* as a source of Fenton's reagent. Biochem Soc Trans 20:110S

Kubatova A, Erbanova P, Eichlerova I, Homolka L, Nerud F, Sasek V (2001) PCB congener selective biodegradation by the white rot fungus *Pleurotus ostreatus* in contaminated soil. Chemosphere 43:207–215

Lawton LA, Robertson PKJ (1999) Physico-chemical treatment methods for the removal of microcystins (cyanobacterial hepatotoxins) from potable waters. Chem Soc Rev 28:217–224

Lundell TK, Makela MR, Hilden K (2010) Lignin-modifying enzymes in filamentous basidiomycetes–ecological, functional and phylogenetic review. J Basic Microbiol 50:5–20

Majeau JA, Brar SK, Tyagi RD (2010) Laccase for removal opf recalcitrant and emerging pollutants. Biores Technol 101:2331–2350

Manji S, Ishihara A (2004) Screening of tetrachlorodibenzo-p-dioxin-degrading fungi capable of producing extracellular peroxidases under various conditions. Appl Microbiol Biotechnol 63:438–444

Marco-Urrea E, Gabarrell X, Sarra M, Caminal G, Vicent T, Reddy CA (2006) Novel aerobic perchloroethylene degradation by the white rot fungus *Trametes versicolor*. Environ Sci Technol 40:7796–7802

Marco-Urrea E, Gabarrell X, Caminal G, Vicent T, Reddy CA (2008a) Aerobic degradation by white rot fungi of trichloroethylene (TCE) and mixtures of TCE and perchloroethylene (PCE). J Chem Technol Biotechnol 83:1190–1196

Marco-Urrea E, Parella T, Gabarrell X, Caminal G, Vicent T, Adinarayana Reddy C (2008b) Mechanistics of trichloroethylene mineralization by the white rot fungus *Trametes versicolor*. Chemosphere 70:404–410

Marco-Urrea E, Aranda E, Caminal G, Guillen F (2009a) Induction of hydroxyl radical production in *Trametes versicolor* to degrade recalcitrant chlorinated hydrocarbons. Biores Technol 100:5757–5762

Marco-Urrea E, Perez-Trujillo M, Caminal G, Vicent T (2009b) Dechlorination of 1, 2, 3- and 1, 2, 4-trichlorobenzene by the white rot fungus *Trametes versicolor*. J Hazard Mater 166:1141–1147

Marco-Urrea E, Perez-Trujillo M, Vicent T, Caminal G (2009c) Ability of white rot fungi to remove selected pharmaceuticals and identification of degradation products of ibuprofen by *Trametes versicolor*. Chemosphere 74:765–772

Marco-Urrea E, Radjenovic J, Caminal G, Petrovic M, Vicent T, Barcelo D (2010) Oxidation of atenolol, propranolol, carbamazepine and clofibric acid by a biological Fenton-like system mediated by the white rot fungus *Trametes versicolor*. Water Res 44:521–532

Martinez D, Larrondo LF, Putnam N, Gelpke MD, Huang K, Chapman J, Helfenbein KG, Ramaiya P, Detter JC, Larimer F, Coutinho PM, Henrissat B, Berka R, Cullen D, Rokhsar D (2004) Genome sequence of the lignocellulose degrading fungus *Phanerochaete chrysosporium* strain RP78. Nat Biotechnol 22:695–700

Masaphy S, Levanon D, Vaya J, Henis Y (1993) Isolation and characterization of a novel atrazine metabolite produced by the fungus *Pleurotus pulmonarius*, 2-chloro-4-ethylamino-6-(1-Hydroxyisopropyl)amino-1, 3, 5-triazine. Appl Environ Microbiol 59:4342–4346

Masaphy S, Henis Y, Levanon D (1996a) Manganese-enhanced biotransformation of atrazine by the white rot fungus *Pleurotus pulmonarius* and its correlation with oxidation activity. Appl Environ Microbiol 62:3587–3593

Masaphy S, Levanon D, Henis Y (1996b) Degradation of atrazine by the lignocellulolytic fungus *Pleurotus pulmonarius* during solid-state fermentation. Biores Technol 56:207–214

Mason MG, Nicholls P, Wilson MT (2003) Rotting by radicals–the role of cellobiose oxidoreductase? Biochem Soc Trans 31:1335–1336

Matheus DR, RV L, Machado KMG (2000) Biodegradation of hexachlorobenzene by basidiomycetes in soil contaminated with industrial residues. World J Microbiol Biotechnol 16:415–421

Moreira PR, Duez C, Dehareng D, Antunes A, Almeida-Vara E, Frère JM, Malcata FX, Duarte JC (2005) Molecular characterization of a versatile peroxidase from a *Bjerkandera* strain. J Biotechnol 118:339–352

Morel M, Ngadin AA, Droux M, Jacquot JP, Gelhaye E (2009) The fungal glutathione *S*-transferase system. Evidence of new classes in the wood-degrading basidiomycete *Phanerochaete chrysosporium*. Cell Mol Life Sci 66:3711–3725

Mori T, Kondo R (2002a) Degradation of 2, 7-dichlorodibenzo-p-dioxin by wood-rotting fungi, screened by dioxin degrading ability. FEMS Microbiol Lett 213:127–131

Mori T, Kondo R (2002b) Oxidation of dibenzo-p-dioxin, dibenzofuran, biphenyl, and diphenyl ether by the white rot fungus *Phlebia lindtneri*. Appl Microbiol Biotechnol 60:200–205

Mougin C, Laugero C, Asther M, Dubroca J, Frasse P (1994) Biotransformation of the herbicide atrazine by the white rot fungus *Phanerochaete chrysosporium*. Appl Environ Microbiol 60:705–708

Mougin C, Pericaud C, Malosse C, Laugero C, Asther C (1996) Biotransformation of the insecticide lindane by the white rot basidiomycete *Phanerochaete chrysosporium*. Pest Sci 47:51–59

Mougin C, Pericaud C, Dubroca J, Asther C (1997a) Enhanced mineralization of lindane in soils supplemented with the white rot basidiomycete *Phanerochaete chrysosporium*. Soil Biol Biochem 29:1321–1324

Mougin C, Pericaud C, Dubroca J, Asther M (1997b) Enhanced mineralization of lindane in soils supplemented with the white rot basidiomycete *Phanerochaete chrysosporium*. Soil Biol Biochem 29:9–10

Murugesan K, Chang YY, Kim YM, Jeon JR, Kim EJ, Chang YS (2010) Enhanced transformation of triclosan by laccase in the presence of redox mediators. Water Res 44:298–308

Nwachukwu EO, Osuji JO (2007) Bioremedial degradation of some herbicides by indigenous white rot fungus, *Lentinus subnudus*. J Plant Sci 2:619–624

Pinedo-Rilla C, Aleu J, Collado IG (2009) Pollutants biodegradation by fungi. Curr Org Chem 13:1194–1214

Pointing SB (2001) Feasibility of bioremediation by white rot fungi. Appl Microbiol Biotechnol 57:20–33

Popp JL, Kirk TK (1991) Oxidation of methoxybenzenes by manganese peroxidase and by Mn^{3+}. Arch Biochem Biophys 288:145–148

Purnomo AS, Kamei I, Kondo R (2008) Degradation of 1, 1, 1-trichloro-2, 2-bis (4-chlorophenyl) ethane (DDT) by brown-rot fungi. J Biosci Bioeng 105:614–621

Purnomo AS, Mori T, Kamei I, Nishii T, Kondo R (2010) Application of mushroom waste medium from *Pleurotus ostreatus* for bioremediation of DDT-contaminated soil. Int Biodeter Biodegrad 64:397–402

Quintero JC, Moreira MT, Feijoo G, Lema JM (2008) Screening of white rot fungal species for their capacity to degrade lindane and other isomers of hexachlorocyclohexane (HCH). Cienc Inv Agr 35:123–132

Raghukumar C (2000) Fungi from marine habitats: an application in bioremediation. Mycol Res 104:1222–1226

Reddy CA (1995) The potential for white rot fungi in the treatment of pollutants. Curr Opin Biotechnol 6:320–328

Reddy CA, D'Souza TM (1994) Physiology and Molecularbiology of the lignin peroxidases of *Phanerochaete chrysosporium*. FEMS Microbiol Rev 13:137–152

Reddy CA, Mathew Z (2001) Bioremediation potential of white rot fungi. In: Gadd GM (ed) Fungi in bioremediation. Cambridge University Press, London, pp 52–78

Reddy GVB, Joshi DK, Aust SD (1997) Degradation of chlorophenoxyacetic acids by the lignin-degrading fungus *Dichomitus squalens*. Microbiol 143:2353–2360

Rodgers CJ, Blanford CF, Giddens SR, Skamnioti P, Armstrong FA, Gurr SJ (2010) Designer laccases: a vogue for high-potential fungal enzymes? Trends Biotechnol 28:63–72

Ruiz-Aguilar GM, Fernández-Sánchez JM, Rodríguez-Vázquez R, Poggi-Varaldo H (2002) Degradation by white rot fungi of high concentrations of PCB extracted from a contaminated soil. Adv Environ Res 6:559–568

Ruiz-Dueñas FJ, Morales M, García E, Miki Y, Martínez MJ, Martínez AT (2009) Substrate oxidation sites in versatile peroxidase and other basidiomycete peroxidases. J Exp Bot 60:441–452

Ryan TP, Bumpus JA (1989) Biodegradation of 2, 4, 5-trichlorophenoxyacetic acid in liquid culture and in soil by the white rot fungus *Phanerochaete chrysosporium*. Appl Microbiol Biotechnol 31:302–307

Sanino F, Filazzola MT, Violante A, Gianfreda L (1999) Fate of herbicides influenced by biotic and abiotic interactions. Chemosphere 39:333–341

Sato A, Watanabe T, Watanabe Y, Harazono K, Fukatsu T (2002) Screening for basidiomycetous fungi capable of degrading 2, 7-dichlorodibenzo-p-dioxin. FEMS Microbiol Lett 213:213–217

Schultz A, Jonas U, Hammer E, Schauer F (2001) Dehalogenation of chlorinated hydroxybiphenyls by fungal laccase. Appl Environ Microbiol 67:4377–4381

Shah MM, Grover TA, Aust SD (1993) Reduction of CCl_4 to the trichloromethyl radical by lignin peroxidase H_2 from *Phanerochaete chrysosporium*. Biochem Biophys Res Commun 191:887–892

Singh BK, Kuhad RC (1999) Biodegradation of lindane (gamma-hexachlorocyclohexane) by the white rot fungus *Trametes hirsutus*. Lett Appl Microbiol 28:238–241

Singh BK, Kuhad RC (2000) Degradation of insecticide lindane (γ-HCH) by white rot fungi *Cyathus bulleri* and *Phanerochaete sordida*. Pest Manag Sci 56:142–146

Subramanian V, Yadav JS (2008) Regulation and heterologous expression of P450 enzyme system components of the white rot fungus *Phanerochaete chrysosporium*. Enzyme Microb Technol 43:205–213

Sugiura T, Yamagishi K, Kimura T, Nishida T, Kawagishi H, Hirai H (2009) Cloning and homologous expression of novel lignin peroxidase genes in the white-rot fungus *Phanerochaete sordida* YK-624. Biosci Biotechnol Biochem 73:1793–1798

Suhara H, Daikoku C, Takata H, Suzuki S, Matsufuji Y, Sakai K, Kondo R (2003) Monitoring of white rot fungus during bioremediation of polychlorinated dioxin-contaminated fly ash. Appl Microbiol Biotechnol 62:601–607

Takada S, Nakamura M, Matsueda T, Kondo R, Sakai K (1996) Degradation of polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans by the white rot fungus *Phanerochaete sordida* YK-624. Appl Environ Microbiol 62:4323–4328

ten Have R, Hartmans S, Teunissen PJ, Field JA (1998) Purification and characterization of two lignin peroxidase isozymes produced by *Bjerkandera* sp. strain BOS55. FEBS Lett 422:391–394

Thomas DR, Carswell KS, Georgiou G (1992) Mineralization of biphenyl and PCBs by the white rot fungus *Phanerochaete chrysosporium*. Biotechnol Bioeng 40:1395–1402

Thurston CF (1994) The structure and function of fungal laccases. Microbiol 140:19–26

Valli K, Wariishi H, Gold MH (1992) Degradation of 2,7-dichlorodibenzo-p-dioxin by the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. J Bacteriol 174:2131–2137

Vanden Wymelenberg V, Minges P, Sabat G, Martinez D, Aerts A, Salamov A, Grigoriev I, Shapiro H, Putnam N, Belinky P, Dosoretz C, Gaskell J, Kersten P, Cullen D (2006) Computational analysis of the *Phanerochaete chrysosporium* v2.0 genome data base and mass spectrometrry identification of peptides in ligninolytic cultures reveal complex mixtures of secreted proteins. Fungal Genet Biol 43:342–356

Vanden Wymelenberg V, Gaskell J, Mozuch M, Kersten P, Sabat G, Martinez D, Cullen D (2009) Transcriptome and secretome analyses of *Phanerochaete chrysosporium* reveal complex patterns of gene expression. Appl Environ Microbiol 75:4058–4068

Vyas BRM, Šašek V, Matucha M, Bubner M (1994) Degradation of 3,3′,4,4′-tetrachlorobiphenyl by selected white rot fungi. Chemosphere 28:1127–1134

Wesenberg D, Kyriakides I, Agatho SN (2003) White rot fungi and their enzymes for the treatment of industrial dye effluents. Biotechnol Adv 22:161–187

Wong DW (2009) Structure and action mechanism of ligninolytic enzymes. Appl Biochem Biotechnol 157:174–209

Yadav JS, Reddy CA (1992) Non-involvement of lignin peroxidases and manganese peroxidases in 2, 4, 5-trichlorophenoxyacetic acid degradation by *Phanerochaete chrysosporium*. Biotechnol Lett 14:1089–1092

Yadav JS, Reddy CA (1993) Mineralization of 2, 4-dichlorophenoxyacetic acid (2, 4-D) and mixtures of 2, 4-D and 2, 4, 5-trichlorophenoxyacetic acid by *Phanerochaete chrysosporium*. Appl Environ Microbiol 59:2904–2908

Yadav JS, Quensen JF, Tiedje JM, Reddy CA (1995a) Degradation of polychlorinated biphenyl mixtures (Aroclors 1242, 1254, and 1260) by the white rot fungus *Phanerochaete chrysosporium* as evidenced by congener-specific analysis. Appl Environ Microbiol 61:2560–2565

Yadav JS, Wallace RE, Reddy CA (1995b) Mineralization of mono- and dichlorobenzenes and simultaneous degradation of chloro- and methyl-substituted benzenes by the white rot fungus *Phanerochaete chrysosporium*. Appl Environ Microbiol 61:677–680

Yadav JS, Bethea C, Reddy CA (2000) Mineralization of trichloroethylene (TCE) by the white rot fungus *Phanerochaete chrysosporium*. Bull Environ Contam Toxicol 65:28–34

Yadav JS, Doddapaneni H, Subramanian V (2006) P450ome of the white rot fungus *Phanerochaete chrysosporium*: structure, evolution and regulation of expression of genomic P450 clusters. Biochem Soc Trans 34:1165–1169

Zhao Y, Yi X (2010) Effects of soil oxygen conditions and soil pH on remediation of DDT-contaminated soil by laccase from white rot fungi. Int J Environ Res Public Health 7:1612–1621

Chapter 3
Microbial Styrene Degradation: From Basics to Biotechnology

Dirk Tischler and Stefan R. Kaschabek

3.1 Introduction

Styrene, the simplest representative of alkenylbenzenes, is one of the most important monomers produced by the chemical industry today. The compound shows a distinct toxicological behavior and is classified as a possible carcinogen due to its metabolism in human via a highly mutagenic epoxide. Considerable amounts of styrene are released by emissions and effluents during production and usage in polymer manufacture and by deposition of industrial wastes. A high chemical reactivity as well as its natural occurrence are reasons for the ubiquitous presence of styrene-catabolic activities among microorganisms. Rapid breakdown of styrene occurs in soils and aquifers under aerobic as well as under anaerobic conditions. As a consequence, styrene can be classified as readily biodegradable. Furthermore, a high volatility and susceptibility to photooxidation prevent bioaccumulation of styrene.

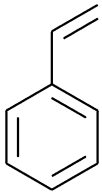
Since first studies in the late 1970s revealed insights about the microbial styrene metabolism in the presence of oxygen, several pathways of aerobic and anaerobic degradation have been reported in the recent years. In addition to basic principles of bacterial carbon metabolism, certain enzymes of aerobic degradation like styrene monooxygenases and styrene oxide hydrolases were found to be of high biotechnological relevance.

This review intends to give a current overview on basics and applied aspects of microbial styrene degradation comprising: (i) physico-chemical and toxicological properties of styrene, (ii) distribution as well as biochemical, genetic, and regulatory

D. Tischler (✉) · S. R. Kaschabek
Department of Biological Sciences, Environmental Microbiology,
TU Bergakademie Freiberg. Leipziger Str. 29,
09599 Freiberg, Germany
e-mail: Dirk-Tischler@email.de

S. N. Singh (ed.), *Microbial Degradation of Xenobiotics*,
Environmental Science and Engineering, DOI: 10.1007/978-3-642-23789-8_3,

Table 3.1 Physico-chemical properties of styrene (CAS no. 100-42-5)

Property	Value	Chemical structure
Molecular formula	C_8H_8	
Molecular weight	104.15 g mol^{-1}	
Boiling point	145.15°C	
Freezing point	−30.6°C	
Flash point (closed cup)	31°C	
Heat of polymerization	–69.8 kJ mol^{-1}	
Density	0.906 g ml^{-1}	
Vapor pressure (25°C)	0.67 kPa	
Solubility in water	300 mg l^{-1}	
Henry partition coefficient (air/water)	0.21	
Octanol/water partition coefficient (K_{ow})	3.02 (log value)	

aspects of identified pathways, and (iii) usage of styrene-degradative consortia and styrene-catabolic enzymes in waste gas treatment and the preparation of chiral building blocks, respectively.

3.2 Physico-Chemical Properties, Toxicological Aspects, Industrial Usage, and Environmental Fate of Styrene

The monoaromatic hydrocarbon styrene was first isolated from natural balsam storax in the 1830s and is also known under the following common synonyms: cinnamene, ethenylbenzene, phenylethene, phenylethylene, and vinylbenzene. Important physico-chemical properties of the flammable colorless oily liquid styrene are summarized in Table 3.1. Styrene monomer has a penetrating sweetish odor and is miscible with most organic solvents in any ratio, but is only slightly soluble in water.

The high reactivity of the exocyclic double bond brings about a distinct tendency of spontaneous polymerization which requires stabilization even at room temperature (e.g. by addition of 4-*tert*-butylcatechol) for long-term storage. This property favors styrene as a monomer and solvent in the industrial production of the polymer polystyrene (PS) and co-polymers like styrene-butadiene rubber (SBR), styrene acrylonitrile copolymer (SAN), and acrylonitrile butadiene styrene resins (ABS). Styrene is also used in combination with unsaturated polyester resins for fiber reinforced synthetics. In 2004, the global styrene demand was reported to be over 24,000 Kt (data calculated from 2006 World Styrene Analysis) (CMAI 2005) making this monomer to one of the most important organic chemicals worldwide.

Due to its widespread usage, styrene has been a subject of extensive toxicological investigation (Gibbs and Mulligan 1997; Rueff et al. 2009). The acute neurotoxic effects of high levels of styrene to human and mammals are related to

prenarcotic central nervous depression which resembles with action of many other solvents which are probably mediated by the direct effect of the lipophilic hydrocarbon on nerve cell membranes (Bond 1989). In contrast, chronic toxicity of styrene may result from the action of reactive metabolites, i.e. its highly reactive epoxide styrene oxide to components of the central nervous system (Mutti 1988; Mutti et al. 1988). This initial metabolite is formed by the action of hepatic microsomal cytochrome P450 monooxygenases and its further metabolism is mediated to a major extent by microsomal epoxide hydrolases (mEH or EH) and different dehydrogenases (Hartmans 1995). The urinary products mandelic acid and phenylglyoxylic acid both represent important biomarkers for styrene exposure (Guillemin and Berode 1988). The International Agency for Research on Cancer (IARC) has classified styrene as carcinogenic to humans (group 2B) and the potential of carcinogenicity largely depends on the ability of the reactive epoxide to covalently modify DNA.

As a consequence of substantial industrial application, the polymer-processing industry represents a major source of anthropogenic styrene release. Furthermore, styrene is released to a lower extent during its production, the disposal of polymers, and combustion processes, of which most important sources are automobile exhausts. In addition to its presence in cigarette smoke, trace levels of styrene are naturally found in certain plants and food (fruits, vegetables, nuts, meat) and are generated from a number of natural processes like the decarboxylation of cinnamic acid (plant decomposition) and the metabolism of microorganisms (Sielicki et al. 1978; Smith 1994; Warhurst and Fewson 1994; Lafeuille et al. 2009). The annual total release of styrene from all above mentioned processes was estimated for the European Union to exceed 23,500 tons and approximately 96% of this load is released directly into the air (European Union Risk Assessment Report Styrene 2002). As a consequence of its high volatility, the atmosphere is the main environmental compartment for the fate of styrene. Photooxidation of styrene by hydroxyl radicals and ozone are the main abiotic routes of degradation and depending on the conditions, half-lives between 3.5 and 9 h were estimated (Alexander 1990). A similar fate can be expected for a substantial amount of styrene dissolved in aqueous systems due to its volatility and its low octanol/water coefficient of 3.02 (log value) which considerably limits the risk of geo-accumulation. Fu and Alexander (1992) have investigated the fate of styrene in different environmental compartments. They determined that 50% styrene is being lost within 3 h in the shallow layers of lake water whereas only 26% gets evaporated within 31 days from 1.5 cm soil depth. In addition, it was shown that styrene, which remained trapped in soil, was a subject of microbial mineralization. Other studies report 87–95% degradation from different types of soil within 16 weeks (US Inventory of Toxic Compounds 2001).

The distribution of styrene metabolism among microorganisms as well as the genetic and biochemical principles will be reviewed in detail in the following headings.

Table 3.2 Distribution of styrene-degrading capabilities among different classes of pro- and eukaryotic microorganisms

Class	Genus	References
Bacteria		
Actinobacteria	*Corynebacterium*	Itoh et al. (1996)
	Gordonia	Alexandrino et al. (2001)
	Mycobacterium	Burback and Perry (1993)
	Nocardia	Hartmans et al. (1990)
	Rhodococcus	Hartmans et al. (1990), Jung and Park (2005), Patrauchan et al. (2008), Tischler et al. (2009), Warhurst et al. (1994), Zilli et al. (2003)
	Streptomyces	Przybulewska et al. (2006)
	Tsukamurella	Arnold et al. (1997)
Bacilli	*Bacillus*	Przybulewska et al. (2006)
Clostridia	*Clostridium*	Grbić-Galić et al. (1990)
α-Proteobacteria	*Sphingomonas*	Arnold et al. (1997)
	Xanthobacter	Hartmans et al. (1989, 1990)
γ-Proteobacteria	*Enterobacter*	Grbić-Galić et al. (1990)
	Pseudomonas	Alexandrino et al. (2001), Beltrametti et al. (1997), Ikura et al. (1997), Kim et al. (2005), Lin et al. (2010), Marconi et al. (1996), O'Connor et al. (1997), Panke et al. (1998), Park et al. (2006a), Rustemov et al. (1992), Velasco et al. (1998)
	Xanthomonas	Arnold et al. (1997)
Sphingobacteria	*Sphinogobacterium*	Przybulewska et al. (2006)
Fungi		
Agaricomycetes	*Bjerkandera*	Braun-Lüllemann et al. (1997)
	Phanerochaete	Braun-Lüllemann et al. (1997)
	Pleurotus	Braun-Lüllemann et al. (1997)
	Trametes	Braun-Lüllemann et al. (1997)
Eurotiomycetes	*Aspergillus*	Paca et al. (2001)
	Exophiala	Cox et al. (1996)
	Penicillium	Cox (1995), de Jong et al. (1990), Paca et al. (2001)
Sordariomycetes	*Gliocladium*	Cox (1995)
	Sporothrix	Cox (1995), René et al. (2010)

3.3 Degradation Pathways

The capability of styrene metabolism seems to be widespread among pro- and eukaryotic microorganisms. A large number of gram-negative and gram-positive bacteria as well as several fungi of the phyli basidiomycota and ascomycota were found to utilize styrene as (sole) source of carbon and energy (Table 3.2). Most frequently, degradation was shown to occur under oxic conditions and a lot of styrene-degrading isolates have initially been found to belong to the genus *Pseudomonas* (Warhurst and Fewson 1994; Mooney et al. 2006b;

O'Leary et al. 2002b). The predominance of styrene degradation among these proteobacterial r-strategists does not automatically reflect its true phylogenetic distribution and can probably be attributed to the rapid growth rate of pseudomonads during conventional enrichment techniques. In fact, more recently the identification of an increasing number of styrene-degrading Actinobacteria of the genera *Corynebacterium*, *Rhodococcus*, *Nocardia*, *Gordonia*, and related ones indicates that the capability of styrene catabolism is a common feature for that class of microorganisms, too.

In general, two major strategies of mineralization have been identified under oxic as well as anoxic conditions which differ with respect to type and location of initial attack. Epoxidation and hydratation of the vinyl side-chain seem to be favored mechanisms of initial attack during aerobic and anaerobic degradation, respectively, whereas dioxygenation and monohydroxylation of the aromatic nucleus are not frequently found under these conditions. There is certain evidence that the degradation pathway acting primarily on the aromatic nucleus has most likely its origin in the breakdown of other aromatic compounds like biphenyl or toluene (Warhurst et al. 1994; Cho et al. 2000; Patrauchan et al. 2008), whereas side-chain attack is more specific and restricted to styrene mineralization. Most microorganisms investigated for styrene degradation were shown to harbor only one single route for styrene breakdown.

Initial processes of aerobic and anaerobic styrene degradation lead to the formation of central intermediates, like 2-hydroxyphenylacetic acid, benzoic acid, 3-vinylcatechol, and 2-ethylhexanol. Since the subsequent conversion of these compounds into intermediates of the TCA cycle is mediated by the regular metabolism, lower degradation pathways are not discussed here.

It should be noted that in addition to the parent compound styrene, a few bacteria are also able to degrade substituted derivatives (Omori et al. 1974; Bestetti et al. 1989) or oligomers (Tuschii et al. 1977; Higashimura et al. 1983). The conversion of substituted derivatives is basically achieved by enzymes of the regular styrene catabolism, while most likely other enzymes are responsible for mineralization of the unsaturated styrene dimer by *Alcaligenes* sp. 559 (Tuschii et al. 1977).

3.3.1 Aerobic Styrene Degradation

Under aerobic conditions, side-chain oxygenation of styrene seems to be the favored mechanism, since most microorganisms investigated in that respect were found to follow this degradation pathway (Hartmans et al. 1990; Cox et al. 1996; Itoh et al. 1996; Beltrametti et al. 1997; Panke et al. 1998; Velasco et al. 1998; Park et al. 2006b). In the first reaction step, styrene is oxygenated into styrene oxide by the action of a monooxygenase (Fig. 3.1). Styrene monooxygenases (SMOs) of bacteria are flavin-dependent, whereas this reaction is typically catalyzed by heme-containing cytochrome P450 monooxygenases in fungi (Cox et al. 1996). Bacterial styrene monooxygenases were shown to be highly enantioselective leading in almost all

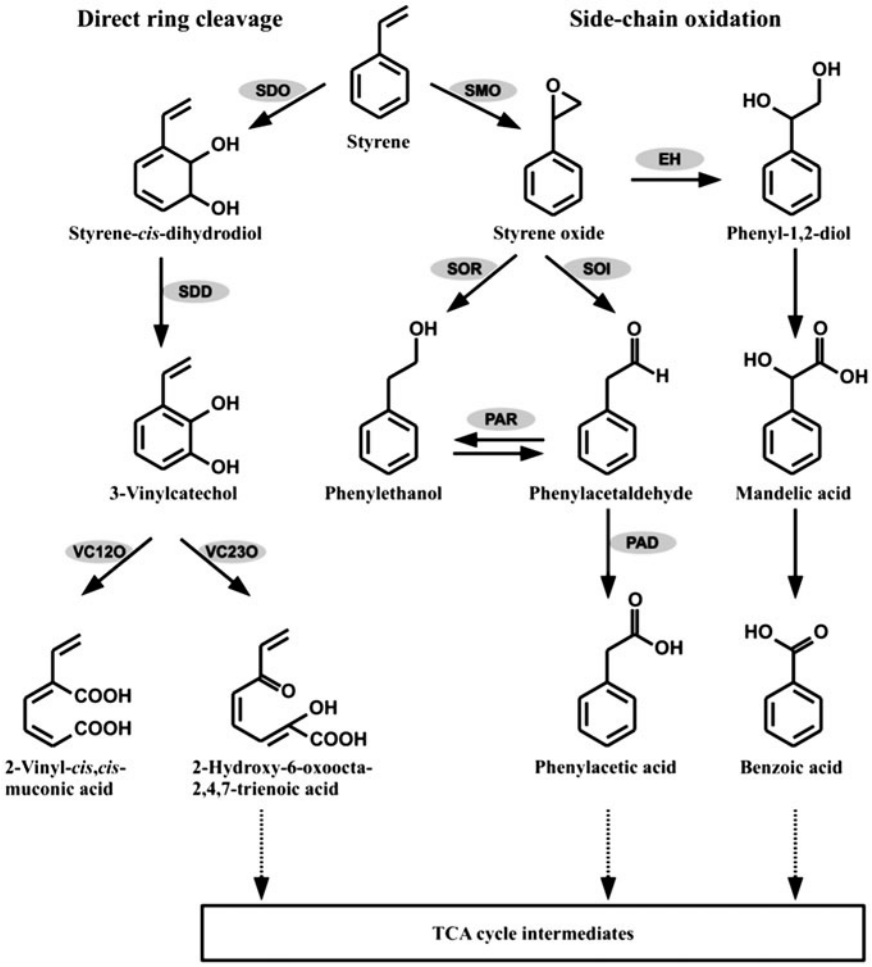


Fig. 3.1 Microbial pathways of aerobic styrene degradation. Characterized enzymes are indicated by their *abbreviation*

cases to the formation of the (*S*)-enantiomer of styrene oxide (Otto et al. 2004; van Hellemond et al. 2007; Tischler et al. 2009). Most commonly, the reactive oxirane is then isomerized to phenylacetaldehyde by a styrene oxide isomerase (SOI). In contrast to cytosolic SMOs, several studies indicated a membrane-bound localization of SOIs in *Pseudomonas*, *Corynebacterium* (Itoh et al. 1997a), and *Rhodococcus* (unpublished). The formed phenylacetaldehyde is then converted to phenylacetic acid by a phenylacetaldehyde dehydrogenase (PAD) (Fig. 3.1). It must be mentioned that *E. coli* strains are able to convert phenylacetaldehyde into 2-phenylethanol, too, which might interfere with studies using whole-cell assays (Beltrametti et al. 1997).

A slight modification of the above pathway comprises the additional action of both a styrene oxide reductase (SOR) and a phenylacetaldehyde reductase (PAR). Styrene oxide is reduced to 2-phenylethanol by SOR and oxidized to phenylacetaldehyde by PAR or another dehydrogenase. In *Pseudomonas fluorescens* ST, this variant was shown to be a side reaction (Marconi et al. 1996), whereas in *Pseudomonas* sp. 305-STR-1-4, *Pseudomonas* sp. Y2, and *Xanthobacter* sp. strain 124X, 2-phenylethanol was identified as one major metabolite (Shirai and Hisatsuka 1979; Hartmans et al. 1989; Utkin et al. 1991). According a current hypothesis, this route might belong to ethylbenzene degradation via 2-phenylethanol and thus reflects an unspecific conversion of styrene by enzymes of this pathway.

As mentioned above, styrene metabolism by bacteria and fungi shares the initial step of monooxygenation. However, all following metabolic reactions in fungi differ (Braun-Lüllemann et al. 1997) and are quite similar to styrene detoxification route in human (Warhurst and Fewson 1994; Rueff et al. 2009). Styrene oxide is hydrolyzed to phenylethan-1,2-diol by the action of an epoxide hydrolase (EH) and oxidized to mandelic acid by a dehydrogenase. Enzymatic decarboxylation then yields benzoic acid. Further, metabolites, like 2-phenylethanol, were detected and might be side products as observed for other organisms.

Initial dioxygenation of the aromatic nucleus and ring cleavage is another type of mechanism through which various bacteria degrade styrene (Bestetti et al. 1989; Hartmans et al. 1989; Warhurst et al. 1994; Patrauchan et al. 2008). In this case, a styrene 2,3-dioxygenase (SDO) introduces two oxygen atoms adjacent to the vinyl group and a styrene *cis*-glycol is formed. Subsequently, a styrene-2,3-dihydrodiol dehydrogenase (SDD) catalyzes re-aromatization to 3-vinylcatechol. These steps are consistent with the peripheral pathways of benzene-, toluene- and ethylbenzene degradation (Smith 1990; Warhurst et al. 1994; Mars et al. 1997) which yield catechol, 3-methylcatechol, and 3-ethylcatechol, respectively, as the central intermediates. Since the involved 2,3-dioxygenases usually show a relatively high substrate tolerance, conversion of styrene may be the result of fortuitous metabolism. The central intermediate 3-vinylcatechol then may undergo *ortho*- or *meta*-cleavage by the action of a vinylcatechol 1,2-dioxygenase (VC12O) or a vinylcatechol 2,3-dioxygenase (VC23O) yielding 2-vinyl-*cis,cis*-muconic acid and 2-hydroxy-6-oxoocta-2,4,7-trienoic acid, respectively. Further degradation of 2-vinyl-*cis,cis*-muconate by *ortho*-pathway fails and as a result this compound accumulates as a dead-end metabolite (Warhurst et al. 1994). A similar observation was made for methylaromatics which are mineralized by most bacteria through the *meta*-cleavage pathway (Marín et al. 2010). For example, if 4-methylcatechol undergoes *ortho*-cleavage, 4-methylmuconolactone accumulates in the growth medium (Knackmuss et al. 1976). Therefore, the *ortho*-cleavage pathway is usually unsuited for the degradation of alkylcatechols.

In contrast, the intensively yellow-colored semialdehyde from *meta*-cleavage of 3-vinylcatechol seems to be subject of further turnover. First indication for the presence of a styrene-catabolic route by *meta*-cleavage may be drawn from the preliminary occurrence of a yellow-colored intermediate from a styrene-growing

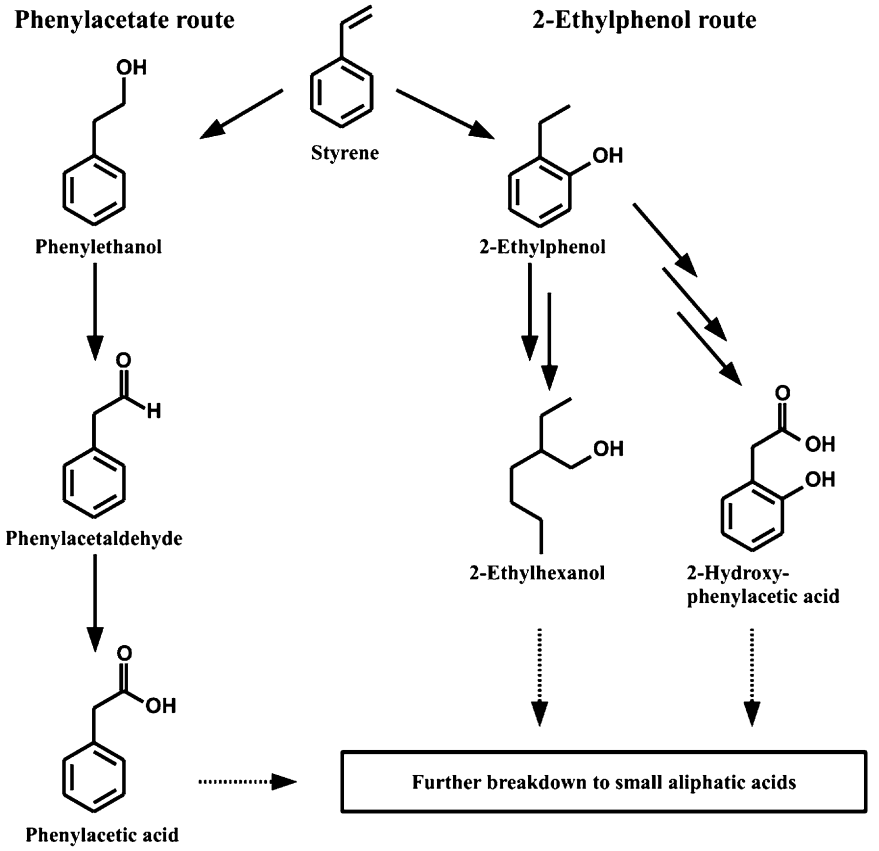


Fig. 3.2 Proposed steps of anaerobic styrene degradation pathways (adapted from Grbić-Galić et al. 1990). The herein shown routes for anaerobic styrene breakdown are based on identified metabolites from pure or mixed cultures. No data about involved enzymes are available so far

culture. However, co-existence of styrene catabolic pathways of side-chain oxygenation and direct ring attack might be possible (Hartmans et al. 1989).

3.3.2 Anaerobic Styrene Degradation

Compared to aerobic metabolism, less information is available on mechanisms of anaerobic styrene breakdown and especially on involved enzymes. Enriched consortia from anaerobic sludge as well as pure bacterial cultures were characterized for their ability to utilize styrene as sole source of carbon and energy (Grbić-Galić et al. 1990; Araya et al. 2000). Till now, three hypothetical pathways were assigned on the basis of identified intermediates rather than on enzymatic activities (Fig. 3.2).

A pathway similar to the aerobic breakdown by side-chain oxygenation was supposed to occur in *Enterobacter* as well as in anaerobic consortia (Grbić-Galić et al. 1990). According to current hypothesis, oxygenation of the vinyl group by a hydratase-catalysed addition of water leads to 2-phenylethanol which is subsequently oxidized by different dehydrogenases via phenylacetaldehyde to phenylacetic acid (Grbić-Galić et al. 1990).

An alternative anaerobic degradation sequence starts with a water-mediated hydroxylation of the aromatic ring, followed by reduction of vinyl side-chain to yield 2-ethylphenol. This intermediate may be subjected to (i) ring cleavage yielding 2-ethylhexanol or (ii) side-chain oxidation yielding 2-hydroxyphenylacetic acid.

Both anaerobic styrene degradation pathways are supposed to be important and seem to occur contemporary in microbial consortia. Pure cultures and microbial communities are able to degrade styrene almost completely under anaerobic conditions into carbon dioxide. Only small amounts of aromatic and alicyclic compounds remain. Most of the anaerobic styrene degradation is still unclear, especially due to the lack of detailed information about involved enzymes and corresponding genes or gene cluster.

3.4 Genetics and Regulatory Mechanisms

Detailed knowledge about the organization of genes and regulatory elements involved in upper styrene degradation by side-chain oxygenation is currently limited to pseudomonads like *P. fluorescens* ST (Marconi et al. 1996; Beltrametti et al. 1997), *P. putida* strain CA-3 (O'Connor et al. 1995, 1997; O'Leary et al. 2001, 2002a), strain S12 (O'Connor et al. 1997; Kantz et al. 2005), and strain SN1 (Park et al. 2006b), and *Pseudomonas* sp. strain LQ26 (Lin et al. 2010), strain VLB120 (Panke et al. 1998; Otto et al. 2004), and strain Y2 (Utkin et al. 1991; Velasco et al. 1998). The styrene-catabolic *sty* genes were found to be both, clustered on the chromosome (Velasco et al. 1998) or on plasmids (Bestetti et al. 1984; Ruzzi and Zennaro 1989) (Fig. 3.3a, b). Regulatory elements (*styS*/*styR*) as well as a styrene transporter (*styE*) were found in direct neighborhood to the catabolic genes *styABCD*. Thus, at least for few *Pseudomonas* strains, a complete *sty(rene)*-operon could be described.

3.4.1 The Gene Cluster styABCD(E) of the Upper Styrene Degradation Pathway

The number and arrangement of genes within the *styABCD(E)* clusters of pseudomonads reflect the necessity and sequence of encoded enzymes, respectively,

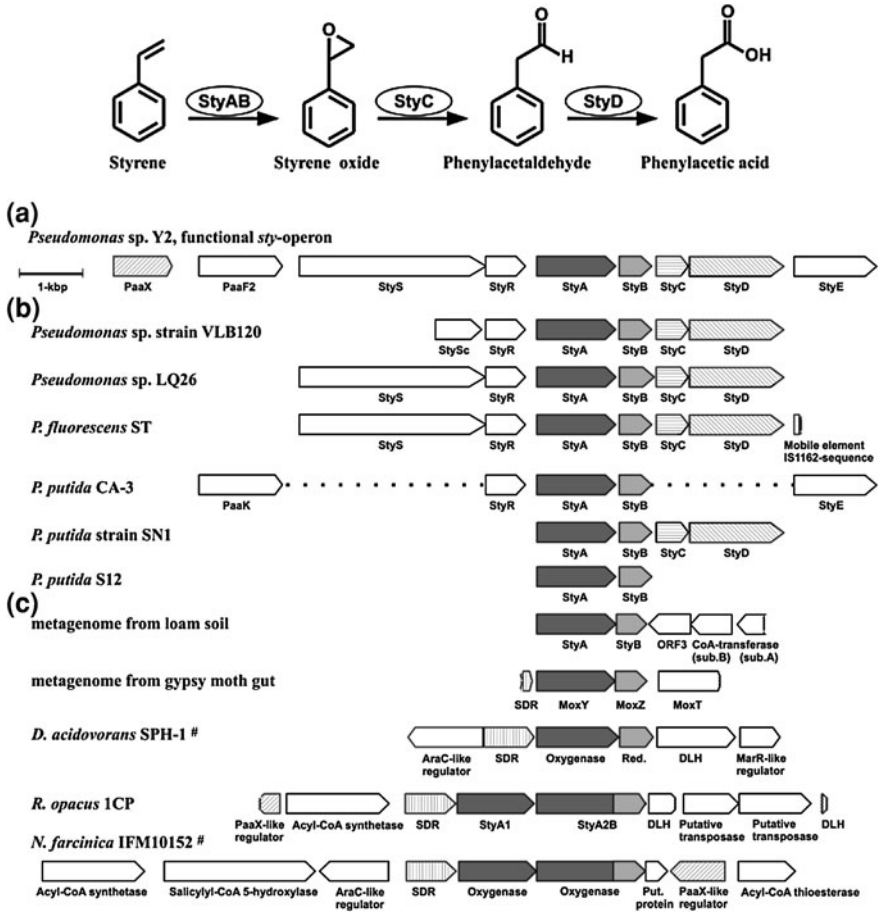


Fig. 3.3 Comparison of the organization of **a** the *styABCDE*-operon from *Pseudomonas* sp. Y2, **b** other (incomplete) styrene-catabolic gene clusters from pseudomonads, and **c** the genetic location of flavin monooxygenases with (# hypothetical) function as styrene monooxygenases. The upper pathway of styrene degradation by side-chain oxygenation is shown at the top and involved gene products are given. Identical types of filling of ORFs indicate similar function of encoded proteins

to catalyze the conversion of styrene into phenylacetic acid (Fig. 3.3a, b). The genes *styA* and *styB* encode the two-component flavin-dependent styrene monooxygenase (SMO, or StyA/StyB) which initially converts styrene to styrene oxide at the expense of NADH. A single gene s*tyC* encodes a styrene oxide isomerase (SOI, or StyC) which catalyzes the intramolecular rearrangement of styrene oxide to phenylacetaldehyde. Finally, *styD* which is located directly downstream to *styC* encodes an NAD- or phenanzine methosulfate-dependent phenylacetaldehyde dehydrogenase (PAD or StyD) oxidizing phenylacetaldehyde into phenylacetic acid, the final product of the upper styrene pathway. An additional gene *styE*

(or *porA*) was found in few pseudomonads to be located directly downstream to the catabolic cluster *styABCD* and high similarities to several membrane-associated ATPase-dependent kinase proteins (Velasco et al. 1998; Mooney et al. 2006a) suggested a function as styrene transporter. This hypothesis was strengthened by the detection of (i) a co-expression of *styE* (*P. putida* CA-3) with *styABCD*, (ii) a styrene-dependent transcription, (iii) a membrane association, and (iv) an increased styrene degradation rate in the presence of additionally overexpressed *styE* copies (Mooney et al. 2006a). Furthermore, basic necessity of the transporter was shown by a *styE*-negative mutant of strain CA-3 which lost its ability to grow on styrene. However, Nikodinovic-Runic and coworkers detected only minor levels of StyE from a styrene-grown pseudomonad under both non-limiting and nitrogen-limiting conditions (Nikodinovic-Runic et al. 2009). These findings implicate that both, membrane diffusion as well as active styrene transport, are important for the uptake of styrene. In addition, it cannot be excluded that other transporters support active styrene uptake.

It is supposed that the upper pathway of styrene degradation may have evolved in a different way as the lower route necessary for phenylacetic acid conversion to TCA cycle intermediates (Ferrández et al. 1998; Olivera et al. 1998; Alonso et al. 2003a; Di Gennaro et al. 2007).

Considering the low number of identified styrene-catabolic gene clusters and their apparently restricted occurrence in pseudomonads, styrene degradation by side-chain oxygenation seems to be not widely distributed in nature. This assumption is currently strengthened by available genome data which did not allow the identification of *styABCD*-like gene clusters from other bacterial phyla. However, taking into account on one hand the widespread ability of styrene utilization (Table 3.2), and on the other hand the preferred isolation of pseudomonads during classical enrichment procedures, current knowledge probably does not reflect the true distribution. In fact, single enzymatic activities or genes with hypothetical function in upper styrene degradation could be found in several other organisms than pseudomonads including gram-positive bacteria.

Starting with the initial flavin-dependent styrene monooxygenase StyA/StyB, the distribution frequency among bacteria is supposed to be generally low (based on representatives per genome, van Berkel et al. 2006). The only gene products showing significant homology to StyA/StyB from pseudomonads and activity on styrene or analogous compounds are found in two metagenoms (Guan et al. 2007; van Hellemond et al. 2007), as well as in the actinobacterium *Rhodococcus opacus* 1CP (Tischler et al. 2009, 2010) (Fig. 3.3c). *In silico*-screening for further representatives yielded several homologous proteins with similarities either to SMOs from pseudomonads or to that one from *Rhodococcus opacus* strain 1CP which was exemplarily shown by van Hellemond et al. (2007) and Tischler et al. (2009) (Fig. 3.3c). Interestingly, only in few Actinobacteria, a novel type of styrene monooxygenase was found so far which will be discussed later.

Biochemical evidence for styrene oxide isomerases was provided for several bacteria like *Xanthobacter* sp. 124X (Hartmans et al. 1989), *Corynebacterium* spp. (Itoh et al. 1996, 1997a), *Rhodococcus opacus* 1CP (Tischler et al. 2009),

Rhodococcus sp. S5, and others (Hartmans et al. 1990). However, none of the corresponding genes has been described so far and homology search of StyC towards available databases did not indicate significant similarities to gene products others than StyC-homologs from pseudomonads.

It should be mentioned that in *Rhodococcus opacus* 1CP, no *styC*-homologous gene could be found in direct neighborhood to the styrene monooxygenase genes *styA1*/*styA2B* (Fig. 3.3c), indicating that the genetic organization of styrene catabolism is different to that in pseudomonads (Tischler et al. 2009).

Phenylacetaldehyde dehydrogenases should be much more common in bacteria, since their substrate phenylacetaldehyde originates from different catabolic pathways apart from styrene degradation. Precursors are phenylpyruvic acid, 2-phenylethylamine, 2-phenylethanol, and phenylmalonic semialdehyde (Ferrández et al. 1997; Long et al. 1997). This assumption is strengthened by homology search of *styD* genes and gene products to the non-redundant genome database which reveals many high-score hits. However, only a few reports about functionally characterized phenylacetaldehyde dehydrogenases are available so far.

3.4.2 The Regulatory System of the Sty-Operon

The two-component regulatory system StyS/StyR of the *sty*-operon from the strains *Pseudomonas putida* CA-3, *Pseudomonas* sp. Y2, and *Pseudomonas fluorescens* ST was investigated in detail (Velasco et al. 1998; Santos et al. 2000; O'Leary et al. 2001, 2002a; Leoni et al. 2003). With respect to the conserved operon organization among pseudomonads (Fig. 3.3a, b), the described features of this regulatory system are supposed to occur and interact similarly in all functional *sty*-operons of pseudomonads.

The gene products of *styS* and *styR,* which are located proximate upstream to *styABCD* (Figs. 3.3 and 3.4), both show similarities to several two-component transduction systems from prokaryotes and eukaryotes (Reizer and Saier 1997). In fact, they were found to positively affect *sty*-operon transcription (Fig. 3.4) and both genes are most likely expressed in a transcription-coupled fashion (O'Leary et al. 2001). On amino acid level, StyS exhibits similarity to sensor kinase proteins, especially to TodS, TutC, and TutS which regulate toluene catabolism in *Pseudomonas* spp. and *Thaurea* sp. (Coschigano and Young 1997; Lau et al. 1997; Leuthner and Heider 1998). A similar two-component regulator was reported for the biphenyl degradation pathway of *Rhodococcus* sp. M5 (Labbé et al. 1997). Close relationship of StyS to the above regulator proteins could also be demonstrated by the ability of styrene to act as an inducer of toluene degradation (Cho et al. 2000; Mosqueda and Ramos 2000). The sensor kinase StyS consists of five functionally different domains: input-1, histidine kinase-1 (HK-1), receiver, input-2, and histidine kinase-2 (HK-2) (Fig. 3.4). Both input domains contain typical motives of PAS-sensing domains (these are signaling modules for changes of oxygen level, redox potential, light, and small ligand concentrations), and are

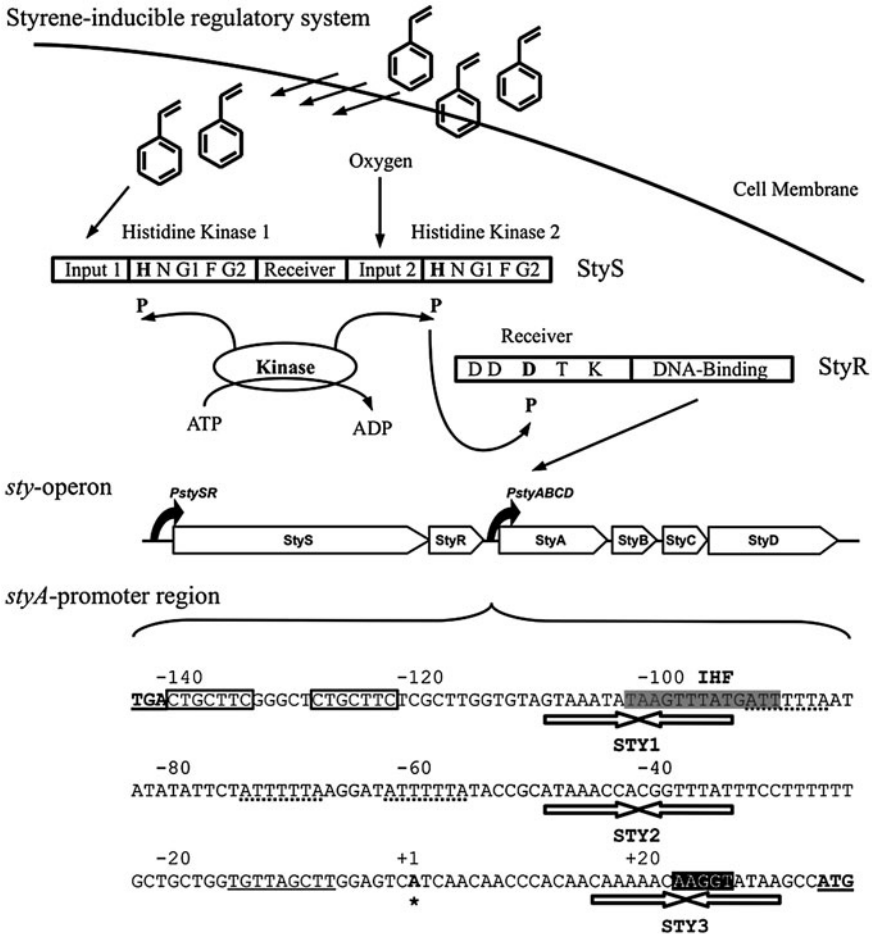


Fig. 3.4 Structure and mechanism of the styrene-inducible regulatory system of the *sty*-operon and a detailed view on the complex *styA*-promoter region (adapted from O'Leary et al. 2002b). The regulatory system (StyS/StyR with conserved motifs, *sty*-operon with indicated promoter sites *PstySR* and *PstyABCD*, and *styA*-promoter region) and its effect on the transcription of styrene-catabolic genes based upon data obtained from styrene-utilizing *Pseudomonas fluorescens* ST. Styrene is sensed by the input 1 domain of StyS and changing oxygen concentrations might be also sensed by another input domain of StyS. Afterwards one of the two histidine kinase domains gets activated by phosphorylation of a histidine residue (*bold 'H'*) and the phosphoryl group is subsequently transferred onto an aspartate residue (*bold 'D'*) of the receiver domain of StyR. This activated response regulator StyR can then bind by its C-terminal DNA-binding domain to one of the three STY-sites of the promoter region. In the presence of styrene as the sole source of carbon, activated StyR binds to STY2 and RNA polymerase and then binds to the promoter region (*underlined*) and initiates transcription of *styABCD*. The complete *styA*-promoter region is outlined, numbered according to the transcriptional start site (+1, *bold 'A'*), and the following elements between StyR-stop and StyA-start codon (*bold faced*) are indicated: the three palindromic sequences STY1, STY2, STY3 (*arrows*), the consensus sequence 5′-WATCAANNNNTTR-3′ (complementary encoded to *styABCD*) for binding of the integration host factor IHF (*gray box*), a directed repeat sequence 5′-CTGCTTC-3′ at the beginning (*boxed*), two 5′-ATTTTTA-3′ motifs (*dotted lines*), and the potential ribosome binding site (*black box*)

expected to sense traces of styrene or degradation intermediates, perhaps as a consequence of an altered redox potential of the cell (Coschigano and Young 1997; Lau et al. 1997; Velasco et al. 1998; Santos et al. 2000). The two histidine kinase domains H←N←G1←F←G2 are highly conserved and harbor a characteristic kinase amino acid motif including a single histidine residue for the phosphoryl group transfer towards the response regulator StyR (Coschigano and Young 1997; Grebe and Stock 1999; Mosqueda and Ramos 2000). Slight differences in their amino acid sequence allow their classification into the kinase superfamilies 1a (HK-1) and 4 (HK-2). The receiver domain belongs to the RA2-receiver superfamily and contains a conserved amino acid motif D←D←S←K typical for bacterial response regulators (Grebe and Stock 1999).

The second half of the *sty*-operon regulatory system is represented by StyR. Like StyS, this protein shows high similarity to two-component regulators, especially those ones of toluene catabolism like TodT and TudC (Lau et al. 1997; Leuthner and Heider 1998). StyR comprises two different domains, an N-terminal regulatory (or receiver) domain (pos. 1 to 127) and a C-terminal DNA-binding domain (pos. 142 to 208), which are joined by a 34-amino acid long Q-linker (O'Leary et al. 2002b). The receiver domain belongs to the RA4-receiver subfamily and harbors the conserved amino acid motif (D←)D←D←T←K (Baikalov et al. 1996; Grebe and Stock 1999), whereas the highly conserved C-terminus shows similarities to family-3 response regulators (Reizer and Saier 1997; Velasco et al. 1998). Thus, StyR belongs to the FixJ/NarL-subfamily of response regulators.

Several studies have demonstrated that StyS-StyR plays a key role within styrene degradation and affects positive as well as negative the expression of catabolic genes (Fig. 3.4) (Panke et al. 1998; Velasco et al. 1998; Santos et al. 2000, 2002; O'Leary et al. 2001, 2002a, b; Leoni et al. 2003, 2005). The presence of styrene is indispensable for the transcription of *styA* and the complete functional *sty*-operon. In most cases, transcripts of the regulatory proteins were found only in presence of styrene (O'Connor et al. 1995; O'Leary et al. 2001). Various carbon sources like phenylacetic acid, glutamate, glucose, and citrate, were found to repress the transcription of catabolic genes even when styrene is present in the culture medium (O'Connor et al. 1995; Santos et al. 2000; O'Leary et al. 2001). Only styrene induces significantly the upper route and phenylacetic acid or its metabolites do not, even at presence of phenylacetic acid in the medium, the *sty*-operon transcription is repressed (O'Leary et al. 2001). Thus, the upper (*sty*-operon) and lower (phenylacetic acid degradation genes) pathway of styrene metabolism are likely to be regulated separately.

Based on identified regulatory elements, the following regulation mechanism was postulated. Styrene is sensed by the sensory input domain of StyS and as a result, one of the two histidine kinase domains is activated by a kinase-catalyzed phosphorylation of conserved histidine residue (H). This phosphoryl group is then transferred onto a conserved aspartic acid residue (D) of the receiver domain of StyR. It was demonstrated that phosphorylated and thus activated StyR binds co-operatively to a palindromic sequence STY2 (Fig. 3.4) of the *styA* promoter region (*PstyABCD*), leading to highly attracted binding of RNA polymerase to a conserved sequence of

the promoter region (5′-TGTTAGCTT-3′). In that case, StyR controls gene transcription of the upper styrene degradation route as an activator, but after translation of catabolic genes, high amounts of phosphorylated StyR may accumulate and act then as a repressor of transcription. The latter effect is caused by binding of activated StyR to a negative regulatory site STY3 within the *styA* promoter region. Another regulatory sequence STY1, located upstream to STY2 and STY3, might affect transcription of catabolic genes positively (presence of styrene) or negatively (presence of glucose or other carbon sources). Additionally, it was demonstrated that an integration host factor (IHF, a small heterodimeric protein) affects *styABCD* transcription due to binding to a consensus sequence in the *styA*-promoter region (5′-WATCAANNNNTTR-3′, complementary encoded to *styABCD*) (Fig. 3.4) (Leoni et al. 2005). A positive role for *PstyABCD* regulation is expected.

Biochemical characterization of the wild-type styrene monooxygenase StyA/StyB from *Pseudomonas* sp. VLB120 indicated that the expression level of the oxygenase subunit StyA exceeds by far that of the NADH:FAD oxidoreductase StyB (Otto et al. 2004). This behavior is similar to a 4-hydroxyphenylacetate 3-monooxygenase (Louie et al. 2003) and probably accounts for the fact that StyB has a much higher specific activity than StyA. Moreover, StyA should not be limited by a molar deficit of StyB since reducing equivalents ($FADH_2$) are transferred mainly by diffusion and a general necessity of StyA/StyB contact is not given (Kantz et al. 2005; Otto et al. 2004). Proteome analysis of *Pseudomonas putida* CA-3 pointed to similar results and indicated that StyA and StyD are by far the most abundant proteins of the upper *sty*-operon which exceed StyB and StyC for at least one order of magnitude (Nikodinovic-Runic et al. 2009).

The regulatory elements *styS-styR* from pseudomonads are the only ones hitherto found to be involved in styrene catabolism. Similar elements are lacking in close neighborhood of all other (putative) styrene monooxygenases identified from metagenomes and different Actinobacteria (Fig. 3.3c). However, a distant localization of related two-component regulatory systems cannot be excluded. Interestingly, two other regulatory elements were found in the neighborhood of (hypothetical) styrene monooxygenases from Actinobacteria: a PaaX- and an AraC-like regulator. The first one is typical for phenylacetic acid degradation which suggests a dependency to styrene degradation. The latter one belongs to the diverse group of AraC/XylS-family transcriptional regulators (Gallegos et al. 1997) frequently involved in the regulatory machinery of aromatic hydrocarbon degradation. A functional link of both elements to SMO regulation is still missing.

3.4.3 Genetic Localization of Single Styrene Monooxygenases in Other Organisms

Despite the fact that the identification of complete styrene-catabolic operons is currently limited to members of the genus *Pseudomonas*, there is certain evidence that styrene degradation by side-chain oxygenation is widespread among bacteria.

This assumption can be drawn since several gram-positive and gram-negative isolates were described to utilize styrene as the sole source of carbon and to produce traces of styrene oxide during this degradation process (Table 3.2) (Hartmans et al. 1989; Arnold et al. 1997; Alexandrino et al. 2001; Przybulewska et al. 2006). In addition, several genes in (meta)genomes and in members of Actinobacteria, which encode for functionally proven or hypothetical styrene monooxygenases, were identified (Fig. 3.3c) (Guan et al. 2007; van Hellemond et al. 2007; Tischler et al. 2009). Besides, the congruence of important amino acid motifs in all of them, the biochemically characterized SMOs from the soil metagenome and from *Rhodococcus opacus* 1CP show a similar behavior and substrate pattern as SMOs from pseudomonads (van Hellemond et al. 2007; Tischler et al. 2009, 2010). None of these SMO genes are part of a *styABCD*-like gene cluster and the virtual absence of SOI- and PAD-like genes raised questions on a functional involvement in styrene degradation. Certain evidence for a role in styrene degradation can be drawn from the observation that each of the novel SMO genes is surrounded by at least one ORF, encoding a protein with function in phenylacetic acid metabolism, like a PaaX-like regulator, a CoA-ligase, a CoA-transferase, a CoA-hydroxylase, and a CoA-thioesterase (Olivera et al. 1998; Di Gennaro et al. 2007; Tischler et al. 2009).

The identification of *styA1/styA2B* from *R. opacus* 1CP and corresponding sets of genes in *Arthrobacter aurescens* TC1, *Nocardia farcinica* IFM10152, and *Streptomyces platensis* CR50 pointed to a novel class of two-component monooxygenases with high similarity to SMOs from pseudomonads. Most remarkably, the gene encoding the FAD:NADH oxidoreductase subunit ("*styB*") is fused with that one of a second version of the oxygenase subunit ("*styA2*") and the corresponding protein StyA2B was shown in *R. opacus* 1CP to act as a single-component self-sufficient monooxygenase with (low) styrene-oxygenating activity (Tischler et al. 2009). Later on, addition of the other oxygenase subunit StyA1 was shown to be necessary for full epoxidation activity and evidence for a specific inter-protein communication was provided (Tischler et al. 2010).

3.4.4 Mobility of Styrene-Catabolic Genes

The *sty*-operon of pseudomonads was shown to be both, chromosomally encoded in *Pseudomonas* sp. strain Y2 (Velasco et al. 1998) as well as located on a plasmid (designated as pEG) in *Pseudomonas fluorescens* ST (Bestetti et al. 1984), which indicates mobility. The plasmid pEG was found to be 37 kbp in size, circular, and self-transmissible. Uptake of pEG by the styrene-negative, plasmid-free *Pseudomonas putida* strain PaW 340 led to a styrene-degrading phenotype and demonstrated mobility of the upper styrene catabolism (Bestetti et al. 1984). Later studies revealed that unidentified DNA-fragments as well as some transposable elements (IS-elements of class IS*21*-like) are encoded on the chromosome as well as on pEG of *P. fluorescens* ST, and that the complete pEG plasmid or

fragments thereof might transpose into the chromosome (Beltrametti et al. 1997, Ruzzi and Zennaro 1989). This assumption was supported by another study of *P. fluorescens* ST, in which the authors reported about the identification of the complete functional *sty*-operon located next to an IS-element on the chromosome (Marconi et al. 1996). However, the IS-element located adjacent to the *sty*-operon was found to be a copy of IS*1162* which can be found two times on the pEG plasmid. Thus, the transposable elements might be responsible for the construction as well as for a kind of mobility (between plasmid and chromosome) of the styrene-catabolic gene cluster. Transposable elements were identified in close proximity of the SMO genes *styA1/styA2B* from *Rhodococcus opacus* 1CP, too, which might be an indication of mobility of these monooxygenases (Fig. 3.3c) (Tischler et al. 2009).

3.4.5 Genetic Aspects of Styrene Catabolism by Direct Ring Cleavage

Compared to the mechanism of side-chain oxygenation, apparently no information about genes and regulatory elements of the direct ring cleavage route are available (Fig. 3.1). From *Pseudomonas putida* MST (Bestetti et al. 1989), *Rhodococcus rhodochrous* NCIMB 13259 (Warhurst et al. 1994), and *Xanthobacter* sp. 124X (Hartmans et al. 1989), only metabolites and enzyme activities were described. However, *meta*-cleavage is a more general pathway for the aerobic degradation of (alkylated) aromatic compounds and occurs in many microorganisms (Knackmuss et al. 1976; Warhurst et al. 1994; Marín et al. 2010). Warhurst and co-workers observed no significant difference in the enzyme activities of the *meta*-cleavage route for styrene compared to similar pathways like that encoded by the TOL plasmid for toluene and xylenes. It probably evolved like *meta*-cleavage pathways from other microorganisms. Interestingly, toluene-grown cells of *Pseudomonas putida* F1 were able to co-metabolize styrene by enzymes of the *meta*-cleavage route of toluene (Cho et al. 2000). First genetic insights were reported for the genome-sequenced *Rhodococcus jostii* RHA1 which confirmed earlier assumptions (Patrauchan et al. 2008). Patrauchan and co-workers found out that strain RHA1 degrades styrene by direct ring attack and *meta*-cleavage, but no gene cluster involved exclusively in styrene degradation was determined. By means of a proteomic approach and targeted gene disruption, responsible genes and products were identified as part of a biphenyl-catabolic gene cluster. The initial activity towards styrene was attributed to a single biphenyl dioxygenase (BPDO). Relevant genes of that biphenyl-catabolic gene cluster are regulated by a two-component regulatory system (BphS/BphT) (Patrauchan et al. 2008). BphS phosphorylates BphT in response to various aromatic substances and the latter one then promotes the transcription of catabolic genes. A regulatory coupling highly similar to BphS/BphT is responsible for transcription of a gene cluster involved in ethylbenzene degradation, too. In presence of both, biphenyl and ethylbenzene, gene clusters are co-expressed, but styrene was found to induce only genes of biphenyl degradation.

This indicates a substrate-dependent regulation of both the biphenyl- and the ethylbenzene gene cluster.

In summary, styrene degradation by direct ring attack occurs rather by an adapted pathway and has not been specifically evolved for the alkenylbenzene than it is the case for the *sty*-operon from pseudomonads.

3.5 Biotechnological Aspects

Styrene-degrading microorganisms and enzymes of styrene-catabolic pathways were shown to be of relevance for the purposes of white (grey) biotechnology.

3.5.1 Styrene Monooxygenases

Perhaps, the most prominent application derived from styrene-degrading bacteria is the production of enantiopure epoxides by means of highly stereoselective styrene monooxygenases (SMOs) (Fig. 3.5). Optically active oxiranes are high-value building blocks for pharmaceutical and agrochemical industry as well as for fine chemistry (Rao et al. 1992; Badone and Guzzi 1994; Besse and Veschambre 1994; Hattori et al. 1995; Schulze and Wubbolts 1999; Breuer et al. 2004). All styrene monooxygenases investigated so far show a strong preference for the formation of (*S*)-isomer of styrene oxide from styrene (enantiomeric excess >98%) (O'Leary et al. 2002b; Mooney et al. 2006b). An oxygenating activity from ethene-grown cells of *Mycobacterium* sp. E3, which was found to convert styrene into (*R*)-styrene oxide (>98% e.e.), probably cannot be assigned as a "true" styrene monooxygenase due to its expression by a different growth substrates. Even more important styrene biotransformation rates of ethene-grown cells were shown to be a factor 40 lower than those ones of styrene-grown pseudomonads expressing an SMO (Nöthe and Hartmans 1994). In fact, other types of monooxygenases are often able to convert styrene into styrene oxide, but high activities and selectivities of real SMOs are never reached (Archelas and Furstoss 1997; Mooney et al. 2006b; O'Leary et al. 2002b).

Despite their high regio- and enantioselectivity, SMOs show a somewhat broad substrate specificity. Substituted styrenes, like monohalogenated derivatives (fluoro-, chloro-, bromostyrene), monomethylstyrenes, and *trans*-β-methylstyrene as well as structurally similar compounds like pyridine analogs, dihydronaphthalene, (substituted) allylbenzenes, indole, and indene are converted to the corresponding epoxides (Bernasconi et al. 2000; Hollmann et al. 2003; van Hellemond et al. 2007; Tischler et al. 2009; Lin et al. 2010). Enantiopure indene oxide is by the way hardly available by chemical synthesis and serves as a precursor of *cis*-1*S*,2*R*-aminoindanol, an intermediate in the production of the anti-HIV-1 drug Crixivan® (indinavir). Furthermore, SMOs are able to oxidize phenylalkyl sulfides

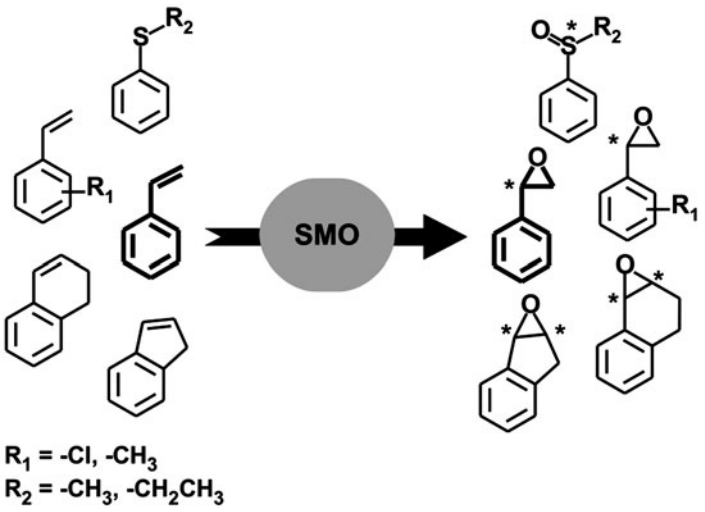


Fig. 3.5 Enantioselective oxygenations catalyzed by styrene monooxygenases (SMOs). *Stars* indicate optically active centers (Tischler et al. 2010)

to sulfoxides and depending on the host, different enantioselectivities are achieved. An SMO from a metagenome was found to yield predominantly the (*R*)-enantiomer of phenylmethyl sulfoxide (>75% e.e.) (van Hellemond et al. 2007), whereas a strong preference of the (*S*)-enantiomer (>99% e.e.) was shown for StyA1/StyA2B from *R. opacus* 1CP (unpublished).

Since the application of SMOs allows the production of a wide range of valuable epoxides, a first pilot-scale biotransformation process was established (Panke et al. 2002). Due to cofactor dependence (NADH, FAD), a whole-cell biotransformation is favored allowing the regeneration of expensive NADH. StyA/StyB from *Pseudomonas* sp. VLB120 were recombinantly expressed in *E. coli* during a 30-L fed-batch to produce enantiopure (*S*)-styrene oxide (307 g, 40% yield). A two-phase extractive fermentation was chosen and subsequently optimized in order to maximize volume productivity and process stability (Panke et al. 2000, 2002; Park et al. 2006a, b). In addition, biocatalysts were modified by approaches of protein engineering (Gursky et al. 2009; Qaed et al. 2010) and different expression systems were analyzed for their suitability (Panke et al. 1999; Han et al. 2006; Park et al. 2006b; Bae et al. 2008, 2010). An evaluation of the optimized process indicated a high degree of competitiveness towards conventional methods of chemical synthesis (Kuhn et al. 2010). The usage of toxic and environmentally harmful bis(2-ethylhexyl)phthalate as organic phase was found to be the major drawback of this type of process.

The use of biofilms instead of two-phase systems may be another approach in order to minimize toxic effects of substrate and product during biotransformations and was demonstrated for a SMO-harboring *Pseudomonas* strain (Gross et al. 2007).

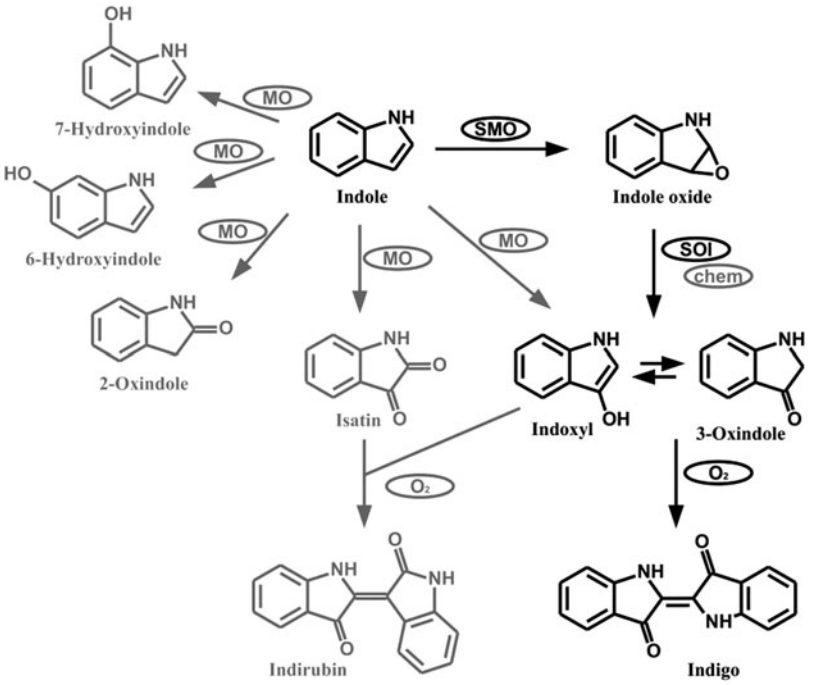


Fig. 3.6 Oxo functionalization of indole and the formation of indigoid dyes (from McClay et al. 2005). SMO/SOI-based oxygenation (*black*) and oxidation of indole (*grey*) catalyzed by various oxygenases (e.g. P450- or toluene monooxygenases; MO) can result in the formation of indigo and indirubin. Indoxyl can be yielded slowly by chemical rearrangement (chem) or faster by SOI activity

In addition to the above mentioned substrates, styrene monooxygenases show a common feature to convert indole to indole oxide. Indole oxide then rearranges spontaneously or by the action of an SOI to indoxyl, followed by spontaneous dimerization to indigo under aerobic conditions (Fig. 3.6) (O'Connor et al. 1997). The expression of SMO/SOI either by styrene-degrading wild-type hosts or by recombinant strains is thus a strategy to produce indigoid dyes. Compared to other oxygenase-dependent transformations as well as to chemical syntheses, the combination of SMO and SOI is more specific and less by-products like hydroxyindole, 2-oxoindole, isatin, or indirubin are formed (McClay et al. 2005), leading to production of highly pure indigo.

3.5.2 Styrene Oxide Hydrolases and Styrene Oxide Isomerases

As mentioned before, epoxide hydrolases (EHs) can be involved in styrene metabolism (Fig. 3.1). More frequently, these enzymes play a role in the

detoxification process of xenobiotics with epoxide-like structures, especially in mammalians. EHs are enantioselective and show a broad substrate spectrum.

Styrene oxide is a model substrate for this enzyme class and most representatives convert solely one enantiomer of racemic styrene oxide into a chiral phenylethan-1,2-diol. Thus, EHs can be used to produce either enantiopure epoxides or enantiopure 1,2-diols (Archer 1997; Archelas and Furstoss 1997; Orru and Faber 1999; Breuer et al. 2004; Kim et al. 2006; Liu et al. 2006). Especially EHs from fungi were subject of considerable research during the past decades because these enzymes can be produced in high amounts and allow the formation of enantiopure compounds with a high value for pharmaceutical industry. Exemplary, the company 'Merck' screened various fungi for enantioselective hydrolase activity on racemic indene oxide to produce (1*S*,2*R*)-indene oxide, which serves as a precursor for the HIV protease inhibitor indinavir (Crixivan®) (Archer 1997). The industrial application of EHs enables 'Merck' to produce this pharmaceutical in high quality.

Compared to styrene oxide hydrolases, less information is available on styrene oxide isomerases (SOIs) which convert styrene oxide to phenylacetaldehyde (Hartmans et al. 1989; Itoh et al. 1997a). In addition, SOIs from *Pseudomonas* species are able to convert various substituted styrene oxides into the corresponding aldehydes or ketones (Miyamoto et al. 2007). During the production of indigo by SMOs, the presence of SOIs yields an almost complete conversion of indole oxide (SMO product) into indoxyl. The absence of isatin prevents the formation of the unwanted by-product indirubin, which is usually formed, if other less specific oxygenases are applied (Fig. 3.6).

However, two major drawbacks of SOI representatives identified so far have to be addressed: (i) SOIs are membrane-bound enzymes and seem to be expressed at very low levels in wild-type microorganisms as well as in recombinant hosts (Hartmans et al. 1989; Itoh et al. 1997a; Nikodinovic-Runic et al. 2009), and (ii) SOIs convert only epoxides with styrene oxide-analogous structure and are exhibiting low enantioselectivity (Itoh et al. 1997a; Miyamoto et al. 2007).

3.5.3 Phenylacetaldehyde Reductases

Phenylacetaldehyde reductases (PAR) from styrene-assimilating bacteria are highly interesting biocatalysts for production of chiral alcohols from various prochiral ketones, *β*-ketoesters, and aldehydes (Itoh et al. 1997b, 2002, 2007; Makino et al. 2007). Itoh and coworkers reported a system utilizing a recombinantly expressed PAR from *Rhodococcus* sp. ST-10 (formerly described as *Corynebacterium* sp. ST-10). On the one hand, it converts ketones and ketoesters to valuable products at the expense of NADH, and on the other hand, it regenerates that reduced cofactor in the presence of 2-propanol. Thus, an additional NADH-regeneration system as for example formate dehydrogenase is not necessary.

Further, enzyme engineering yielded mutants with broader substrate spectrum which are less affected by high amounts of substrates (e.g. 2-propanol).

3.5.4 Production of Substituted Catechols

Substituted *ortho*-diphenols are useful building blocks for dyes, polymers, and pharmaceuticals. Certain isomers are only hardly available from conventional chemical syntheses and hence biocatalytic preparation strategies are becoming of increasing interest. Catechols are important intermediates of the peripheral degradation of aromatic hydrocarbons typically by the action of a dioxygenase and a dihydrodiol dehydrogenase. Their further metabolization is achieved during the central *ortho*- and *meta*-cleavage pathway by intradiol-acting- (C12Os) or extradiol-acting dioxygenases (C23Os), respectively. It is well known that C23Os are specifically inhibited by 3-fluoro- and 3-chlorocatechol by a so-called "suicide inactivation", leading to an accumulation of catechols by the action of initial dioxygenases/dehydrogenases (Bartels et al. 1984; Mars et al. 1997). Taking into consideration the toxic behavior of accumulated catechols for the host, this phenomenon represents a simple route to produce various catechol derivatives.

Since *R. rhodochrous* NCIMB 13259 was shown to degrade styrene by direct ring attack and *meta*-cleavage (Fig. 3.1), the inhibitory effect of 3-fluorocatechol was exploited by Warhurst and co-workers to obtain 3-vinylcatechol (Warhurst et al. 1994). The initial styrene dioxygenase and dihydrodiol reductase of a styrene-grown culture of strain NCIMB 13259 both exhibit a broad substrate tolerance allowing the conversion of toluene and ethylbenzene into 3-methylcatechol and 3-ethylcatechol, respectively.

3.5.5 Formation of p-Hydroxystyrene

The introduction of genes encoding a phenylalanine/tyrosine ammonia lyase (from *Rhodotorula glutinis*) and a *p*-coumaric acid decarboxylase (from *Lactobacillus plantarum*) into *E. coli* WWQ51.1 recently allowed the construction of a pathway for the formation of *p*-hydroxystyrene from the central metabolite tyrosine during growth of that strain on glucose (Qi et al. 2007). Product toxicity was overcome by a two-phase fermentation system in which the biotransformation takes place in the aqueous phase and *p*-hydroxystyrene accumulates in the organic phase. Later on, the host was changed from *E. coli* to a more solvent-tolerant *P. putida* strain lacking styrene monooxygenase and feruloyl-coenzym A synthethase, yielding an increased product formation (Verhoef et al. 2009). *p*-Hydroxystyrene is a valuable target for chemical industry (especially in polymer production) and as a potential substrate for styrene-catabolic enzymes, it might serve as a source for other interesting compounds, like *p*-hydroxystyrene oxide and *p*-hydroxyphenylacetaldehyde. A combination of the above pathway with certain activities of initial

styrene metabolism would allow the formation of these compounds starting from glucose and avoiding the use of toxic styrene.

3.5.6 Formation of Polyhydroxyalkanoates

Many bacteria produce polyhydroxyalkanoates (PHAs) as carbon-based storage compounds under conditions of unbalanced nutrient supply (Anderson and Dawes 1990; Madison and Huisman 1999). Styrene is used as a carbon source in that respect and can be utilized by *P. putida* CA-3 in order to produce PHAs during nitrogen starvation (Ward et al. 2005). Yields of up to 23% of the cell dry weight were obtained and the polymer was shown to consist of the monomers 3-hydroxyhexanoate, 3-hydroxyoctanoate, and 3-hydroxydecanoate in a ratio of 3:22:75. Two other pseudomonads were shown to produce PHAs in lower yields under similar conditions too. Combination of this microbial ability with a process of thermal polystyrene cleavage yielding crude styrene monomer may represent an attractive strategy for the production of biodegradable polymers from recalcitrant polystyrene wastes (Ward et al. 2006).

3.5.7 Biological Waste Gas Treatment

Since a few decades, the principle of biological waste gas treatment has been used in order to remove harmful volatile organic compounds (VOCs) from gas streams. Especially for the removal of low concentrations of biodegradable VOCs from large volumes of waste gases, the application of biofilters, trickling biofilters, and bioscrubbers represent a cost-effective alternative to methods of physico-chemical treatment as for example active carbon filters and waste gas combustion (Deshusses 1997; Delhomenie and Heitz 2005; Malhautier et al. 2005).

All biofiltration techniques, of which the biofilter is the most simple and widely distributed one, depend on the degradative potential of a complex microbial consortium. The degradative capability is either a result of prolonged adaptation of the available microflora (present on the surface of the organic packing material) or may be supported by the addition of microorganisms of certain metabolic properties. Several studies showed the removal of readily biodegradable volatile organic compounds i.e. styrene (Table 3.3) by a simple adaptation and enrichment of naturally available microbial consortia (Corsi and Seed 1995; Arnold et al. 1997; Juneson et al. 2001). However, the application of specialized strains of bacteria or fungi with styrene-catabolic activities was found to be advantageous for the treatment of waste gases containing this kind of hydrocarbon as a major component (Cox and Deshusses 1999; Jang et al. 2004, 2006; Jung and Park 2005; Cox et al. 1997; René et al. 2010).

Table 3.3 Styrene-metabolizing consortia and single representatives of the microflora of various biofilters for VOC removal

Microflora	Filter: carrier material	Reference
Mixed cultures, *Gordonia* sp. strain D7, *Pseudomonas* sp. strain D26	Biofilter: crushed wood and bark compost, 74% water content	Alexandrino et al. (2001)
Inoculated with activated sludge: *Tsukamurella* sp., *Pseudomonas* sp., *Sphingomonas* sp., *Xanthomonas* sp., γ-Proteobacteria	Biofilter: peat, about 10% burned clay and plastic filling; low-soluble nutrient mix and lime added to adjust nutrient content and pH	Arnold et al. (1997)
Biofilm dominated by the fungus *Exophiala jeanselmei*	Biofilter: Perlite	Cox et al. (1997)
Inoculum: garden soil in 0.015% (v/v) Tween-80	Biofilter: six types of activated carbon, polyurethane, and Perlite	Cox et al. (1993)
Long-term adapted mixed microbial culture	Biofilter: Perlite	Weigner et al. (2001)
Activated sludge	Trickle-bed air biofilter: coal particles	Lu et al. (2001)
Pseudomonas sp. IS-3	Biofilter: cubic polyurethane, minimal salt medium supplied	Kim et al. (2005)
Pseudomonas sp. SR-5	Biofilter: peat and/or ceramic, nutrient solution supplied	Jang et al. (2004, 2006)
Pseudomonas putida ST201	Packed tower biofilter: ceramic	Okamoto et al. (2003)
Rhodococcus pyridinovorans PYJ-1	Biofilter: compost	Jung and Park (2005)
Rhodococcus rhodochrous AL NCIMB 13259	Biofilter: peat and glass beads, water content and pH regulated	Zilli et al. (2003)
Sporothrix variecibatus	Biofilter: sieved Perlite	René et al. (2010)

3.5.8 Biosensors

Biosensor systems for a broad range of toxic aromatics including BTEX compounds are available in order to measure the relative bioavailability of these hydrocarbons in contaminated groundwater, soil, and air, as well as to assess the degree of bioremediation and removal. The *xylR*-, *xylS*-, or *tod*- regulators in combination with different reporter systems have been frequently used for these purposes showing a somewhat broad detection specificity (Keane et al. 2002; Rodriguez-Mozaz et al. 2006). A construction, in which the styrene-inducible regulator gene *styR* was combined with the reporter gene of beta-galactosidase (*lacZ*), recently allowed the formation of a styrene-sensitive biosensor (Alonso et al. 2003b). However, styrene oxide, phenylacetaldehyde, 2-phenylethanol, and toluene were shown to act as alternative inducers.

3.6 Conclusions

Despite of an extensive industrial usage of styrene which brings about significant anthropogenic releases in the environment, the ecological threat of this hydrocarbon is considerably limited due to its high volatility, its distinct chemical reactivity, and a pronounced susceptibility against biological transformations. In mammals and human, these transformations involve an initial cytochrome P450-mediated oxygenation yielding a mutagenic epoxide which, as a proven carcinogen, causes the most hazardous risk of chronic styrene exposure. Among pro- and eukaryotic microorganisms, styrene-catabolic activities were found to be wide-spread under aerobic and anaerobic conditions. Dioxygenation of the aromatic nucleus, followed by an extradiol-cleavage pathway of the resulting catechol as well as side-chain oxygenation of styrene to the central intermediate phenylacetic acid were found to be the two major mechanisms of mineralization, allowing microorganisms to utilize this hydrocarbon as the sole source of carbon and energy. Especially the latter pathway could be elucidated in members of the genus *Pseudomonas* on biochemical and genetic level. In addition, regulation of the so called *sty*-operon comprising the genes of a styrene monooxygenase StyA/StyB, a styrene oxide isomerase StyC, and a phenylacetaldehyde dehydrogenase StyD was characterized in detail. More recent studies have indicated a phylogenetically more diverse distribution of styrene monooxygenases among Actinobacteria. Interestingly, these enzymes differ from those ones typically found in pseudomonads in respect of an unusually fused structure as well as an absence of neighbored genes of the *sty*-operon. StyA1/StyA2B of *Rhodococcus opacus* 1CP is the first biochemically characterized representative of this group of flavin-dependent monooxygenases and evidence was provided for a novel transfer mechanism of reduced FAD between FAD:NADH oxidoreductase- and epoxidizing monooxygenase subunit.

In contrast to the mechanism of side-chain oxygenation, styrene catabolism by initial ring dioxygenation and *meta*-cleavage is a less specific degradation route since it is frequently observed for the mineralization of other (alkylated) aromatic hydrocarbons like benzene, toluene, xylene, and biphenyl. A degradation pathway for the latter compound enables *Rhodococcus jostii* RHA1 to grow on styrene.

Knowledge on the mechanisms and the distribution of styrene-catabolic pathways is not only interesting from a scientific point of view, but also relevant in respect of a biotechnological applicability. Styrene is an important volatile organic component of industrial waste gases and hence styrene-degrading microorganisms are of relevance in the field of biological waste gas treatment. Several styrene-catabolic enzymes like styrene monooxygenase, styrene oxide hydrolase, and styrene oxide isomerase are attractive biocatalytic tools. They can be applied to the highly enantioselective epoxidation of styrene into (*S*)-styrene oxide, to the resolution of racemic styrene oxide coupled with the preparation of enantiopure vicinal diols, and to the preparation of pure aromatic aldehydes, respectively.

Despite the amount of available information on microbial styrene degradation, a number of questions and speculations remain unanswered. In order to face two of them: (i) it seems that important mechanisms of aerobic styrene catabolism, e.g. by sequential monooxygenation of the aromatic nucleus, have not been traced out till yet, and (ii) it should not wonder if abundance of the *sty*-operon could be shown for others members of the class of *Gammaproteobacteria* than pseudomonads since a mobility of these genes has already been shown. Elucidation of these questions is not only interesting from an evolutionary point of view, but also it may lead to the identification of novel biocatalysts and further biotechnological applications.

Acknowledgment The authors are grateful to the team of the Department Environmental Microbiology, TU Bergakademie Freiberg, under supervision of Prof. Michael Schlömann for substantial and critical discussion. A special thank is dedicated to Janosch Gröning for his unremitting assistance. The corresponding author was supported by two predoctoral fellowships from Deutsche Bundesstiftung Umwelt (DBU) and Fulbright.

References

Alexander M (1990) The environmental fate of styrene. SIRC Rev 1:33–42

Alexandrino M, Knief C, Lipski A (2001) Stable-isotope-based labeling of styrene-degrading microorganisms in biofilters. Appl Environ Microbiol 67:4796–4804

Alonso S, Bartolomé-Martín D, del Alamo M, Díaz E, García JL, Perera J (2003a) Genetic characterization of the styrene lower catabolic pathway of *Pseudomonas* sp. strain Y2. Gene 319:71–83

Alonso S, Navarro-Llorens JM, Tormo A, Perera J (2003b) Construction of a bacterial biosensor for styrene. J Biotechnol 102:301–306

Anderson AJ, Dawes EA (1990) Occurrence, metabolism, metabolic role, and industrial use of bacterial polyhydroxyalkanoates. Microbiol Rev 54:450–472

Araya A, Chamy R, Mota M, Alves M (2000) Biodegradability and toxicity of styrene in the anaerobic digestion process. Biotechnol Lett 22:1477–1481

Archelas A, Furstoss R (1997) Synthesis of enantiopure epoxides through biocatalytic approaches. Annu Rev Microbiol 51:491–525

Archer IVJ (1997) Epoxide hydrolases as asymmetric catalysts. Tetrahedron 53:15617–15662

Arnold M, Reittu A, Von Wright A, Martikainen PJ, Suihko ML (1997) Bacterial degradation of styrene in waste gases using a peat filter. Appl Microbiol Biotechnol 48:738–744

Badone D, Guzzi U (1994) Synthesis of the potent and selective atypical beta-adrenergic agonist SR 59062 A. Bioorg Med Chem Lett 16:1921–1924

Bae J-W, Shin S, Raj SM, Lee SE, Lee S-G, Jeong Y-J, Park S (2008) Construction and characterization of a recombinant whole-cell biocatalyst of *Escherichia coli* expressing styrene monooxygenase under the control of arabinose promoter. Biotechnol Bioprocess Eng 13:69–76

Bae J-W, Doo E-H, Shin S-H, Lee S-G, Jeong Y-J, Park J-B, Park S (2010) Development of a recombinant *Escherichia coli*-based biocatalyst to enable high styrene epoxidation activity with high product yield on energy source. Process Biochem 45:147–152

Baikalov I, Schröder I, Kaczor-Grzeskowiak M, Grzeskowiak K, Gunsalus RP, Dickerson RE (1996) Structure of the *Escherichia coli* response regulator NarL. Biochemistry 35:11053–11061

Bartels I, Knackmuss HJ, Reineke W (1984) Suicide inactivation of catechol 2,3-dioxygenase from *Pseudomonas putida* mt-2 by 3-halocatechols. Appl Environ Microbiol 47:500–505

Beltrametti F, Marconi AM, Bestetti G, Galli E, Ruzzi M, Zennaro E (1997) Sequencing and functional analysis of styrene catabolism genes from *Pseudomonas fluorescens* ST. Appl Environ Microbiol 63:2232–2239

Bernasconi S, Orsini F, Sello G, Colmegna A, Galli E, Bestetti G (2000) Bioconversion of substituted styrenes to the corresponding enantiomerically pure epoxides by a recombinant *Escherichia coli* strain. Tetrahedron Lett 41:9157–9161

Besse P, Veschambre H (1994) Chemical and biological synthesis of chiral epoxides. Tetrahedron 50:8885–8927

Bestetti G, Galli E, Ruzzi M, Baldacci G, Zennaro E, Frontali L (1984) Molecular characterization of a plasmid from *Pseudomonas fluorescens* involved in styrene degradation. Plasmid 12:181–188

Bestetti G, Galli E, Benigni C, Orsini F, Pelizzoni F (1989) Biotransformation of styrenes by a *Pseudomonas putida*. Appl Microbiol Biotechnol 30:252–256

Bond JA (1989) Review of the toxicology of styrene. Crit Rev Toxicol 19:227–249

Braun-Lüllemann A, Majcherczyk A, Huttermann A (1997) Degradation of styrene by white-rot fungi. Appl Microbiol Biotechnol 47:150–155

Breuer M, Ditrich K, Habicher T, Hauer B, Kesseler M, Stuermer R, Zelinski T (2004) Industrial methods for the production of optically active intermediates. Angew Chem Intl Ed 43: 788–824

Burback BL, Perry JJ (1993) Biodegradation and biotransformation of groundwater pollutant mixtures by *Mycobacterium vaccae*. Appl Environ Microbiol 59:1025–1029

Cho MC, Kang D-O, Yoon BD, Lee K (2000) Toluene degradation pathway from *Pseudomonas putida* F1: substrate specificity and gene induction by 1-substituted benzenes. J Ind Microbiol Biotech 25:163–170

CMAI (2005) In: Chemical market associates, Inc. 2006 World styrene analysis. Houston, Texas, USA, Nov 2005

Corsi RL, Seed L (1995) Biofiltration of BTEX: media, substrate, and loadings effects. Environ Prog 14:151–158

Coschigano PW, Young LY (1997) Identification and sequence analysis of two regulatory genes involved in anaerobic toluene metabolism by strain T1. Appl Environ Microbiol 63:652–660

Cox HHJ (1995) Styrene removal from waste gas by the fungus *Exophiala jeanselmei* in a biofilter. PhD Thesis, University of Groningen, The Netherlands

Cox HH, Deshusses MA (1999) Biomass control in waste air biotrickling filters by protozoan predation. Biotechnol Bioeng 62:216–224

Cox HHJ, Houtman JHM, Doddema HJ, Harder W (1993) Enrichment of fungi and degradation of styrene in biofilters. Biotechnol Lett 15:737–742

Cox HH, Faber BW, Heiningen WNV, Radhoe H, Doddema HJ, Harder W (1996) Styrene metabolism in *Exophiala jeanselmei* and involvement of a cytochrome P-450-dependent styrene monooxygenase. Appl Environ Microbiol 62:1471–1474

Cox HH, Moerman RE, van Baalen S, van Heiningen WN, Doddema HJ, Harder W (1997) Performance of a styrene-degrading biofilter containing the yeast *Exophiala jeanselmei*. Biotechnol Bioeng 53:259–266

de Jong E, Beuling EE, van der Zwan RP, de Bont JAM (1990) Degradation of veratryl alcohol by *Penicillium simplicissimum*. Appl Microbiol Biotechnol 34:420–425

Delhomenie M-C, Heitz M (2005) Biofiltration of air: a review. Crit Rev Biotechnol 25:53–72

Deshusses MA (1997) Biological waste air treatment in biofilters. Curr Opin Biotechnol 8: 335–339

Di Gennaro P, Ferrara S, Ronco I, Galli E, Sello G, Papacchini M, Bestetti G (2007) Styrene lower catabolic pathway in *Pseudomonas fluorescens* ST: identification and characterization of genes for phenylacetic acid degradation. Arch Microbiol 188:117–125

European Union Risk Assessment Report Styrene (2002) Styrene, Part I–Environment. European Communities, Luxembourg

Ferrández A, Prieto MA, García JL, Díaz E (1997) Molecular characterization of PadA, a phenylacetaldehyde dehydrogenase from *Escherichia coli*. FEBS Lett 406:23–27

Ferrández A, Miñambres B, García B, Olivera ER, Luengo JM, García JL, Díaz E (1998) Catabolism of phenylacetic acid in *Escherichia coli*. Characterization of a new aerobic hybrid pathway. J Biol Chem 273:25974–25986

Fu MH, Alexander M (1992) Biodegradation of styrene in samples of natural environments. Environ Sci Technol 26:1540–1544

Gallegos MT, Schleif R, Bairoch A, Hofmann K, Ramos JL (1997) Arac/XylS family of transcriptional regulators. Microbiol Mol Biol Rev 61:393–410

Gibbs BF, Mulligan CN (1997) Styrene toxicity: an ecotoxicological assessment. Ecotoxicol Environ Saf 38:181–194

Grbić-Galić D, Churchman-Eisel N, Mraković I (1990) Microbial transformation of styrene by anaerobic consortia. J Appl Bacteriol 69:247–260

Grebe TW, Stock JB (1999) The histidine protein kinase superfamily. Adv Microb Physiol 41:139–227

Gross R, Hauer B, Otto K, Schmid A (2007) Microbial biofilms: new catalysts for maximizing productivity of long-term biotransformations. Biotechnol Bioeng 98:1123–1134

Guan C, Ju J, Borlee BR, Williamson LL, Shen B, Raffa KF, Handelsman J (2007) Signal mimics derived from a metagenomic analysis of the gypsy moth gut microbiota. Appl Environ Microbiol 73:3669–3676

Guillemin MP, Berode M (1988) Biological monitoring of styrene: a review. Am Ind Hyg Assoc J 49:497–505

Gursky L, Nikodinovic-Runic J, Feenstra K, O'Connor K (2009) In vitro evolution of styrene monooxygenase from *Pseudomonas putida* CA-3 for improved epoxide synthesis. Appl Microbiol Biotechnol 85:995–1004

Han JH, Park MS, Bae JW, Lee EY, Yoon YJ, Lee S-G, Park S (2006) Production of (*S*)-styrene oxide using styrene oxide isomerase negative mutant of *Pseudomonas putida* SN1. Enzyme Microb Technol 39:1264–1269

Hartmans S (1995) Microbial degradation of styrene. In: Biotransformations: microbiological degradation of health risk compounds. Elsevier Sci 32:227–238

Hartmans S, Smits JP, van der Werf MJ, Volkering F, de Bont JAM (1989) Metabolism of styrene oxide and 2-phenylethanol in the styrene-degrading *Xanthobacter* strain 124X. Appl Environ Microbiol 55:2850–2855

Hartmans S, van der Werf MJ, De Bont JAM (1990) Bacterial degradation of styrene involving a novel flavin adenine dinucleotide-dependent styrene monooxygenase. Appl Environ Microbiol 56:1347–1351

Hattori K, Nagano M, Kato T, Nakanishi I, Imai K, Kinoshita T, Sakane K (1995) Asymmetric synthesis of FR165914: a novel beta-3-adrenergic agonist with a benzocycloheptene structure. Bioorg Med Chem Lett 5:2821–2824

Higashimura T, Sawamoto M, Hiza T, Karaiwa M, Tsuchii A, Suzuki T (1983) Effect of methyl substitution on microbial degradation of linear styrene dimers by two soil bacteria. Appl Environ Microbiol 46:386–391

Hollmann F, Lin P-C, Witholt B, Schmid A (2003) Stereospecific biocatalytic epoxidation: the first example of direct regeneration of a FAD-dependent monooxygenase for catalysis. J Am Chem Soc 125:8209–8217

Ikura Y, Yoshida Y, Kudo T (1997) Physiological properties of two *Pseudomonas mendocina* strains which assimilate styrene in a two-phase (solvent-aqueous) system under static culture conditions. J Ferment Bioeng 83:604–607

Itoh N, Yoshida K, Okada K (1996) Isolation and identification of styrene-degrading *Corynebacterium* strains, and their styrene metabolism. Biosci Biotechnol Biochem 60:1826–1830

Itoh N, Hayashi K, Okada K, Ito T, Mizuguchi N (1997a) Characterization of styrene oxide isomerase, a key enzyme of styrene and styrene oxide metabolism in *Corynebacterium* sp. Biosci Biotech Biochem 61:2058–2062

Itoh N, Morihama R, Wang J, Okada K, Mizuguchi N (1997b) Purification and characterization of phenylacetaldehyde reductase from a styrene-assimilating *Corynebacterium* strain, ST-10. Appl Environ Microbiol 63:3783–3788

Itoh N, Matsuda M, Mabuchi M, Dairi T, Wang J (2002) Chiral alcohol production by NADH-dependent phenylacetaldehyde reductase coupled with in situ regeneration of NADH. Eur J Biochem 269:2394–2402

Itoh N, Nakamura M, Inoue K, Makino Y (2007) Continuous production of chiral 1,3-butanediol using immobilized biocatalysts in a packed bed reactor: promising biocatalysis method with an asymmetric hydrogen-transfer bioreduction. Appl Microbiol Biotechnol 75:1249–1256

Jang JH, Hirai M, Shoda M (2004) Styrene degradation by *Pseudomonas* sp. SR-5 in biofilters with organic and inorganic packing materials. Appl Microbiol Biotechnol 65:349–355

Jang JH, Hirai M, Shoda M (2006) Enhancement of styrene removal efficiency in biofilter by mixed cultures of *Pseudomonas* sp. SR-5. J Biosci Bioeng 102:53–59

Juneson C, Ward OP, Singh A (2001) Microbial treatment of a styrene-contaminated air stream in a biofilter with high elimination capacities. J Ind Microbiol Biotechnol 26:196–202

Jung I-G, Park C-H (2005) Characteristics of styrene degradation by *Rhodococcus pyridinovorans* isolated from a biofilter. Chemosphere 61:451–456

Kantz A, Chin F, Nallamothu N, Nguyen T, Gassner GT (2005) Mechanism of flavin transfer and oxygen activation by the two-component flavoenzyme styrene monooxygenase. Arch Biochem Biophys 442:102–116

Keane A, Phoenix P, Ghoshal S, Lau PCK (2002) Exposing culprit organic pollutants: A review. J Microbiol Methods 49:103–119

Kim J, Ryu HW, Jung DJ, Lee TH, Cho K-S (2005) Styrene degradation in a polyurethane biofilter inoculated with *Pseudomonas* sp. IS-3. J Microbiol Biotechnol 15:1207–1213

Kim HS, Lee OK, Lee SJ, Hwang S, Kim SJ, Yang S-H, Park S, Lee EY (2006) Enantioselective epoxide hydrolase activity of a newly isolated microorganism, *Sphingomonas echinoides* EH-983, from seawater. J Mol Catal B: Enzym 41:130–135

Knackmuss H-J, Hellwig M, Lackner H, Otting W (1976) Cometabolism of 3-methylbenzoate and methylcatechols by a 3-chlorobenzoate utilizing *Pseudomonas*: accumulation of (+)-2,5-dihydro-4-methyl- and (+)-2,5-dihydro-2-methyl-5-oxo-furan-2-acetic acid. Eur J Appl Microbiol 2:267–276

Kuhn D, Kholiq MA, Heinzle E, Bühler B, Schmid A (2010) Intensification and economic and ecological assessment of a biocatalytic oxyfunctionalization process. Green Chem 12: 815–827

Labbé D, Garnon J, Lau PC (1997) Characterization of the genes encoding a receptor-like histidine kinase and a cognate response regulator from a biphenyl/polychlorobiphenyl-degrading bacterium, *Rhodococcus* sp. strain M5. J Bacteriol 179:2772–2776

Lafeuille J-L, Buniak M-L, Vioujas M-C, Lefevre S (2009) Natural formation of styrene by cinnamon mold flora. J Food Sci 74:276–283

Lau PC, Wang Y, Patel A, Labbé D, Bergeron H, Brousseau R, Konishi Y, Rawlings M (1997) A bacterial basic region leucine zipper histidine kinase regulating toluene degradation. Proc Natl Acad Sci USA 94:1453–1458

Leoni L, Ascenzi P, Bocedi A, Rampioni G, Castellini L, Zennaro E (2003) Styrene-catabolism regulation in *Pseudomonas fluorescens* ST: phosphorylation of StyR induces dimerization and cooperative DNA-binding. Biochem Biophys Res Commun 303:926–931

Leoni L, Rampioni G, Stefano VD, Zennaro E (2005) Dual role of response regulator StyR in styrene catabolism regulation. Appl Environ Microbiol 71:5411–5419

Leuthner B, Heider J (1998) A two-component system involved in regulation of anaerobic toluene metabolism in *Thauera aromatica*. FEMS Microbiol Lett 166:35–41

Lin H, Qiao J, Liu Y, Wu Z-L (2010) Styrene monooxygenase from *Pseudomonas* sp. LQ26 catalyzes the asymmetric epoxidation of both conjugated and unconjugated alkenes. J Mol Catal B: Enzym 67:236–241

Liu Z, Michel J, Wang Z, Witholt B, Li Z (2006) Enantioselective hydrolysis of styrene oxide with the epoxide hydrolase of *Sphingomonas* sp. HXN-200. Tetrahedron: Asymmetry 17:47–52

Long MT, Bartholomew BA, Smith MJ, Trudgill PW, Hopper DJ (1997) Enzymology of oxidation of tropic acid to phenylacetic acid in metabolism of atropine by *Pseudomonas* sp. strain AT3. J Bacteriol 179:1044–1050

Louie TM XS, Xie Xun L (2003) Coordinated production and utilization of FADH2 by NADP(H)-flavin oxidoreductase and 4-hydroxyphenylacetate 3-monooygenase. Biochemistry 42:7509–7517

Lu C, Lin M-R, Lin J (2001) Removal of styrene vapor from waste gases by a trickle-bed air biofilter. J Hazard Mater B82:233–245

Madison LL, Huisman GW (1999) Metabolic engineering of poly (3-hydroxyalkanoates): from DNA to plastic. Microbiol Mol Biol Rev 63:21–53

Makino Y, Dairi T, Itoh N (2007) Engineering the phenylacetaldehyde reductase mutant for improved substrate conversion in the presence of concentrated 2-propanol. Appl Microbiol Biotechnol 77:833–843

Malhautier L, Khammar N, Bayle S, Fanlo J-L (2005) Biofiltration of volatile organic compounds. Appl Microbiol Biotechnol 68:16–22

Marconi AM, Beltrametti F, Bestetti G, Solinas F, Ruzzi M, Galli E, Zennaro E (1996) Cloning and characterization of styrene catabolism genes from *Pseudomonas fluorescens* ST. Appl Environ Microbiol 62:121–127

Marín M, Pérez-Pantoja D, Donoso R, Wray V, González B, Pieper DH (2010) Modified 3-oxoadipate pathway for the biodegradation of methylaromatics in *Pseudomonas reinekei* MT1. J Bacteriol 192:1543–1552

Mars AE, Kasberg T, Kaschabek SR, van Agteren MH, Janssen DB, Reineke W (1997) Microbial degradation of chloroaromatics: use of the *meta*-cleavage pathway for mineralization of chlorobenzene. J Bacteriol 179:4530–4537

McClay K, Boss C, Keresztes I, Steffan RJ (2005) Mutations of toluene-4-monooxygenase that alter regiospecificity of indole oxidation and lead to production of novel indigoid pigments. Appl Environ Microbiol 71:5476–5483

Miyamoto K, Okuro K, Ohta H (2007) Substrate specificity and reaction mechanism of recombinant styrene oxide isomerase from *Pseudomonas putida* S12. Tetrahedron Lett 48:3255–3257

Mooney A, O'Leary ND, Dobson ADW (2006a) Cloning and functional characterization of the *styE* gene, involved in styrene transport in *Pseudomonas putida* CA-3. Appl Environ Microbiol 72:1302–1309

Mooney A, Ward PG, O'Connor KE (2006b) Microbial degradation of styrene: biochemistry, molecular genetics, and perspectives for biotechnological applications. Appl Microbiol Biotechnol 72:1–10

Mosqueda G, Ramos JL (2000) A set of genes encoding a second toluene efflux system in *Pseudomonas putida* DOT-T1E is linked to the *tod* genes for toluene metabolism. J Bacteriol 182:937–943

Mutti A (1988) Styrene exposure and serum prolactin. J Occup Med 30:481–482

Mutti A, Falzoi M, Romanelli A, Bocchi MC, Ferroni C, Franchini I (1988) Brain dopamine as a target for solvent toxicity: effects of some monocyclic aromatic hydrocarbons. Toxicology 49:77–82

Nikodinovic-Runic J, Flanagan M, Hume AR, Cagney G, O'Connor KE (2009) Analysis of the *Pseudomonas putida* CA-3 proteome during growth on styrene under nitrogen-limiting and non-limiting conditions. Microbiology 155:3348–3361

Nöthe C, Hartmans S (1994) Formation and degradation of styrene oxide stereoisomers by different microorganisms. Biocatalysis 10:219–225

O'Connor K, Buckley CM, Hartmans S, Dobson AD (1995) Possible regulatory role for nonaromatic carbon sources in styrene degradation by *Pseudomonas putida* CA-3. Appl Environ Microbiol 61:544–548

O'Connor KE, Dobson AD, Hartmans S (1997) Indigo formation by microorganisms expressing styrene monooxygenase activity. Appl Environ Microbiol 63:4287–4291

Okamoto K, Izawa M, Yanase H (2003) Isolation and application of a styrene-degrading strain of *Pseudomonas putida* to biofiltration. J Biosci Bioeng 95:633–636

O'Leary ND, O'Connor KE, Duetz W, Dobson ADW (2001) Transcriptional regulation of styrene degradation in *Pseudomonas putida* CA-3. Microbiology 147:973–979

O'Leary ND, Duetz WA, Dobson ADW, O'Connor KE (2002a) Induction and repression of the *sty* operon in *Pseudomonas putida* CA-3 during growth on phenylacetic acid under organic and inorganic nutrient-limiting continuous culture conditions. FEMS Microbiol Lett 208: 263–268

O'Leary ND, O'Connor KE, Dobson ADW (2002b) Biochemistry, genetics and physiology of microbial styrene degradation. FEMS Microbiol Rev 26:403–417

Olivera ER, Miñambres B, García B, Muñiz C, Moreno MA, Ferrández A, Díaz E, García JL, Luengo JM (1998) Molecular characterization of the phenylacetic acid catabolic pathway in *Pseudomonas putida* U: the phenylacetyl-CoA catabolon. Proc Natl Acad Sci USA 95: 6419–6424

Omori T, Jigami Y, Minoda Y (1974) Microbial oxidation of α-methylstyrene and *β*-methylstyrene. Agr Biol Chem 38:409–415

Orru RV, Faber K (1999) Stereoselectivities of microbial epoxide hydrolases. Curr Opin Chem Biol 3:16–21

Otto K, Hofstetter K, Roethlisberger M, Witholt B, Schmid A (2004) Biochemical characterization of StyAB from *Pseudomonas* sp. strain VLB120 as a two-component flavin-diffusible monooxygenase. J Bacteriol 186:5292–5302

Paca J, Koutsky B, Maryska M, Halecky M (2001) Styrene degradation along the bed height of perlite biofilter. J Chem Technol Biotechnol 76:873–878

Panke S, Witholt B, Schmid A, Wubbolts MG (1998) Towards a biocatalyst for (*S*)-styrene oxide production: characterization of the styrene degradation pathway of *Pseudomonas* sp. strain VLB120. Appl Environ Microbiol 64:2032–2043

Panke S, De Lorenzo V, Kaiser A, Witholt B, Wubbolts MG (1999) Engineering of a stable whole-cell biocatalyst capable of (*S*)-styrene oxide formation for continuous two-liquid-phase applications. Appl Environ Microbiol 65:5619–5623

Panke S, Wubbolts MG, Schmid A, Witholt B (2000) Production of enantiopure styrene oxide by recombinant *Escherichia coli* synthesizing a two-component styrene monooxygenase. Biotechnol Bioeng 69:91–100

Panke S, Held M, Wubbolts MG, Witholt B, Schmid A (2002) Pilot-scale production of (*S*)-styrene oxide from styrene by recombinant *Escherichia coli* synthesizing styrene monooxygenase. Biotechnol Bioeng 80:33–41

Park J-B, Bühler B, Habicher T, Hauer B, Panke S, Witholt B, Schmid A (2006a) The efficiency of recombinant *Escherichia coli* as biocatalyst for stereospecific epoxidation. Biotechnol Bioeng 95:501–512

Park MS, Bae JW, Han JH, Lee EY, Lee S-G, Park S (2006b) Characterization of styrene catabolic genes of *Pseudomonas putida* SN1 and construction of a recombinant *Escherichia coli* containing styrene monooxygenase gene for the production of (*S*)-styrene oxide. J Microbiol Biotechnol 16:1032–1040

Patrauchan MA, Florizone C, Eapen S, Gómez-Gil L, Sethuraman B, Fukuda M, Davies J, Mohn WW, Eltis LD (2008) Roles of ring-hydroxylating dioxygenases in styrene and benzene catabolism in *Rhodococcus jostii* RHA1. J Bacteriol 190:37–47

Przybulewska K, Wieczorek A, Nowak A (2006) Isolation of microorganisms capable of styrene degradation. Polish J Environ Stud 15:777–783

Qaed AA, Lin H, Tang D-F, Wu Z-L (2010) Rational design of styrene monooxygenase mutants with altered substrate preference. Biotechnol Lett (Online publication)

Qi WW, Vannelli T, Breinig S, Ben-Bassat A, Gatenby AA, Haynie SL, Sariaslani FS (2007) Functional expression of prokaryotic and eukaryotic genes in *Escherichia coli* for conversion of glucose to p-hydroxystyrene. Metab Eng 9:268–276

Rao AVR, Gurjar MK, Kaiwar V (1992) Enantioselective catalytic reductions of ketones with new four membered oxazaborolidines: Application to (*S*)-Tetramisole. Tetrahedron: Asymmetry 3:859–862

Reizer J, Saier MH (1997) Modular multidomain phosphoryl transfer proteins of bacteria. Curr Opin Struct Biol 7:407–415

René ER, Veiga MC, Kennes C (2010) Biodegradation of gas-phase styrene using the fungus *Sporothrix variecibatus*: impact of pollutant load and transient operation. Chemosphere 79:221–227

Rodriguez-Mozaz S, Lopez de Alda MJ, Barceló D (2006) Biosensors as useful tools for environmental analysis and monitoring. Anal Bioanal Chem 386:1025–1041

Rueff J, Teixeira JP, Santos LS, Gaspar JF (2009) Genetic effects and biotoxicity monitoring of occupational styrene exposure. Clin Chim Acta 399:8–23

Rustemov SA, Golovleva LA, Alieva RM, Baskunov BP (1992) New pathway of styrene oxidation by a *Pseudomonas putida* culture. Microbiologica 61:1–5

Ruzzi M, Zennaro E (1989) pEG plasmid involved in styrene degradation: molecular dimorphism and integration of a segment into the chromosome. FEMS Microbiol Lett 50:337–343

Santos PM, Blatny JM, Bartolo ID, Valla S, Zennaro E (2000) Physiological analysis of the expression of the styrene degradation gene cluster in *Pseudomonas fluorescens* ST. Appl Environ Microbiol 66:1305–1310

Santos PM, Leoni L, Bartolo ID, Zennaro E (2002) Integration host factor is essential for the optimal expression of the *styABCD* operon in *Pseudomonas fluorescens* ST. Res Microbiol 153:527–536

Schulze B, Wubbolts MG (1999) Biocatalysis for industrial production of fine chemicals. Curr Opin Biotechnol 10:609–615

Shirai K, Hisatsuka K (1979) Production of β-phenethyl alcohol from styrene by *Pseudomonas* 305-STR-1–4. Agric Biol Chem 43:1399–1406

Sielicki M, Focht DD, Martin JP (1978) Microbial transformations of styrene and [14C] styrene in soil and enrichment cultures. Appl Environ Microbiol 35:124–128

Smith MR (1990) The biodegradation of aromatic hydrocarbons by bacteria. Biodegradation 1:191–206

Smith MR (1994) The physiology of aromatic hydrocarbons degrading bacteria. In: Ratledge C (ed) Biochemistry of microbial degradation. Kluwer, Dordrecht, pp 347–378

Tischler D, Eulberg D, Lakner S, Kaschabek SR, van Berkel WJH, Schlömann M (2009) Identification of a novel self-sufficient styrene monooxygenase from *Rhodococcus opacus* 1CP. J Bacteriol 191:4996–5009

Tischler D, Kermer R, Gröning JAD, Kaschabek SR, van Berkel WJH, Schlömann M (2010) StyA1 and StyA2B from *Rhodococcus opacus* 1CP: A multifunctional styrene monooxygenase system. J Bacteriol 192:5220–5227

Tuschii A, Suzuki T, Takahara Y (1977) Microbial degradation of styrene oligomer. Agric Biol Chem 41:2417–2421

US Inventory of Toxic Compounds (2001) TRI92. Toxics release inventory public data. Office of Pollution Prevention and Toxics, US EPA, Washington, DC, 94. Available at http://www.epa.gov

Utkin I, Yakimov M, Matveeva L, Kozlyak E, Rogozhin I, Solomon Z, Bezborodov A (1991) Degradation of styrene and ethylbenzene by *Pseudomonas* species Y2. FEMS Microbiol Lett 77:237–242

van Berkel WJH, Kamerbeek NM, Fraaije MW (2006) Flavoprotein monooxygenases, a diverse class of oxidative biocatalysts. J Biotechnol 124:670–689

van Hellemond EW, Janssen DB, Fraaije MW (2007) Discovery of a novel styrene monooxygenase originating from the metagenome. Appl Environ Microbiol 73:5832–5839

Velasco A, Alonso S, Garcia JL, Perera J, Diaz E (1998) Genetic and functional analysis of the styrene catabolic cluster of *Pseudomonas* sp. strain Y2. J Bacteriol 180:1063–1071

Verhoef S, Wierckx N, Westerhof RGM, de Winde JH, Ruijssenaars HJ (2009) Bioproduction of p-hydroxystyrene from glucose by the solvent-tolerant bacterium *Pseudomonas putida* S12 in a two-phase water-decanol fermentation. Appl Environ Microbiol 75:931–936

Ward PG, de Roo G, O'Connor KE (2005) Accumulation of polyhydroxyalkanoate from styrene and phenylacetic acid by *Pseudomonas putida* CA-3. Appl Environ Microbiol 71:2046–2052

Ward PG, Goff M, Donner M, Kaminsky W, O'Connor KE (2006) A two step chemo-biotechnological conversion of polystyrene to a biodegradable thermoplastic. Environ Sci Technol 40:2433–2437

Warhurst AM, Fewson CA (1994) A review. Microbial metabolism and biotransformation of styrene. J Appl Bacteriol 77:597–606

Warhurst AM, Clarke KF, Hill RA, Holt RA, Fewson CA (1994) Metabolism of styrene by *Rhodococcus rhodochrous* NCIMB 13259. Appl Environ Microbiol 60:1137–1145

Weigner P, Páca J, Loskot P, Koutský B, Sobotka M (2001) The start-up period of styrene degrading biofilters. Folia Microbiol 46:211–216

Zilli M, Converti A, Di Felice R (2003) Macrokinetic and quantitative microbial investigation on a bench-scale biofilter treating styrene-polluted gaseous streams. Biotechnol Bioeng 83:29–38

Chapter 4
Bacterial Decolorization and Degradation of Azo Dyes

Poonam Singh, Leela Iyengar and Anjali Pandey

4.1 Introduction

Color has always been a part of human life since long. All colorants, until the middle of nineteenth century, were of natural origin. Manufacturing of synthetic dyes started in 1856 and the first compound to be synthesized was aniline purple. By the beginning of the twenteeth century, synthetic dyes almost completely replaced natural compounds. Synthetic dyes represent an important class of industrial chemicals, which are used extensively in textile, leather tanning, paper production, food technology, agriculture, light harvesting array, coloring and pharmaceuticals (Dafale et al. 2010).

All aromatic compounds absorb electromagnetic energy, but only those that absorb the visible wavelength region ($\sim$350–700 nm) are colored. Dyes contain chromophoric groups—decolorised electron systems with conjugated double bonds and auxochromes that cause or intensify the color of the chromophore. Usual chromophores are –C=C–, –C=N–, –C=O, –N=N–, $–NO_2$ and quinonoid rings. Examples of auxochromes are $–NH_2$, $–SO_3H$, –COOH and –OH (Christie 2001). Dyes may be classified according to types of chromophores in their structure or as per the method of application. Based on their chemical structures, dyes can be classified into 20–30 different types. Over one million tonnes of synthetic dyes are produced worldwide every year for dyeing and printing. Azo dyes, which are aromatic compounds with one or more –N=N– groups, constitute

P. Singh
Division of Toxicology, Central Drug Research Institute, Lucknow, India

L. Iyengar
Centre for Post Graduate Studies, Jain University, Bangalore, India

A. Pandey (✉)
Department of Chemistry, Indian Institute of Technology, Kanpur, India
e-mail: anjali@iitk.ac.in

S. N. Singh (ed.), *Microbial Degradation of Xenobiotics*,
Environmental Science and Engineering, DOI: 10.1007/978-3-642-23789-8_4,

around 50% of synthetic dyes used in commercial applications (Ollgaard et al. 1999; Stolz 2001; Zollinger 1991).

In textile dyeing, all applied dyes do not bind to the fabric and large amounts are lost in wastewater. Depending on the class of the dye and application, the loss in wastewaters can vary from 2% for basic dyes to as high as 50% for reactive dyes, leading to severe contamination of surface and ground waters in the viscinity of dyeing industries (Ganesh et al. 1994; O'Neill et al. 1999). Many dyes are visible in water at concentrations as low as 1 mg/L. Textile- processing wastewaters with dye content in the range of 10–200 mg/L are highly colored. Release of colored effluents into the surface water leads to aesthetic problems, obstructs light penetration and oxygen transfer into water bodies, hence affecting aquatic life (Öztürk and Abdullah 2006; Vandevivere et al. 1998; Weisburger 2002). Some of the dyes and their degradation products are carcinogenic in nature (Levine 1991). A review of the mutagenicity of effluents showed that textile and other dye-related industries produce consistently more potent wastewaters when compared to other industrial discharges (Houk 1992). Studies by Rajaguru et al. (2002) and Umbuzeiro et al. (2005) have shown that azo dyes contribute to mutagenic activity of ground and surface waters polluted by textile effluents. Thus, the removal of color from textile effluent has been a major concern.

Dyes are photolytically stable and thus highly persistent in natural environments. For instance, the half life of hydrolysed Reactive Blue (RB 19) is reported to be 46 years at pH 7 and 25°C (Hao et al. 2000). Azo compounds are xenobiotic in nature, as only one natural azo compound i.e. 4–4′ dihydroxy azo benzene, has been reported so far (Gill and Strauch 1984). Hence, they can be expected to be recalcitrant to biodegradation. The conventional methods of wastewater treatment and/or dilution with domestic wastewater followed by treatment are not sufficient to alleviate dye related hazards. Hence, alternative methods are required to detoxify the dyes in wastewater.

Physiochemical methods, such as adsorption, coagulation, chemical oxidation, photodegradation or membrane filtration, are used for the color removal from dye containing effluents (Vandervivere et al. 1998; Swaminathan et al. 2003; Behnajady et al. 2004; Wang et al. 2004; Golab et al. 2005; Lopez-Grimau and Gutierrez 2005). Many of these methods have severe limitations, such as large amount of sludge formation, incomplete removal, possible formation of hazardous products, requirement of safe sludge disposal, high energy input and the cost.

Advanced oxidation processes (AOP) and biological methods have been considered as better technologies for treatment of dye containing wastewaters. AOP utilizes strong oxidizing agents and/or catalysts (TiO_2, ZnO_2, Mn, Fe) in the presence or absence of an irradiation source. These involve mainly the generation of (OH) radical for the destruction of refractory and hazardous pollutants (Vandevivere et al. 1998; Alaton et al. 2002; Al-Kdasi et al. 2004). This process is highly cost intensive and energy consuming. These constraints have led to the consideration of biological methods as an attractive option for the treatment of these wastewaters. Biological methods are generally considered environment friendly as they can lead to complete mineralization of organic pollutant at low cost.

It is generally observed that dyes resist biodegradation in conventional activated sludge treatment units (Vandervivere et al. 1998). Ekici et al. (2001) tested the stability of amines formed from selected azo dye metabolites in activated sludge. They were relatively stable under wastewater treatment conditions. However, microorganisms are highly versatile and have developed enzyme systems for the degradation of many xenobiotics, including azo dyes, nitro aromatics and haloorganics, after long exposure. These microorganisms, which include fungi, bacteria, yeast and algae, can decolorize and even completely mineralize many azo dyes under certain environmental conditions (Banat et al. 1996; Razo-Flores et al. 1997a, 1997b; Van der Zee et al. 2001a). Many reviews are available on the physiochemical and microbiological methods for decolorization of azo dyes (Banat et al. 1996; Delee et al. 1998, Vandevivere et al. 1998; O'Neill et al. 1999; McMullan et al. 2001; Stolz 2001; Rai et al. 2005; Van der Zee and Villaverde 2005; Dos Santos et al. 2007; Dafale et al. 2010; Srinivasan and Veeraraghavan 2010; Saratale et al. 2011).

This chapter has focus on the bacterial decolorization of azo dyes under different redox conditions, mechanisms by which bacteria decolorize azo dyes, fate of aromatic amines generated by this reaction and its application for the treatment of wastewaters.

4.2 Decolorization of Azo Dyes by Bacteria

Anaerobic decolorization of azo dyes was first investigated using intestinal anaerobic bacteria (Walker and Ryan 1971). Later studies showed that many bacterial genera, which include anaerobic, facultatively anaerobic and aerobic, reduce –N=N– bond under different redox conditions (Oxyspring et al. 1996; Chang and Lin 2000). Reductive cleavage of the –N=N– bond is the initial step of the bacterial degradation of azo dyes by bacteria, both in the presence or absence of oxygen.

4.2.1 Azo Dye Decolorization Under Methanogenic Conditions

Anaerobic methanogenesis, from complex organic compounds, requires the coordinated participation of many different trophic groups of bacteria, which include acidogenic, acetogenic and methanogenic bacteria (Kasper and Wuhrmann 1978). It has been widely reported that azo dyes are gratuitously reduced by anaerobic sludges, anaerobic sediments and anaerobic enrichment cultures (Brown and Hamburger 1987; Weber and Wolfe 1987; Chung et al. 1992; Manu and Choudhary 2003). Dye decolorization, under methanogenic conditions, is dependent on the type of primary electron donor. Acetate and other volatile fatty acids are normally poor electron donors, whereas ethanol, glucose, H_2/CO_2 and formate

are more effective electron donors for dye reduction (Tan et al. 1999; Dos Santos et al. 2003; Dos Santos 2005; Pearce et al. 2006). Complex substrates, such as whey and tapioca, have also been used for dye decolorization under methanogenic conditions (Chinwetkitvanich et al. 2000; Willetts et al. 2000; Talarposhti et al. 2001; Yoo et al. 2001; Isik and Sponza 2005a; Van der Zee and Villaverde 2005).

Extensive studies have been carried out to determine the role of diverse groups of bacteria in the decolorization of azo dyes under methanogenic conditions. It is currently accepted that azo dye reduction is due to a co-metabolic reaction, in which the reducing equivalents formed during the degradation of primary substrates can be chemically transferred to the azo dyes. In anaerobic consortia, reducing equivalents are formed by fermentative bacteria and consumed by methanogens to form methane. However, it might happen that some methanogens utilize the reducing equivalents towards dye reduction instead of methanogenesis. Thus, fermentative bacteria and methanogenic archaea may play an important role in the reduction of azo dyes. Carliell et al. (1996) and Razo-Flores et al. (1997a) have associated the decolorization with methanogens, whereas studies by other investigators showed that acidogenic as well as methanogenic bacteria contribute to dye decolorization (Chinwetkitvanich et al. 2000; Bras et al. 2001; Dos Santos et al. 2006). Recently, Firmino et al. (2010) assessed decolorization of Congo Red as well as real textile wastewater in one- and two-stage anaerobic treatment systems. Results indicated that fermentative bacteria in acidogenic reactor played a major role in dye decolorizaton. Further the minimum electron donor concentration required to sustain dye reduction was much higher than the stoichiometric amount. Additionally, a decrease on the hydraulic retention time (from 24 to 12 h) did not significantly affect decolorisation, indicating that electron transfer was not a concern. Experiments with real textile wastewater showed low decolorisation efficiencies in both systems, most likely due to the presence of dyes not susceptible to reductive decolorisation under these experimental conditions. Use of molecular methods to characterize the microbial populations in anaerobic-baffled reactors treating industrial dye waste showed that members of the γ-proteobacteria, together with sulfate reducing bacteria populations were prominent members of mixed bacterial populations. Along with these, a methanogenic population dominated by *Methanosaeta* species and *Methanomethylovorans hollandica* contributed to the treatment of industrial wastewater (Plumb et al. 2001).

Sulphate reducing bacteria (SRB) proliferate under anaerobic conditions if the electron acceptor is present. Available literature indicates that the decolorization in the presence of SRB is due to extracellular chemical reduction with sulphide (Chinwetkitvanich et al. 2000; Yoo et al. 2000; Diniz et al. 2002). The chemical contribution of other biogenic reductants for the decolourisation of azo dyes under anaerobic conditions may involve cysteine, ascorbate or Fe^{2+} (Yoo 2002; Van der Zee et al. 2003).

Reduction under anaerobic conditions appears to be nonspecific as most of the azo compounds, with varied structures, were decolorized, although the rate of decolorization was dependent on the added organic carbon source as well as the dye structure (Bromley-Challenor et al. 2000; Stolz 2001). Further, there was no

correlation between decolorization rate and molecular weight indicating that decolorization is not a specific process and cell permeability is not important for decolorization. Thus, anaerobic azo dye decolorization is a fortuitous process, where dye might act as an acceptor of electrons supplied by carriers of the electron transport chain. Alternatively, decolorization might be attributed to non-specific extracellular reactions occurring between reduced compounds generated by the anaerobic biomass (Van der Zee et al. 2001a). The kinetics of decolorization under methanogenic conditions have been studied by various investigators (Carliell et al. 1994; Weber 1996; Van der Zee et al. 2001a; Isik and Sponza 2005a, b). With a few specific dyes, autocatalysis by quinone- like compounds, formed during azo dye reduction, contributes significantly to the overall reduction process (Van der Zee et al. 2001b, Méndez-Paz et al. 2003). For example, formation of 1-amino-2-naphthol during reduction of AO7 accelerated the decolorization by several times. A few attempts have been made to study the correlation of anaerobic biodegradability and the electrochemical characteristics of azo dyes in details. However, rapid decolorization was observed when the ORP values dropped below –50 mV (Bromley-Challenor et al. 2000; Guo et al. 2006).

4.2.2 Azo Dye Decolorization Under Anoxic/Microaerophylic Conditions

By definition, anoxic condition refers to the absence of oxygen, where as microaerophylic environments require very low level of dissolved oxygen (DO), but not completely oxygen-free. Azo dye decolorization under anoxic conditions has been reported by mixed, defined microbial consortia as well as pure bacterial cultures (Nigam et al. 1996; Kapdan et al. 2000; Padmavathy et al. 2003; Moosvi et al. 2005). Several pure bacterial strains belonging to *Pseudomonas*, *Aeromonas*, *Bacillus*, *Enterococcus*, *Lactobacillus*, *Proteus*, *Shewanella*, *Rhodopseudomonas* and other bacterial genera decolorized azo dyes under microaerophylic conditions (Chen et al. 1999, 2003a; Chang et al. 2001; Yu et al. 2001; Li et al. 2004; Pearce et al. 2006; Rajamohan and Karthikeyan 2006; Bafana and Chakarbarti 2008; Wang et al. 2008; Silveria et al. 2009). Azo dye decolorization, under these conditions, generally requires complex organic sources, such as yeast extract or peptone or a combination of complex organic source and carbohydrate (Chen et al. 2003a; Khehra et al. 2005). Unlike dye decolorization under methanogenic conditions, where glucose is the preferred substrate, its suitability for anoxic dye decolorization by facultative anaerobes and fermenting bacteria, seems to vary, depending on the bacterial culture. Decolorization of Mordant Yellow 3 by *Sphingomonas xenophaga* Strain BN6 was greatly enhanced by glucose, whereas significant decrease in azo dye decolorization, has been reported in the presence of *P. leuteola*, *Aeromonas* sp., *Shewanella putrefaciens* and a few other mixed cultures (Haug et al. 1991; Kapdan et al. 2000; Chang et al. 2001; Chen et al. 2003a; Khalid et al. 2008a). Negative effect of glucose on anoxic decolorization

has been attributed either to the decrease in pH due to acid formation or to catabolic repression which commonly occurs in bacterial systems. This often causes an inhibition of the transcription of cyclic-AMP dependent genes, which may include gene encoding for azo reductase (Chen et al. 2003a).

Effect of chemical structure, on azo dye decolorization by *Aeromonas hydrophila* and *Pseudomonas luteola*, was investigated by Hsueh et al. (2009) and Hsueh and Chen (2008). Properties of substituent group on aromatic ring could affect the efficiency of decolorization. The azo dyes with electron-withdrawing groups (e.g., sulfo group) at para and ortho to azo bond could be more preferred for color removal than those at meta position. However, due to steric hindrance of ortho substituent groups, azo dyes with para substituent could be more favorable than those with ortho substituent for decolorization. Thus, the ranking of the position for the electron-withdrawing substituent in azo dyes for decolorization was para > ortho > meta. Regarding the electronic effect, azo dyes with stronger electron-withdrawing group (e.g., sulfo group) at specific positions (e.g., at para) could be more easily decolorized than those with a carboxyl group. Thus, their study suggested that both the positions of substituents on the aromatic ring and the electronic characteristics of substituents in azo dyes all significantly affected biodecolorization by these organisms.

Wastewaters from textile processing and dye-stuff manufacture industries contain substantial amounts of salts in addition to azo dye residues. There are a few reports on salinity effects on dye-degrading bacteria (Dafale et al. 2008; Khalid et al. 2008b; Tan et al. 2009a, b). Studies with four azo dyes in the presence of varying concentrations of NaCl (0–100 g L^{-1}) with *Shewanella putrefaciens* strain AS96 showed that under static, low oxygen conditions, the bacterium decolorized 100 mg dye L^{-1} at salt concentrations up to 60 g NaCl L^{-1}. There was an inverse relationship between the velocity of the decolorization reaction and salt concentration over the range between 5 and 60 g NaCl L^{-1} and at dye concentrations between 100 and 500 mg L^{-1}. High-performance liquid chromatography analysis demonstrated the presence of 1-amino-2-naphthol, sulfanilic acid and nitroaniline as the major metabolic products of the azo dyes, which could be further degraded by a shift to aerobic conditions. These findings show that *Shewanella* could be effective for the treatment of dye-containing industrial effluents containing high concentrations of salt (Khalid et al. 2008b). A salt-tolerant bacterium was isolated from the surface soil of a pharmaceutical factory, which could efficiently decolorize azo dyes. The strain was identified as *Exiguobacterium* sp. according to its morphological characteristics and 16S rRNA gene sequence analysis (Tan et al. 2009a). Dynamics of microbial community for X-3B wastewater decolorization during adaptation to high salt as well as metal ions is reported by Tan et al. (2009b). Results of sequencing batch tests showed that the microbial community could decolorize high concentration of dye (1100 mg L^{-1}) even in the presence of high salt concentrations (150 g L^{-1} NaCl) and some metal ions, such as Mg^{2+}, Ca^{2+} (1–10 mmol L^{-1}) and Pb^{2+} (1 mmol L^{-1}). 16S rDNA-based molecular analysis techniques demonstrated that the microbial community shifted during the acclimatization process affected by salt or metal ions. Some stains similar to *Bacillus*, *Sedimentibacter*,

Pseudomonas, *Clostridiales*, *Streptomyces* and some uncultured clones were present during the dynamic succession. Dye decolorization, by mixed as well as pure cultures, has been reviewed by Pearce et al. (2003). Azo dye decolorization by halophilic and halotolerant microorganisms has been recently reviewed by Amoozegar et al. (2010).

4.2.3 Azo Dye Decolorization Under Aerobic Conditions

Several bacterial strains, that can aerobically decolorize azo dyes, have been isolated during the past few years. Many of these strains require organic carbon sources, as they cannot utilize dye as the growth substrate (Stolz 2001; Kodam et al. 2005). *P. aeruginosa* decolorized a commercial tannery and textile dye, Navitan Fast blue S5R, in the presence of glucose under aerobic conditions. This organism was also able to decolorize various other azo dyes (Nachiyar and Rajkumar 2003). There are only very few bacteria that are able to grow on azo compounds as the sole carbon source. These bacteria cleave –N=N– bonds reductively and utilize amines as the source of carbon and energy for their growth. Such organisms are specific to their substrate. Examples of bacterial strains with this trait are *Xenophilus azovorans* KF 46 (previously *Pseudomonas* sp. KF46) and *Pigmentiphaga kullae* K24 (previously *Pseudomonas* sp. K24), which can grow aerobically on carboxy-orange I and carboxy-orange II, respectively (Zimmermann et al. 1982; Kulla et al. 1983). These organisms, however, could not grow on structurally analogous sulfonated dyes, acid orange 20 (Orange I) and AO7. Coughlin et al. (1999) have reported the isolation of a *Sphingomonas* sp, strain 1CX, an obligate aerobe, which can grow on an azo dye, AO7, as the sole carbon, energy and nitrogen source. This strain degraded only one of the component amines (1-amino 2-naphthol) formed during AO7 decolorization. 4-aminobenzesulfonate (4-ABS) degradation, however, required the additional presence of an unidentified strain, SAD4i (Coughlin et al. 2003). Sphingomonas ICX could also decolorize several azo dyes consisting of either 1-amino-2-naphthol or 2-amino-1-naphthol coupled via the azo bond to a phenyl or naphthyl moiety (Coughlin et al. 1999). Similar azo dyes, such as AO6 or AO20, which lack these structures, were not decolorized. Three bacterial strains, that could utilize either AO7 or acid red 88 as sole carbon sources, were isolated from soil and sewage samples and were identified as *Bacillus* sp. OY1-2, *Xanthomonas* sp. NR25-2 and *Pseudomonas* sp. PR41-1 (Sugiura et al. 1999). Four bacterial species have been isolated using methyl red as the sole carbon source. Two of these strains have been identified as *Vibrio logei* and *P. nitroreducens.* Amine products were not detected in the culture medium, indicating their degradation (Adedayo et al. 2004). Recently, Ruiz-Arias et al. (2010) have reported that three bacterial strains, pertaining to the genera *Pseudomonas*, *Arthrobacter* and *Rhizobium* could decolorize AO7.

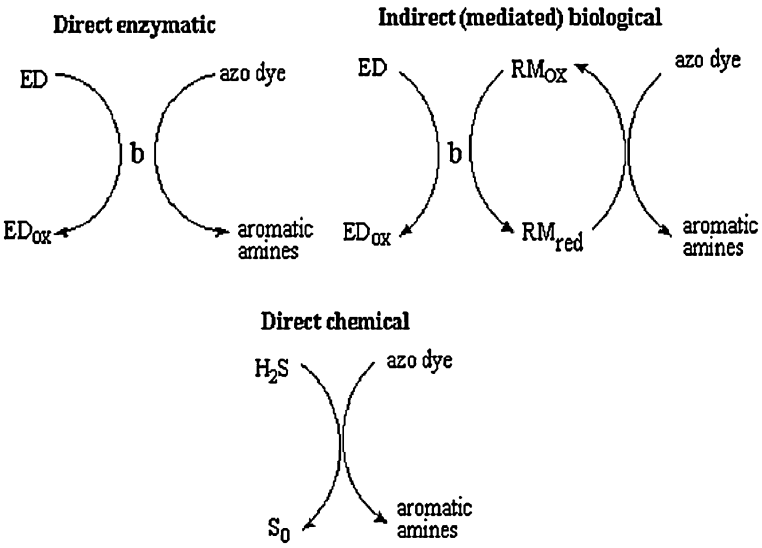


Fig. 4.1 Schematic representation of different mechanisms of anaerobic azo dye reduction. *RM* Redox mediator, *ED* Electron donor, *b* baceria (enzymes). *Source* Pandey et al. (2007)

4.2.4 Mechanism of Azo Dye Reduction

The azo bond (–N=N–) cleavage involves a transfer of four-electrons which proceeds through two stages at the azo linkage. Two electrons are transferred to the azo dye in each stage, which acts as final electron acceptor. The reduction may involve different mechanisms, such as enzymatic (Zimmermann et al. 1982, 1984; Rafii et al. 1990; Haug et al. 1991; Suzuki et al. 2001; Maier et al. 2004), involvement of low molecular weight redox mediators (Van der zee and Cervantes 2009), chemical reduction by biogenic reductants like sulphide (Yoo et al. 2001; Diniz et al. 2002) or a combination of these. Further the location of the reaction can be intracellular or extracellular (Zimmermann et al. 1982; Rafii et al. 1990). Different mechanism of anaerobic azo dye reduction are given in Fig. 4.1.

4.2.4.1 Role of Azoreductases in Decolorization of Azo Dyes

Enzymes that catalyze the reduction of azo groups are termed azoreductases. Utilizing NADH and/or NADPH as an electron donor, azoreductase can decolorize azo dyes into corresponding aromatic amines by reductive cleavage of azo bonds. There are two types of azo reductases: oxygen sensitive, secreted by anaerobic or under microaerophyllic conditions and oxygen insensitive azoreductases present in obligate aerobic bacteria. The presence of azoreductases in anaerobic bacteria,

that decolorized sulfonated azo dyes during growth on solid or complex media, was first reported by Rafii et al. (1990). These strains belonged mainly to the genera *Clostridium* and *Eubacterium.* Azoreductases from these strains were oxygen–sensitive and were produced constitutively and released extracellularly. Later investigations made with *C. perfringens* showed that azo dye reduction is catalyzed by an enzyme presumed to be flavin adenine dinucleotide dehydrogenase, which can also reduce nitro aromatic compounds (Rafii and Cerniglia 1995). Immunoelectron microscope analysis showed that the enzyme was secreted as it was synthesized (Rafii and Cerniglia 1995). The gene for this enzyme from *C. perfringens* has been cloned and expressed in *Escherichia coli* (Rafii and Coleman 1999). In spite of such extensive studies on oxygen-sensitive azo reductases from anaerobic bacteria by Rafii and coworkers, the source of NADH necessary for the extracellular enzyme activity is still not clear.

Role of azoreductases for the decolorization of azo dyes under anaerobic conditions has been recently reviewed (Sandhya 2010). Bacterial oxygen-insensitive azoreductases can be classified into two types: one is monomeric flavin-free enzymes containing a putative NAD(P)H binding motif and the other is polymeric flavin-dependent enzymes. However, based on structure, flavin dependency, and dinucleotide preference, azoreductases can be classified into three groups (Chen et al. 2010). Phylogenetic analysis has also complemented the grouping scheme at the molecular level.

The genes encoding oxygen-insensitive flavin dependent azoreductases have been cloned from *Bacillus* sp. OY1-2, *Escherichia coli, Enterococcus faecalis*, *Rhodobacter sphaeroides, Pseudomonas aeruginosa* and *Staphylococcus aureus.* Biochemical characteristics and protein structures of many bacterial FMN-dependent azoreductases have been recently determined (Chen et al. 2005; Ito et al. 2006; Liu et al. 2007; Wang et al. 2007). Two monomeric flavin-free azoreductases from *Xenophilus azovorans* KF46F (Zimmermann et al. 1982) and *Pigmentiphaga kullae* K24 (Zimmermann et al. 1984; Blumel and Stolz 2003) have been described. Polymeric azoreductases are strikingly different from monomeric flavin-free azoreductases with respect to both their structural and biochemical requirements for catalytic process. Three monomeric flavin-free azoreducatses, two from *Pigmentiphaga kullae* K24 and one from Xenophilus *azovorans* KF46F show very narrow substrate specificity (Blumel et al. 2002; Blumel and Stolz 2003; Chen et al. 2010). On the other hand, polymeric flavin-dependent azoreductase families can catalyze reduction of substrates which vary in both chemical nature and size (Chen 2006; Ooi et al. 2009). It is to be mentioned that purified oxygen insensitive azo reductases, from *Enterobacter agglomerans* and *Pseudomonas aeruginosa*, are monomers and the former enzyme shows a broad substrate range (Moutaouakkil et al. 2003; Nachiyar and Rajakumar 2005). However both these enzymes have not been characterized for the presence of flavin.

As the presence of azo compounds in the environment are due mainly to human activities, reduction of azo dyes may not be the primary roles of these enzymes. Most azo compounds are generally very polar and/or large molecules,

which render them difficult to diffuse through the cell membrane. Moreover, the reduction products of azo compounds from azoreductases (i.e., corresponding aromatic amines) are usually more toxic than azo substrates. Thus, the physiological role of azoreductase has recently been a subject of debate. Recent studies by Liu et al. (2009) showed that the physiological role of *Escherichia coli* azoreductase AzoR is a quinone reductase providing resistance to thiol-specific stress caused by electrophilic quinones. Based on the phylogeny of azoreductase enzyme from different organisms and comparing it with the small subunit rRNA-based phylogeny of the organisms, Bafana and Chakrabarti (2008) have reported that the two phylogenies were found to be incongruent, indicating several events of lateral transfer of azoreductase gene between phylogenetically diverse organisms. Azoreductases have been shown to reduce azo compounds via a Ping Pong Bi Bi mechanism (Liu et al. 2007). The currently accepted mechanism requires two cycles of NADPH-dependent reduction of FMN to FMNH2, which reduces the azo substrate to a hydrazine in the first cycle, and reduces the hydrazine to two amines in the second cycle. The mechanism is supported by the detection of the predicted hydrazine intermediate by mass spectrometry in reaction mixtures (Bin et al. 2004). Recently, Ryan et al. (2010) have proposed a novel mechanism, where the second reduction step occurs independently of the enzyme.

Recent studies have shown that there is a significant induction of enzymes, laccase, veratryl alcohol oxidase and DCIP (2,6-dichlorophenol-indophenol) reductase, in dye degrading bacteria. This suggests that apart from azoreductase, these enzymes may also have an active role in dye decolorization (Senan and Abraham 2004; Olukanni et al. 2010; Saratale et al. 2010; Swati et al. 2010).

4.2.4.2 Reductive Decolorization of Azo Dye in the Presence of Redox Mediators

Redox mediators are known to be effective for the reductive transformation of many contaminants by bacteria under anaerobic conditions (Van der Zee 2001b, Dos Santos et al. 2003, 2007). Their role in azo dye decolorization has been extensively studied and has been reviewed recently by Van der Zee and Cervantes (2009). The compounds, that accelerate the electron transfer from a primary electron donor to a terminal electron acceptor, are known as redox mediators. They will only be effective for azo dye reduction, if they lower the reaction's activation energy (Van der Zee and Cervantes 2009). For this purpose, their E0′ should ideally be in between those of the two eventual half reactions, the reduction of an azo dye and the oxidation of a primary electron donor. Reductive azo dye decolorization occurs in two steps in presence of redox mediators, the first step is a non-specific enzymatic mediated reduction. The second step is a chemical reoxidation of the mediator by the azo dyes (Keck et al. 1997). It is well documented that the redox mediators act to accelerate the transfer of reducing equivalents to the terminal electron acceptor, minimize the stearic hindrance of dye molecule and decrease the activation energy of the chemical reaction and enhance the reaction

rate by one to several orders of magnitude (Bragger et al. 1997; Van der Zee et al. 2000; Moir et al. 2001; Cervantes 2002, 2006; Dos Santos 2005; Costa et al. 2010). Flavin-based compounds, like FAD, FMN and Riboflavin, as well as quinone-based compounds, like AQS, AQDS and lawsone are well known redox mediators (Semde et al. 1998; Cervantes et al. 2000; Rau et al. 2002; Field and Brady 2003; Dos Santos et al. 2004, 2005; Encinas-Yocupicio et al. 2003). Since most azo compounds are generally polar and/or large molecules, their permeability to cell membrane is low. As a result, the reducing activity cannot be dependant on the intracellular uptake of the dye (Robinson et al. 2001). It is demonstrated that the azo reductase activity of cell extracts can be much higher than that of intact cells and that the cell membrane forms a barrier for dyes and mediators (Kudlich et al. 1997; Russ et al. 2000). Hence, mediated azo dye reduction by *Sphingomonas xenophaga*, is presumably associated with the membrane-bound respiratory chain enzyme NADH: ubiquinone oxidoreductase (Kudlich et al. 1997; Rau et al. 2002). Several other species may also use similar membrane-associated enzyme systems. Another mechanism could be the participation of intracellular enzymes, which reduces a mediator. Reduced mediator diffuses through the cell membrane and transfers electrons to the dye. Such an example of red ox mediator is Lawsone, the only quinone with a mediator-feasible E0′ that could be reduced by the enzymes (Rau and Stolz 2003).

Recently, immobilized redox mediators for azo dye reduction have been reported by several investigators. Anthraquinone, immobilized by entrapment in calcium alginate (CA), Polyvinyl alcohol (PVA)-H_3BO_3 and agar, was used repeatedly for the decolorization of azo dyes by salt tolerant bacteria. Results showed that the decolorization rate of CA immobilized anthraquinone was retained over 90% of their original value (Guo et al. 2007). The anthraquinone and quinone-reducing consortium were co-immobilized by entrapment in calcium alginate. The co-immobilized beads exhibited higher decolorization rate for many kinds of azo dyes (Su et al. 2009). Quinoid redox mediators, including 1,2-naphthoquinone-4-sulfonate and anthraquinone-2,6-disulfonate, were adsorbed on anion exchange resins in order to explore their catalytic effects on the reductive decolorization of azo dyes by anaerobic granular sludge. Immobilized quinones preserved their catalytic properties once adsorbed on the surface of resins. Addition of immobilized quinones to sludge incubations increased the rate of decolorization of azo dyes by 8.8-fold as compared to controls lacking quinines (Cervantes et al. 2010). Other matrix used for immobilizing redox mediators include polyurethane foam, metal oxides nanopartcles, polypyrrole (PPy) on activated carbon felt (Alvarez et al. 2010; Jing et al. 2009; Lu et al. 2010).

Van der Zee et al. (2003) have reported that activated carbon, which is known to have quinone groups at its surface, enhanced dye decoloraization. This was probably one of the first examples of biocatalysis mediated by activated carbon. Recently, Mezohegyi et al. (2007) showed that both electron-mediating capability and specific surface area of activated carbon contribute to higher reduction rates of AO7.

4.2.4.3 Azo Dye Decolorization by Biogenic Inorganic Compounds

Chemical contribution to reductive decolorisation of azo dyes may involve biogenic reducing agents, such as cysteine, sulphide, ascorbate or Fe^{2+} that are formed as end products of metabolic reactions under anaerobic conditions (Yoo 2002; Van der Zee et al. 2003). Both biological and chemical reduction of azo dyes are accelerated by the addition of redox mediators, such as quinine and flavin structures (Cervantes et al. 2007). It has been seen that H_2S generation by SRB resulted in the extracellular decolorization of acid dye, reactive orange-96 (Yoo et al. 2000; Diniz et al. 2002). Diniz et al. (2002) have also reported that azo bond cleavage in the presence of a strain of SRB, with lactate as the carbon source, is extra cellular and non enzymatic. Albuquerque et al. (2005) investigated the decolorization of Remazol Brilliant Violet 5R or an acid dye (Acid Orange 7) under different experimental conditions to assess the role of biogenic sulfide. It was observed that the chemically mediated reduction of the azo bond coupled to biological sulfate reduction seemed to account for the high decolorization of both dyes. Based on the analysis and demonstration in upflow anaerobic sludge bed reactors, the relative importance of chemical dye reduction mechanisms in high rate anaerobic bioreactors was shown to be small due to the high biomass levels in the reactors (Van der Zee et al. 2003).

4.3 Biodegradation of Aromatic Amines

Aromatic amines formed from reductive decolorization of azo dyes, either by biotic or abiotic processes, are generally toxic and are known or suspected carcinogens (Ekici et al. 2001). Further, they may undergo transformations, when they are released into water bodies. These may be more or less toxic than the parent compounds. Thus, the total degradation of the products formed is the only solution for abating the toxicity of these xenobiotics. In this section, mineralization of aromatic amines under different red-ox conditions, is briefly discussed.

4.3.1 Fate of Aromatic Amines Under Anaerobic Conditions

Mineralization of a few simple aromatic amines has been reported under methanogenic conditions. Complete mineralization of azodisalicylate was observed under methanogenic conditions (Razo-Flores et al. 1997a). 5-aminosalicylate (5-ASA), initially formed from dye reduction, was further degraded to CO_2 and CH_4. Partial mineralization of mordent Orange 1 in a continuous UASBR was reported by Donlon et al. (1997). The azo dye was reductively cleaved to p-phenylenediamine and 5-ASA. Out of which, 5-ASA was mineralized, while p-phenylenediamine got accumulated. Complete mineralization of 5-ASA as well

as 2-aminobenzoic acid (2ABA) by methanogenic sludge was reported by Kalyuzhnyl et al. (2000). A strain of *Citrobacter freundii* has been isolated from 5-ASA degrading methanogenic consortium. Methanogenic enrichment culture degraded 5-ASA to CH_4, CO_2, NH_4, whereas *Citrobacter freundii* strain WA1 reduced 5-ASA with simultaneous deamination to 2-hydroxy benzyl alcohol during anaerobic growth in the presence of other organic substrates, such as pyruvate or glucose (Savelieva et al. 2004) Decolorization and partial degradation of Acid Orange (AO6), AO7 and Acid Orage 52 by methanogenic sludge has been reported by Yemashova et al. (2004). A complete bioreduction of all these azo dyes was observed in expanded graular sludge bioreactor. 4-ABS and 1-amino-2-naphthol were persistent under anaerobic conditions, whereas 2,4-dihydroxyaniline was completely mineralized under anaerobic conditions. N,N-di,ethylphenylediamine formed from Acid orange-52 was transformed to 1,4-phenylenediamine. Many reports have shown that sulfonated aromatic amines, which are constituents of many azo dyes, are non biodegradable under methanogenic conditions (Razo-Flores et al. 1996; Tan et al. 1999). However, recent report by González-Gutiérrez et al. (2009) has shown that even aminonaphthylsulfonates were partially degraded under methanogenic conditions. Degradation was dependent on reactions such as hydroxylation, carboxylation and redox reactions carried out by enzymes and by means of the interactions with the activated carbon as a carrier and with the substrate, dextrose.

4.3.2 Degradation of Aromatic Amines Under Anoxic/Microaerophylic Conditions

There are a few reports on the degradation of aromatic amines under anoxic conditions. Drzyzga and Blotevogel (1997) reported that diphenylamine (DPA) was co-metabolically degraded in anoxic sediment–water batch enrichments. In gas chromatography-mass spectrometry (GC–MS) measurements, aniline was identified as a major breakdown product of the diphenylamine structure. The fate of the other carbon ring system remained unclear, because neither benzene or other ring cleavage products of diphenylamine were observed. *Bacillus fusiformis* KMK 5 could tolerate and degrade azo dyes, Disperse Blue 79 (DB79) and Acid Orange 10 (AO10) under anoxic conditions. A complete mineralization of DB79 and AO10 at the concentration of 1.5 g/l was observed within 48 h (Kolekar et al. 2008). The complete biodegradation of azo dye, Fast Acid Red GR was observed by *Shewanella decolorationis* S1 under microaerophilic conditions (Xu et al. 2007). *S. decolorationis* S12 could use a range of carbon sources for azo dye decolorization, including lactate, formate, glucose and sucrose, with lactate being the optimal carbon source. Sulfonated aromatic amines were not detected during the biotransformation of Fast Acid Red GR, while H_2S was formed. The decolorizing products, aniline, 1,4-diaminobenzene and 1-amino-2-naphthol, were completely degraded under these conditions. This strain was capable of azo dye decolorization

at high rates even under anaerobic conditions in the presence of Fe(III). In fact, the dye decolorization was enhanced by the presence of Fe(III). When 1 mM Fe(III) was added, the methyl red decolorizing efficiency was 72.1% after incubation for 3 h, whereas the decolorizing efficiency was only 60.5% in Fe(III)-free medium. However, amines were not degraded (Xu et al. 2007). A strain of photosynthetic bacterium, *Rhodopseudomonas palustris* W1, isolated from a lab-scale anaerobic moving bed biofilm reactor treating textile effluent decolorized Reactive Black 5 (RB5) and partially degrade amines under anaerobic condition (Wang et al. 2008). Based on the proposed pathway for partial mineralization, this is probably first example of ring cleaveage of naphthyl and benzene amino sulfonates under anaerobic conditions.

4.3.3 Fate of Aromatic Amines Under Aerobic Conditions

General degradation pathways for benzene and naphthyl amines under aerobic conditions involve the participation of either mono or dioxygenases (specific hydroxylases) leading to the formation of dihydroxyaromatic compounds (Bayley and Barbour 1984). The dihydroxylated compounds are subsequently dearomatized by the cleavage of aromatic ring, which is catalysed by specific dioxygenases. Ring cleavage can occur either at ortho or meta position to the hydroxyl groups, leading to the formation of intermediates of central metabolic routes, such as tricaboxylic acid cycle. A group of aromatic amines, that are more difficult to degrade even under aerobic conditions, are represented by aryl sulfonates. Presence of sulfonate group on aromatic ring not only confers the xenobiotic character, but also recalcitrant nature to these compounds, as not many aromatic sulfonates are known among natural compounds (Alexander and Lustigham 1996). Further, polar nature of sulfonyl group requires highly specific transport enzymes for their entry into the cell, thus rendering these compounds resistant to biodegradation by bacteria utilizing normal aromatics. Aminobenzene sulfonates (ABS) and aminonaphthyl sulfonates (ANS) are structural components of many azo dyes.

Extensive studies have been carried out on aniline degradation. It is reported that a novel microorganism, *Delftia acidovorans* strain HY99 is capable of aerobic and anaerobic degradation of aniline (Kahng et al. 2000). Strain HY99 was found to aerobically metabolize aniline via catechol and 2-hydroxymuconic semialdehyde intermediates, and via p-aminobenzoate under anaerobic conditions linked to nitrate reduction.

There have been a few reports on the bacterial consortia and pure cultures, which can utilize ABS and ANS sulfonates, as sole carbon and energy sources (Nortemann et al. 1986; Thurnheer et al. 1986; Locher et al. 1989; Perei et al. 2001; Singh et al. 2008; Wang et al. 2009). There appears to be very few reports on 2-ABS degradation. A pure cuture, *Alcaligenes* sp. (Jahnke et al. 1990) and a bacterial consortium (Singh et al. 2008) degraded 2-ABS. 4-ABS is the most susceptible for biodegradation (Tan et al. 2005; Carvalho et al. 2008).

A consortium consisting of *Hydrogenophaga palleroni* (Strain S1) as well as *Agrobacterium radiobacter* (Strain S2) and few bacterial strains degrade 4-ABS (Feigal and Knackmuss 1993; Dangmann et al. 1996). Mineralization of 4-ABS by a bacterial strain PNS-1, *Agrobacterium* sp. has been reported by Singh et al. (2006). Two bacterial strains (ICX and SAD4i) were isolated from the laboratory scale Rotating Drum Bioreactor, were able to mineralize in co-culture up to 90% of added AO7 under aerobic conditions (Coughlin et al. 2002). During mineralization of AO7, strain ICX reduced the azo bond under aerobic conditions and also degraded the resulting cleavage product 1-amino-2-naphthol. Interestingly, strain SAD4i consumed the other cleavage product, i.e. sulfanilic acid. Not many reports are available on 3-aminobenzenesulphonate (3-ABS) degradation (Kolbener et al. 1994). Nachiyar et al. (2007) have reported that *Pseudomonas aeruginosa* CLRI BL22, which can decolorize Navitan Blue under aerobic conditions, can mineralize 3-ABS. Unlike 2- and 4-ABS degrading strains, which can utilize ABS as the sole carbon and energy source, *Pseudomonas aeruginosa* required glucose for 3-ABS degradation. Barsing et al. (2011) investigated the mineralization of aromatic amines resulting from decolorization of azo dyes by a novel bacterial consortium (TJ-2). Three bacterial strains in the consortium, were identified as *Pseudomonas pseudoalcaligenes*, *Pseudomonas citronellolis* and *Pseudomonas testosterone*). It was observed that aromatic amine mineralization depended upon the structure of aromatic amine. Para- and meta-hydroxy substituted aromatic amines were easily mineralized as compared to ortho-substituted which undergoes autoxidation when exposed to oxygen.

A few bacterial cultures, utilizing naphthyl amines as the sole organic carbon source, have been also reported. *Sphingomonas sp*. strain ICX decolorized AO7 and degraded 1-amino-2-naphthol (Coughlin et al. 1999), while *P. aeruginosa* degraded 1,4-diaminonaphthalene (Nachiyar and Rajkumar 2004). Sulfonated naphthylamines are among the most common products of bacterial decolorization of azo dyes. Pure cultures belonging to the genera *Pseudomonas*, *Moraxella* and *Arthrobacter,* which can either degrade 2-aminonaphthyl sulfonate (2ANS) or utilize it as sulfur source, have been isolated (Nortemann et al. 1986; Wittich et al. 1988; Ohe et al. 1990; Rozgaj and Glancer 1992).

Extensive studies have been carried on the degradation of 6-aminonaphthyl-2-sulfonate (6A2NS) (Keck et al. 2006). *Comamonas testosteroni* A3 grew on naphthalene-2-sulfonate, but not on substituted naphthalenesulfonates, as the sole source of carbon and energy. The most intensively studied bacterial strain with the ability to degrade naphthalenesulfonates is *Sphingomonas xenophaga BN6*, which degrades various amino- and hydroxynaphthalenesulfonates to the corresponding amino- or hydroxysalicylic acids (Stolz 1999). A complete mineralization requires a co-culture consisting of *Sphingomonas xenophaga* BN6 as well as *Pesudomonas* sp. BN9 or other bacterial strains, which degrade substituted salicylic acids. In contrast to the degradation of (substituted) naphthalenesulfonic acids to the corresponding salicylates, degradation of (substituted) salicylates requires different metabolic pathways (Keck et al. 2006). So far, only one bacterial strain (*Pseudaminobacter salicylatoxidans* BN12) is known to completely degrade

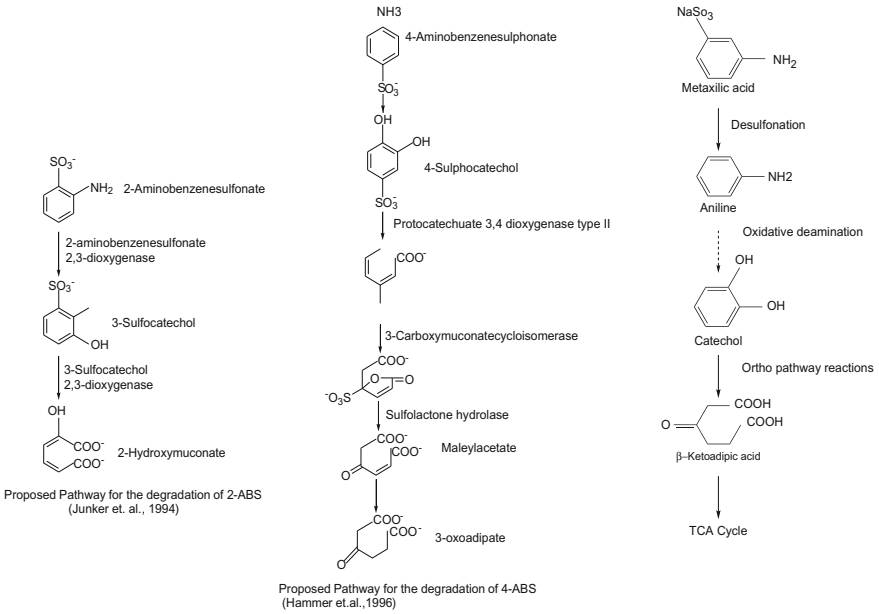


Fig. 4.2 Proposed pathways for the degradation of aminobenzene sulfonates (ABS)

6A2NS (Kampfer et al. 1999). Available literature on the degradation of sulfonated aromatic amines (SAA) shows that cultures generally have a narrow substrate range. Mixtures of aromatic sulfonates can only be degraded by mixed bacterial consortia, which harbor diverse activities for congeneric substrates and metabolites (Hopper 1991). Proposed pathways for ABS and ANS degradation are shown in Figs. 4.2 and 4.3.

There have been many reports in recent years where aromatic amines are either not detected or not formed in stoitiometric quantities after dye reduction, indicating their degradation. However, metaboloic pathways are still unknown in many cases. Two sulfonated aromatic amines (benzene-based and naphthalene-based), formed from decolorization of remazol brilliant violet 5R during anaerobic phase of Sequential batch reactor, were degraded in the aerobic phase (Cinar et al. 2008). Activity of two aerobic enzymes, catechol 1,2-dioxygenase and catechol 2,3-dioxygenase, were measured as indicators of aromatic amine biodegradation.

4.3.4 The Autoxidation of Aromatic Amines

A few aromatic amines, formed during the anaerobic reduction of the azo dyes, are unstable under aerobic conditions and undergo auto-oxidation reactions. Examples are 5-ASA, phenylenediamine, o-amino hydroxy and o-dihydroxy naphthalenes

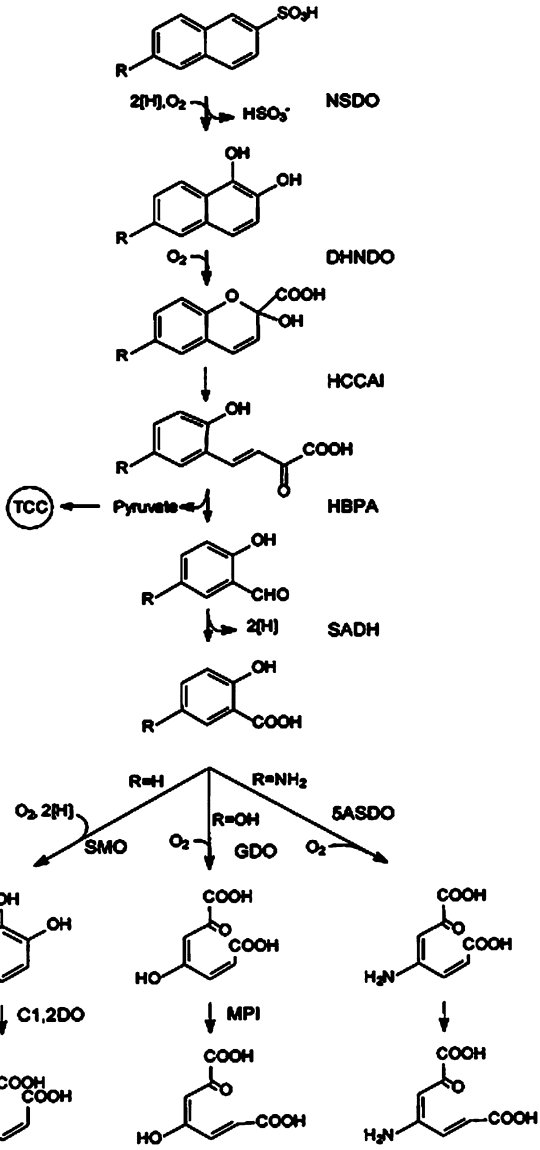


Fig. 4.3 Proposed pathways for the degradation of substituted naphthalene-sulfonates. *NSDO* naphthalenesulfonate dioxygenease; *DHNDO* 1,2-dihydroxynaphthalene dioxygenase; *HCCAI* 2-hydroxychromene-2-carboxylate isomersae; *HBPA* 2′-hydroxybenzal-pyruvate aldolase; *SADH* salicylaldehyde dehydrogenase; *SMO* salicylate 1-monooxygenase; *GDO* gentisate 1,2-dioxygenase; 5*ASDO* 5-aminosalicylate 1,2-dioxygenase; *C*1 2*DO* catechol 1,2-dioxygenase; *MPI* maleylpyruvate isomerase; *TCC* tricarboxylic acid cycle. *Source* Hinter et al. (2001)

(Jensen et al. 1993; Kudlich et al. 1999). Oxygen reacts with aromatic products, via free radical reactions, leading to the formation of undesirable colored oligomers and polymers which may be toxic and mutagenic. Further, these polymers are more recalcitrant to biodegradation. Thus, an effective mineralization of azo dyes, using anaerobic decolorization followed by aerobic degradation of amines, can only be possible, if the aerobic biodegradation rate for amines is much higher than the autoxidation rate.

4.4 Toxicity Assessment of Azo Dyes and their Degradation Products

The main aim of the azo dye containing wastewater treatment is the reduction of associated toxicity. Many reports cite textile wastewater as a significant contributor to toxic load on aquatic ecosystems (Rajaguru et al. 2002; Umbuzeiro et al. 2005; Puvaneshwari et al. 2006). Many in vivo and in vitro tests are available for toxicity assessment (Farre and Barcelo 2003). They include: (1) Animal assays: fish, rat, mice and Daphnia are generally used test organisms in the animal assays; (2) The Comet Assay, which is also called as single cell gel electrophoresis (SCGE), is a sensitive and rapid technique for quantifying and analyzing DNA damage to individual cells (Rajaguru et al. 2003); (3) Ames *Salmonella*/microsome mutagenicity assay, which is a short-term bacterial reverse mutation assay specifically designed to detect a wide range of chemical substances that can produce genetic damage that leads to gene mutations; (4) The biosensor test, which utilises a modified strain of the *Saccharomyces cerevisiae* with the gene encoding green fluorescent protein (GFP) linked to the inducible promoter of the DNA damage responsive RAD54 gene. On exposure to a genotoxin, the production of GFP is up-regulated in parallel with RAD54, and the resulting cellular fluorescence provides a measure of genotoxicity. Acute toxicity is simultaneously determined by monitoring relative total growth of the cell culture during incubation (Keenan et al. 2007); (5) The bioluminescence inhibition assay is based on a marine gram negative bacterium, *Vibrio fischeri*. Light production is directly proportional to the metabolic activity of the bacterial population and any inhibition of enzymatic activity causes a corresponding decrease in bioluminescence. The assay provides a measure of sub-lethal response (Parvez et al. 2005). Apart from these, many other assays, which include plant bioassasys, bacterial assays based on growth, enzyme inbition etc., can be used to assess the toxicity of azo dye prior to and after decolorization and degradation. A few recent references on bacterial decolourization, mineralization and enzyme toxicity has been given in Table 4.1.

4.5 Application of Biological (Bacterial) Methods for Azo Dye Containing Wastewaters

A wide range of structurally diverse dyes is used in the textile industry in the same unit. Although there are many reports available on dye decolorization under aerobic conditions in the presence of either glucose and or yeast extract, most of these are limited to few specific dyes. On the other hand, decolorization in the absence of oxygen is non-specific. As compared to methanogenic consortia, anoxic decolorization requires high concentrations of co-substrates, such as yeast extract or peptone, thus making the process economically unviable for industrial-scale application unless alternate cheaper sources are identified (Moosvi et al. 2005).

Table 4.1 Studies on bacterial decolorization, mineralization, enzymes and toxicity studies

Bacteria	Dye	Red-ox condition	Comments	References
Staphylococus arlettae	RY107, RR198, RB5, DB71	Microaerophilic-aerobic	Toxicity studies	Elisangela et al. (2009a)
Klebsiella sp.	RY107, RR198, DB71	Microaerophilic-aerobic		Elisangela et al. (2009b)
Pseudomonas aeruginosa	Remazol Orange	Microaerophilic	Degradation pathway proposed	Sarayu and Sandhya (2010)
Pseudomonas, Arthrobacter, and *Rhizobium*	AO7	Aerobic	Batch and continuously operated multistage packed-bed BAC reactor	Ruiz-Arias et al. (2010)
Cardiobacterium homini and *Pseudomonas stutzeri*	Direct black 38	Anoxic	Release of benzidine and its subsequent degradation to aminobiphenyl	Bafana et al. (2007)
Bacillus velezensis	Direct Red 28	Aerobic	Azoreducatase isolation and toxicity studies	Bafana and Chakrabarti (2008)
Pseudomonas putida mt-2	Acid yellow 17	Aerobic	Degradation of amines Toxicity studies	Mansour et al. (2009)
Shewanella putrefaciens strain AS96	Four azo dyes	Microaerophyllic-aerobic	Amine degradation Tolerant to high saline conditions	Khalid et al. (2008a, b)
Exiguobacterium sp.	X-3B	Anaerobic	Salt tolerant bacteria Effect of red-ox mediator	Tan et al. (2009a, b)
Galactomyces geotrichum	Methyl red	Aerobic	Induction of various enzymes Phytotoxicity studies	Jadhav et al. (2008)
Micrococcus glutamicus and *Proteus vulgaris*	Sulfonated reactive dye Green HE4B and other dyes	Aerobic	Phytotoxicity and microbial toxicity studies. Table on the performance of various microbial consortia	Saratale et al. (2010, 2011)
Halomonas sp. strain GTW	Five azo dyes	Anoxic	Salt tolerant	Guo et al. (2007)

(continued)

Table 4.1 (continued)

Bacteria	Dye	Red-ox condition	Comments	References
Pseudomonas desmolyticum	Red HE7B	Aerobic	Induction of Extracellular Peroxidase and other enzymes. Amine degradation	Kalme et al. (2007)
Microbial Consortium	Methyl orange	Aerobic	Response surface methodology. Induction of various enzymes Phytotoxicity studies	Ayed et al. (2010)
Rhodopseudomonas palustris W1	Reactive Black 5	Anaerobic	Salt tolerant. Proposed degradative pathway for aminonaphthylsulfonate	Xingzu et al. (2008)

Except for few, aromatic amines formed from decolorization of azo dyes are recalcitrant to biodegradation under anaerobic conditions and require aerobic conditions. Thus, bacterial decolorization and degradation of azo dyes leading to non-toxic end products, can only be achieved using sequential red-ox (anaerobic–aerobic) conditions (Dafale et al. 2010). Especially aerobic reactors can be bio-augmented with specific isolated bacterial cultures (Khalid et al. 2010).

Maintaining high cell density is required for achieving high dye decolorization and degradation in biological reactors. This is possible by immobilizing bacteria on specific surfaces or encapsulating them in gel matrix. Various support media, such as ceramic materials, granular activated carbon, polyurethane foams as well as alginate, polyvinylalcohol for encapsulation of bacterial cells, have been reported. Use of immobilized cells system in azo dye decolorization has been reported by several researchers (Chen et al. 2003b; He et al. 2004; Moutaouakkil et al. 2004; Singh et al. 2006; Barragan et al. 2007; Chen and Lin 2007; Zeroual et al. 2007). Cell entrapment or biofilm on support materials may provide a low-oxygen environment in the inside of the material becomes anaerobic constituting the perfect environment for the decolorizing bacteria to degrade the azo dye. Aerobic decolorization and mineralization of a sulfonated Phenylazonaphthol dye by a Bacterial Community Immobilized in a Multistage Packed-Bed Biologic Activated Carbon Reactor was recently reported by Ruiz-Arias et al. (2010).

Different reactor configurations used for anaerobic/aerobic steps and their efficiencies, have been excellently reviewed by Van der Zee and Villaverde (2005). These include anaerobic high-rate reactors, such as upflow anaerobic sludge blanket, fixed film, rotating biological contactors and anaerobic baffled reactors for anaerobic processes and activated sludge and rotating biological contactors for aerobic treatment (Van der Zee and Villaverde 2005).

Different red-ox conditions can be provided in two separate reactors. Decolorized effluent from anaerobic reactor can be fed to aerobic reactor for amine removal. Anaerobic and aerobic environments can also be provided in a single reactor for different periods (Cinar and Demiröz 2010). Integrated decolorization and amine degradation is only possible in reactors with biofilms on support material or with immobilized microbes entrapped in a gel matrix (Field et al. 1995; Kudlich et al. 1996). The fate of aromatic amines has been specifically addressed by a few investigators. Some of these studies show partial or complete removal of many aromatic amines in the aerobic stage. As reviewed by Pinheiro et al. (2004), various substituted aminobenzene, aminonaphthalene and aminobenzidine compounds have been found to be aerobically biodegradable. However, they generally require an enrichment of specialized cultures. Recently, various combinations of physical, chemical and biological methods have been used for the treatment of dye containing wastewaters, which was recently reviewed by Lu and Liu (2010).

Still reports on pilot-scale and full-scale implementation of anaerobic–aerobic biological treatment are still scarce. Delee et al. (1998) have reviewed the reports on full-scale and pilot-scale plants and some of their limitations. A two-stage fixed bed pilot plant for on-site anaerobic decolorization of textile wastewater was reported by Georgiou et al. (2005). Anaerobic (with facultative anaerobic bacterial

culture)– aerobic sequential system was used for color and COD removal from real textile wastewater at pilot scale (Kapdan and Alparslan 2005). A pilot-scale (300 m^3/d) wastewater reclamation system was tested for printing and dyeing wastewater treatment from a textile factory using anoxic–aerobic conditions. Treated wastewater was filtered by sub-filter technology (Patent: CN1308024A). The average COD, color and turbidity removal efficiencies were 91, 92.5 and 90.9%, respectively (Lu et al. 2009). A three-stage pilot-scale moving-bed biofilm reactor (MBBRs, anaerobic–anaerobic–aerobic in series) was investigated to treat textile dyeing wastewater. Each reactor was filled with 20% (v/v) of polyurethane-activated carbon (PU-AC) as carrier for biological treatment (Park et al. 2010). Recent studies with real wastewaters have shown that the addition of an anaerobic/anoxic unit prior to activated sludge significantly improves the effluent quality with respect to color. There appears to be only one report on the successful full-scale implementation of anaerobic technology in combination with aerobic membrane technology for the treatment of more than 1000 L wastewaters per day from textile processing industry (Stolz 2001, VA Tech WABAG 2003).

4.6 Conclusions

Azo dyes constitute 60–70% of synthetic dyes used in commercial application for textile dyeing. The discharge of highly colored synthetic dyes effluents to receiving water bodies is an indicator of severe water pollution. The treatment of azo-dye-containing wastewaters still presents a technical challenge for us. As regulations are becoming more and more stringent, there is urgent need for technically feasible and cost-effective methods. Under methanogenic and anoxic conditions, bacteria can decolorize many azo dyes. The rate of decolorization can be enhanced in the presence of red-ox mediators. Recently there have been many reports on the dye decolorization by bacterial cultures in the presence of oxygen. Oxygen insensitive azo reductases, key enzymes for aerobic azo bond reduction, have been isolated from many sources and characterized. Degradation of many amines including sulfonated aromatic amines, requires the presence of specialized cultures. Many have a very narrow substrate range. Hence, there is a requirement for developing microbial consortia that harbor genes for the rapid degradation of mixtures of aromatic amines. Thus, anaerobic–aerobic conditions are required for decolorization and degradation of azo dyes. Although there are extensive literature available on sequential/integrated anaerobic–aerobic systems at laboratory scale, reports on the evaluation of pilot scale and full scale units for amine removal and toxicity assessment are still scarce. It may be necessary to use integrated processes, which may include chemical and biological, to achieve the required degree of treatment of dye-containing wastewaters, so that regulatory standards (set to avoid the toxicity to humans and animals) can be met.

References

Adedayo O, Javadpour S, Taylor C, Anderson WA, Moo-Young M (2004) Decolourization and detoxification of methyl red by aerobic bacteria from a wastewater treatment plant. World J Microbiol Biotechnol 20:545–550

Alaton IA, Balcioglu IA, Bahnemann DW (2002) Advanced oxidation of a reactive dyebath effluent: comparision of O_3, H_2O_2/UV-C and TiO_2/UV - A process. Water Res 36:1143–1154

Albuquerque MGE, Lopes AT, Serralheiro ML, Novais JM, Pinheiro HM (2005) Biological sulphate reduction and redox mediator effects on azo dye decolourisation in anaerobic-aerobic sequencing batch reactors. Enzyme Microb Technol 36:790–799

Alexander M, Lustigham BK (1996) Effect of chemical structure on microbial degradationn of substituted benzenes. J Agric Food Chem 14:410–413

Al-Kdasi A, Idris A, Saed K, Guan CT (2004) Treatment of textile wastewater by advanced oxidation processes—a review. Global Nest Intl J 6:221–229

Alvarez LH, Perez-Cruz MA, Rangel-Mendez JR, Cervantes FJ (2010) Immobilized redox mediator on metal-oxides nanoparticles and its catalytic effect in a reductive decolorization process. J Hazard Mater 184:268–272

Amoozegar MA, Hajighasemi M, Hamedi J, Asad S, Ventosa A (2010) Azo dye decolorization by halophilic and halotolerant microorganisms. Ann Microbiol. doi: 10.1007/s13213-010-0144-y

Ayed L, Khelifi E, Jannet HB, Miladi H, Cheref A, Achour S, Bakhrouf A (2010) Response surface methodology for decolorization of azo dye methyl orange by bacterial consortium: produced enzymes and metabolites characterization. Chem Eng J 165:200–208

Bafana A, Chakrabarti T (2008) Lateral gene transfer in phylogeny of azoreductase enzyme. Comput Biol Chem 32:191–197

Bafana A, Devi SS, Krishnamurthi K, Chakrabarti T (2007) Kinetics of decolourisation and biotransformation of direct black 38 by *C. hominis* and *P. stutzeri*. App Microbiol Biotechnol 74:1145–1152

Banat IM, Nigam P, Singh D, Merchant R (1996) Microbiol degradation of textile—dye containing effluents: a review. Biores Technol 58:217–227

Barragan BE, Costa C, Carmen Marquez C (2007) Biodegradation of azo dyes by bacteria inoculated on solid media. Dyes Pigment 75:73–81

Barsing P, Tiwari A, Joshi T, Garg S (2011) Application of a novel bacterial consortium for mineralization of sulphonated aromatic amines. Biores Technol 102:765–771

Bayley RC, Barbour MG (1984) Degradation of aromatic compounds by the meta and gentisate pathways: Biochemistry and regulation. In: Gibson DT (ed) Microbial degradation of organic compounds. Marcel Dekker, New York, pp 253–294

Behnajaday MA, Modirshahla N, Shokri M (2004) Photodestruction of Acid Orange 7 (AO7) in aqueous solutions by UV/H_2O_2: influence of operational parameters. Chemosphere 55:129–134

Bin Y, Jiti Z, Jing W, Cuihong D, Hongman H, Zhiyong S, Yongming B (2004) Expression and characteristics of the gene encoding azoreductase from *Rhodobacter sphaeroides* AS1.1737. FEMS Microbiol Lett 236:129–136

Blumel S, Stolz A (2003) Cloning and characterization of the gene coding for the aerobic azoreductase from *Pigmentiphaga kullae* K24. Appl Microbiol Biotechnol 62(2–3):186–190

Blumel S, Knackmuss HJ, Stolz A (2002) Molecular cloning and characterization of the gene coding for the aerobic azoreductase from Xenophilus azovorans KF46F. Appl Environ Microbiol 68(8):3948–3955

Bragger JL, Lloyd AW, Soozandehfar SH, Bloom.field SF, Marriott C, Martin GP (1997) Investigations into the azo reducing activity of a common colonic microorganism. Intl J Pharm 157:61–71

Bras R, Ferra MIA, Pinheiro HM, Goncalves IC (2001) Batch tests for assessing decolourisation of azo dyes by methanogenic and mixed cultures. J Biotechnol 89:155–162

Bromley-Challenor KCA, Knapp JS, Zhang Z, Gray NCC, Hetheridge MJ, Evans MR (2000) Decolorization of an azo dye by unacclimated activated sludge under anaerobic conditions. Water Res 34:4410–4418

Brown D, Hamburger B (1987) The degradation of dyestu.s: part III–investigations of their ultimate degradability. Chemosphere 16:1539–1553

Carliell CM, Barclay SJ, Naidoo N, Buckley CA, Mulholland DA, Senior E (1994) Anaerobic decolorisation of reactive dyes in conventional sewage treatment processes. Water SA 20:341–344

Carliell CM, Barclay N, Buckley CA (1996) Treatment of exhausted reactive dye bath effluent using anaerobic digestion laboratory and full-scale trials. Water SA 22:225–233

Carvalho MC, Pereira C, Gonc-alves IC, Pinheiro HM, Santosa AR, Lopesa, Ferra MI (2008) Assessment of the biodegradability of a monosulfonate azo dye and aromatic amines. Intl Biodeter Biodegrad 62:96–103

Cervantes FJ (2002) Quinones as Electron Acceptors and Redox Mediators for the Anaerobic Biotransformation of Priority Pollutants. Agrotechnology and Food Sciences, Sub-department of Environmental Technology. Wageningen University, Wageningen, The Netherlands, p 166

Cervantes FJ (2006) Catalytic effects of different redox mediators on the reductive decolorization of azo dyes. Water Sci Technol 54:165–170

Cervantes FJ, van der Velde S, Lettinga G, Field JA (2000) Competition between methanogenesis and quinone respiration for ecologically important substrates in anaerobic consortia. FEMS Microbiol Ecol 34:161–171

Cervantes FJ, Enrı′quez JE, Arvayo GE, Razo-Flores E, Field JA (2007) Biogenic sulphide plays a major role on the riboflavin-mediated decolourisation of azo dyes under sulphate-reducing conditions. Chemosphere 68:1082–1089

Cervantes FJ, Garcia-Espinosa A, Moreno-Reynosa MA, Rangel-Mendez JR (2010) Immobilized redox mediators on anion exchange resins and their role on the reductive decolorization of azo dyes. Environ Sci Technol 44(5):1747–1753

Chang JS, Lin YC (2000) Fed-batch bioreactor strategies for microbial decolorization of azo dye using a *Pseudomonas leuteola* strain. Biotechnol Pro 16:979–985

Chang JS, Chou C, Lin Y, Ho J, Hu TL (2001) Kinetic Characteristics of bacterial azo-dye decolorization by *Pseudomonas luteola*. Water Res 35:2041–2850

Chen H (2006) Recent advances in azo dye degrading enzyme research. Curr Protein Pept Sci 7(2):101–111

Chen H, Feng J, Kweon O, Xu H, Cerniglia CE (2010) Identification and molecular characterization of a novel flavin-free NADPH preferred azoreductase encoded by azoB in *Pigmentiphaga kullae* K24. BMC Biochem 11:13

Chen H, Hopper SL, Cerniglia CE (2005) Biochemical and molecular characterization of an azoreductase from *Staphylococcus aureus*, a tetrameric NADPH-dependent flavoprotein. Microbiology 151:1433–1441

Chen JP, Lin YS (2007) Decolorization of azo dye by immobilized Pseudomonas luteola entrappe in alginate-silicate sol-gel beads. Process Biochem 42:934–942

Chen KC, Huang WT, Wu JY, Houng JY (1999) Microbial decolorization of azo dyes by *Proteus mirabilis*. J Ind Microbiol Biotechnol 23:686–690

Chen KC, Wu JY, Huang CC, Liang YM, Hwang SJ (2003a) Decolorization of azo dye by using PVA immobilized microorganisms. J Biotechnol 101:241–252

Chen KC, Wu JY, Liou DJ, Hwang SJ (2003b) Decolorization of textile dyes by newly isolated bacterial strains. J Biotechnol 101:57–68

Chinwetkitvanich S, Tuntoolvest M, Panswad T (2000) Anaerobic decolorization of reactive dyebath effluents by a two stage UASB system with Tapioca as co-substrate. Water Res 34:2223–2232

Christie R (2001) Colour chemistry. The Royal Society of Chemistry, Cambridge, UK

Chung KT, Stevens SEJ, Cerniglia CE (1992) The reduction of azo dyes by the intestinal microflora. Crit Rev Microbiol 18:175–197

Cinar O, Demiröz K (2010) Biodegradation of azo dyes in anaerobic-aerobic sequencing batch reactors in biodegradation of azo dyes series. In: Atacag Erkurt H (ed) The handbook of environmental chemistry, vol 9, 1st edn. Springer, Berlin

Cinar O, Yasa S, Kertmen M, Demiroz K, Yigit NO, Kitis M (2008) Effect of cycle time on biodegradation of azo dye in sequencing batch reactor. Proc Saf Environ Protect 86:455–460

Costa MC, Mota S, Nascimento RF, Dos Santos AB (2010) Anthraquinone-2,6-disulfonate (AQDS) as a catalyst to enhance the reductive decolourisation of the azo dyes Reactive Red 2 and Congo Red under anaerobic conditions. Biores Technol 101(1):105–110

Coughlin MF, Kinkle BK, Bishop PL (1999) Degradation of azo dyes containing amino naphthol by Sphingomonas sp strain ICX. J Ind Microbiol Biotechnol 23:341–346

Coughlin MF, Kinkle BK, Bishop PL (2002) Degradation of acid orange 7 in an aerobic biofilm. Chemosphere 46:11–19

Coughlin MF, Kinkle BK, Bishop PL (2003) High performance degradation of azo dye acid orange 7 and sulfanilic acid in a laboratory scale reactor after seeding with cultured bacterial strains. Water Res 37:2757–2763

Dafale N, Rao NN, Meshram SU, Wate SR (2008) Decolorization of azo dyes and simulated dye bath wastewater using acclimatized microbial consortium-biostimulation and halo tolerance. Biores Technol 99:2552–2558

Dafale N, Wate SR, Meshram SU, Rao NN (2010) Bioremediation of wastewater containing azo dyes through sequential anaerobic-aerobic bioreactor system and its biodiversity. Environ Rev 18:21–36

Dangmann E, Stolz A, Kuhm AE, Hammer A, Feigel B, Noisommit-Rizzi M, Rizzi M, Knackmuss H-J (1996) Degradation of 4-aminobenzenesulfonate by a two-species bacterial coculture. Physiological interactions between *Hydrogenophaga palleronii* S1 and *Agrobacterium radiobacter* S2. Biodegradation 7:223–229

Delee W, O'Niell C, Hawkcs FR, Pinheiro HM (1998) Anaerobic treatment of textile effluents: a reviw. J Chem Technol Biotechnol 73:323–325

Diniz PE, Lopes AT, Lino AR, Serralheiro ML (2002) Anaerobic reduction of a sulfonated azo dye Congo Red by Sulphate reducing bacteria. Appl Biochem Biotechnol 97:147–163

Donlon B, Razo-Flores E, Luijten M, Swarts H, Lettinga G, Field JA (1997) Detoxification and partial mineralization of the azo dye mordant orange 1 in a continuous upflow anaerobic sludge-blanket reactor. Appl Microbiol Biotechnol 47:83–90

Dos Santos AB (2005) Reductive decolourisation of dyes by thermophilic anaerobic granular sludge Sub-department of Environmental Technology. Wageningen University, Wageningen, p 176

Dos Santos AB, Bisschops IAE, Cervantes FJ, Van Lier JB (2004) Effect of different redox mediators during thermophilic azo dye reduction by anaerobic granular sludge and comparative study between mesophilic (30°C) and thermophilic (55°C) treatments for decolourisation of textile wastewaters. Chemosphere 55:1149–1157

Dos Santos AB, Cervantes FJ, van Lier JB (2006) Potentials of high-temperature anaerobic treatment and redox mediators for the reductive decolorization of azo dyes from textile wastewaters. Water Sci Technol 54(2):151–156

Dos Santos AB, Cervantes FJ, van Lier JB (2007) Review paper on current technologies for decolourisation of textile wastewaters: Perspectives for anaerobic biotechnology. Biores Technol 98:2369–2385

Dos Santos AB, Cervantes FJ, Yaya-Beas RE, van Lier JB (2003) Effect of redox mediator, AQDS, on the decolorization of a reactive azo dye containing triazine group in a thremophilic anaerobic EGSB reactor. Enzyme Microbiol Technol 33:942–951

Dos Santos AB, Madrid MP, Stams AJM, Van Lier JB, Cervantes FJ (2005) Azo dye reduction by mesophilic and thermophilic anaerobic consortia. Biotechnol Progr 21:1140–1145

Drzyzga O, Blotevogel KH (1997) Microbial degradation of diphenylamine under anoxic conditions. Curr Microbiol 35:343–347

Ekici P, Leupold G, Parlar H (2001) Degradability of selected azo dye metabolites in activated sludge systems. Chemosphere 44:721–728

Elisangela F, Andrea Z, Fabiana F, Isis SS, Artur C, Lucia RD (2009a) Microaerophilic–aerobic sequential decolourization/biodegradation of textile azo dyes by a facultative *Klebsiella* sp. strain VN-31. Process Biochem 44:446–452

Elisangela F, Andrea Z, Fabio DG, Cristiano R, Regina DL, Artur C (2009b) Biodegradation of textile azo dyes by a facultative *Staphylococcus arlettae* strain VN-11 using a sequential microaerophilic/aerobic process. Intl Biodeter Biodegrad 63:280–288

Encinas-Yocupicio AA, Razo-Flores E, Sánchez Diaz F, Dos Santos AB, Field JA, Farre M, Barcelo D (2003) Toxicity testing of wastewater and sewage sludge by biosensors, bioassays and chemical analysis. Trends Anal Chem 22(5):299–310

Farre M, Barcelo D (2003) Toxicity testing of wastewater and sewage sludge by biosensors, bioassays and chemical analysis. Trends Anal Chem 22(5):299–310

Feigal BJ, Knackmuss HJ (1993) Syntropic interactions during degradation of 4-aminobenzenesulfonic acid by a two species bacterial culture. Arch Microbiol 159:124–130

Field JA, Brady J (2003) Riboflavin as a redox mediator accelerating the reduction of azo dye mordant yellow 10 by anaerobic granular sludge. Water Sci Technol 48:187–193

Field JA, Stams AJM, Kato M, Schraa G (1995) Enhanced biodegradation of aromatic pollutant in coculture of anaerobic and aerobic bacterial consortia. Antonie Van Leeuwenhoek 67:47–77

Firmino PIM, da Silva MER, Cervantes FJ, dos Santos AB (2010) Colour removal of dyes from synthetic and real textile wastewaters in one- and two-stage anaerobic systems. Biores Technol 101:7773–7779

Ganesh R, Boardman GD, Michelson D (1994) Fate of azo dyes in sludges. Water Res 28:1367–1376

Georgiou D, Hatires J, Aivasidis A (2005) Microbial immobilization in a two-stage fixed bed reactor pilot plant for on-site anaerobic decolorization of textile wastewater. Enzyme Microbial Technol 37:597–605

Gill M, Strauch RJ (1984) Constituents of *Agaricus xanthodermus* Genevier: The first naturally endogenous azo compound and toxic phenoloc metabolites Zeitschrift fur Naturforschung C. J Biosci 39c:1027–1029

Golab V, Vinder A, Simonic M (2005) Efficiency of the coagulation/flocculation method for the treatment of dye bath effluent. Dyes Pigments 67:93–97

González-Gutiérrez LV, González-Alatorre G, Escamilla-Silva EM (2009) Proposed pathways for the reduction of a reactive azo dye in an anaerobic fixed bed reactor. World J Microbiol Biotechnol 25:415–426

Guo J, Zhou J, Wang D, Tian C, Wang P, Salah Uddin M, Yu Hui (2007) Biocalalyst effects of immobilized anthraquinone on the anaerobic reduction of azo dyes by the salt-tolerant bacteria. Water Res 41:426–432

Guo J, Zhou J, Wang D, Xiang X, Yu H, Tian C, Song Z (2006) Correlation of anaerobic biodegradability and the electrochemical chatacteristic of azo dyes. Biodegradation 17:341–346

Hao OJ, Kim H, Chang PC (2000) Decolorization of wastewater. Crit Rev Env Sci Technol 30:449–505

Haug W, Schmidt A, Nortemann B, Hempel DC, Stolz A, Knackmuss HJ (1991) Mineralization of the sulfonated azo dye Mordant Yellow 3 by a 6-aminonaphthalene-2-sulfonate-degrading bacterial consortium. Appl Environ Microbiol 57:3144–3149

He F, Hu WR, Li YZ (2004) Investigation of isolation and immobilization of a microbial consortium for decoloring of azo dye 4BS. Water Res 38:3596–3604

Hinter J-P, Lechner C, Riegret U, Kuhm AE, Storm T, Reemtsma T, Stolz A (2001) Direct ring fission of salicylate by a salicylate 1,2-Dioxygensae activity from Pseudaminobacter salicylatoxidans. J Bacteriol 183:6936–6942

Hopper SW (1991) Biodegradation of sulfonated aromatics. In: Chaudhry GR (ed) Biodegradation and bioremediation of toxic themicals. Chapman and Hall, London, pp 169–182

Houk VS (1992) The genotoxicity of industrial wastes and effluents: A review. Mutat Res 277:91–138

Hsueh CC, Chen BY (2008) Exploring effects of chemical structure on azo dye decolorization characteristics by *Pseudomonas luteola*. J Hazard Mater 154:703–710

Hsueh CC, Chen BY, Yen CY (2009) Understanding effects of chemical structure on azo dye decolorization characteristics by *Aeromonas hydrophila*. J Hazard Mater 167:995–1001

Isik M, Sponza DT (2005a) Effects of alkalinity and co-substrate on the performance of an upflow anaerobic sludge blanket (UASB) reactor through decolorization of Congo Red azo dye. Biores Technol 96:633–643

Isik M, Sponza DT (2005b) Substrate removal kinetics in an upflow anaerobic sludge blanket reactor decolorizing simulated textile wastewater. Process Biochem 40:1189–1193

Ito K, Nakanishi M, Lee WC, Sasaki H, Zenno S, Saigo K, Kitade Y, Tanokura M (2006) Three-dimensional structure of AzoR from Escherichia coli An oxidereductase conserved in microorganisms. J Biol Chem 281:20567–20576

Jadhav SU, Kalme SD, Govindwar SP (2008) Biodegradation of Methyl red by Galactomyces geotrichum MTCC 1360. Intl Biodeter Biodegrad 62(2):135–142

Jahnke M, Banna T, Klintworth R, Auling G (1990) Mineralisation of Orthanilic acid is a plasmid asociated trait in Alcaligenes sp. O-1. J Gen Microbiol 136:2241–2249

Jensen J, Cornett C, Olsen CE, Bondesen S, Christensen J, Christensen LA, Tjornelund J, Hansen SH (1993) Identification of oxidation products of 5-aminosalicylic acids in faeces and the study of their formation in vitro. Biochem Pharm 45:1201–1209

Jing W, Lihua L, Zhou J, Hong L, Liu G, Jin R, Yang F (2009) Enhanced biodecolorization of azo dyes by electropolymerization-immobilized redox mediator. J Hazard Mater 168(2–3): 1098–1104

Junker F, Leisinger T, Cook AM (1994) 3 Sulphocatechol 2,3 dioxygenase and other dioxygenases (E.CI.13.II.2 and ECI.14.12.) in the degradative pathway of 2-aminobenzene-sulfonic acid and 4-toluenesulphonic acids in Alcaligenes sp. strain. Microbiology 140: 1713–1722

Kahng HY, Kukor JJ, Oh KH (2000) Characterization of strain HY99, a novel microorganism capable of aerobic and anaerobic degradation of aniline. FEMS Microbiol Lett 190:215–221

Kalme SD, Parshetti GK, Jadhav SU, Govindwar SP (2007) Biodegradation of benzidine based dye Direct Blue-6 by Pseudomonas desmolyticum NCIM 2112. Biores Technol 98(7):1405–1410

Kalyuzhnyl S, Sklyar V, Mosolova T, Kucherenko I, Russkova Z, Degtyaryova N (2000) Methanogenic biodegradation of aromatic amines. Water Sci Technol 42:363–370

Kampfer P, Muller C, Mau M, Neef A, Auling G, Busse HJ, Osborne AM, Stolz A (1999) Description of Pseudaminobacter gen Nov. with two new species P. salicylatoxidans sp. nov. and P. defluvii sp. nov. Intl J Syst Bacteriol 149:887–897

Kapdan IK, Alparslan S (2005) Application of anaerobic–aerobic sequential system to real textile wastewater for color and COD removal. Enz Microb Technol 36:273–279

Kapdan IK, Kargi F, McMullan G, Marchant R (2000) Decolorization of textile dye stuffs by a mixed bacterial consortium. Biotechnol Lett 22:1179–1181

Kasper HF, Wuhrmann K (1978) Kinetic parameters and relative turnover of some important catabolic reactions in digesting sludge. Appl Environ Microbiol 36:1–7

Keck A, Conradt D, Mahler A, Stolz A, Mattes R, Klein J (2006) Identification and functional analysis of the genes for naphthalenesulfonate catabolism by Sphingomonas xenophaga BN6. Microbiology 152(7):1929–1940

Keck A, Klein J, Kudlich M, Stolz A, Knackmuss HJ, Mattes R (1997) Reduction of azo dyes by redox mediators originating in the naphthalenesulfonic acid degradation pathway of *Sphingomonas* sp. strain BN6. Appl Environ Microbiol 63:3684–3690

Keenan PO, Knight AW, Billinton N, Cahill PA, Dalrymple IM, Hawkyard CJ, Stratton-Campbell D, Walmsley RM (2007) Clear and present danger? The use of a yeast biosensor to monitor changes in the toxicity of industrial effluents subjected to oxidative colour removal treatments. J Environ Monit 9:1394–1401

Khalid A, Arshad M, Crowley DE (2008a) Accelerated decolorization of structurally different azo dyes by newly isolated bacterial strains. Appl Microbiol Biotechnol 78:361–369

Khalid A, Arshad M, Crowley DE (2008b) Decolorization of azo dyes by *Shewanella* sp. under saline conditions. Appl Microbiol Biotechnol 79(6):1053–1059

Khalid A, Arshad M, Crowley DE (2010) Bioaugmentation of azo dyes in biodegradation of azo dyes series. In: Atacag Erkurt H (ed) The handbook of environmental chemistry, vol 9, 1st edn. Springer, Berlin

Khehra MS, Saini HS, Sharma DK, Chadha BS, Chimni SS (2005) Decolorization of various azo dyes by bacterial consortia. Dyes Pigment 67:55–61

Kodam KM, Soojhawon I, Lokhande PD, Gawai KR (2005) Microbial decolorization of reactive azo dyes under aerobic conditions. World J Microbiol Biotechnol 21:367–370

Kolbener P, Baumann U, Cook AM, Leisinger T (1994) 3-Nitrobenzenesulfonicacid and 3-aminobenzenesulfonic acid in a laboratory trickling filter: Biodegradability with different activated sludges. Water Res 28:1855–1860

Kolekar YM, Pawar SP, Gawai KR, Lokhande PD, Shouche YS, Kodam KM (2008) Decolorization and degradation of Disperse Blue 79 and Acid Orange 10, by *Bacillus fusiformis* KMK5 isolated from the textile dye contaminated soil. Biores Technol 99(18): 8999–900

Kudlich M, Bishop P, Knackmuss HJ, Stolz A (1996) Synchronous anaerobic and aerobic degradation of the sulfonated azo dye Mordant Yellow 3 by immobilized cells from a naphthalenesulfonate-degrading mixed culture. Appl Microbiol Biotechnol 46:597–603

Kudlich M, Hetheridge MJ, Knackmuss HJ, Stolz A (1999) Autoxidation reactions of different aromatic o-aminohydroxynaphthalenes that are formed during the anaerobic reduction of sulfonated azo dyes. Environ Sci Technol 33:869–901

Kudlich M, Keck A, Klein J, Stolz A (1997) Localization of the enzyme system involves in anaerobic reduction of azo dyes by *Sphingomonas* sp. strain BN6 and e.ect of artificial redox mediators on the rate of azo dye reduction. Appl Environ Microbiol 63:3691–3694

Kulla HG, Klausener F, Mayer U, Ludeke B, Leisinger T (1983) Interference of aromatic sulfo groups in the microbial degradation of the azo dyes Orange I and Orange II. Arch Microbiol 135:1–7

Levine WG (1991) Metabolism of azo dyes: Implication for detoxification and activation. Drug Metabol Rev 23:253–309

Li JG, Lalman JA, Biswas N (2004) Biodegradation of Red B dye by Bacillus sp. OY1–2. Environ Technol 25:1167–1176

Liu G, Zhou J, Fu QS, Wang J (2009) The *Escherichia coli* Azoreductase AzoR Is Involved in Resistance to Thiol-Specific Stress Caused by Electrophilic Quinones. J Bacteriol 191:6394–6400

Liu G, Zhou J, Lv H, Xiang X, Wang J, Zhou M, Qv Y (2007) Azoreductase from Rhodobacter sphaeroides AS1.1737 is a flavodoxin that also functions as nitroreductase and flavin mononucleotide reductase. Appl Microbiol Biotechnol 76(6):1271–1279

Locher HH, Thurnheer T, Leisinger T, Cook AM (1989) 3-nitrobenzenesulufonate, 3-amino-benzenesulfonate and 4-aminobenzenesulfonate as sole carbon sources for bacteria. Appl Environ Microbiol 55:492–494

Lopez-Grimau V, Guitierrez MC (2005) Decolorization of simulated reactive dyebath effluents by electrochemical oxidation assisted by UV light. Chemosphere 62:106–112

Lu H, Zhou J, Wang J, Si W, Teng H, Liu G (2010) Enhanced biodecolorization of azo dyes by anthraquinone-2-sulfonate immobilized covalently in polyurethane foam. Biores Technol 101:7185–7188

Lu X, Liu L, Yang B, Chen J (2009) Reuse of printing and dyeing wastewater in processess assessed by pilot-scale test using combined biological process and sub-filter technology. J Clean Prod 17:111–114

Lu X, Liu R (2010) Treatment of azo dye-containing wastewater using integrated processes in biodegradation of azo dyes. Handb Environ Chem 9:133–155

Maier J, Kandelbaner A, Eracher A, Cavaco-Paulo A, Gubitz GM (2004) A new alkali-thermostable azoreductase from *Bacillus* sp. strain SF. Appl Environ Microbiol 70:837–844

Mansour HB, Mosrati R, Corroler D, Ghedira K, Barillier D, Chekir-Ghedira L (2009) Mutagenicity and genotoxicity of acid yellow 17 and its biodegradation products. Drug Chem Toxicol 32:222–229

Manu B, Chaudhari S (2003) Decolorization of indigo and azo dyes in semicontinuous reactors with long hydraulic retention time. Process Biochem 38:1213–1221

McMullan G, Meehan C, Conneely A, Kirby N, Robinson T, Nigam P, Banat IM, Merchant R, Smyth WF (2001) Microbial decolorization and degradation of textile dyes. Appl Microbiol Biotechnol 56:81–87

Méndez-Paz D, Omil F, Lema LM (2003) Modeling of the Acid Orange 7 anaerobic biodegradation. Water Sci Technol 48:133–139

Mezohegyi G, Kolodkin A, Castro UI, Bengoa C, Stuber F, Font J, Fabregat A (2007) Effective anaerobic decolorization of azo dye acid orange 7 in continuous upflow packed-bed reactor using biological activated carbon system. Ind Eng Chem Res 46:6788–6792

Moir D, Masson S, Chu I (2001) Structure-activity relationship study on the bioreduction of azo dyes by Clostridium paraputrificum. Environ Toxicol Chem 20:479–484

Moosvi S, Keharia H, Madamawar D (2005) Decolorization of textile dye reactive violet by a newly isolated bacterial consortium. RVM 11.1. World J Microbiol Biotechnol 21:667–672

Moutaouakkil A, Zeroual Y, Dzayri FZ, Talbi M, Lee K, Blaghen M (2003) Purification and partial characterization of azoreductase from *Enterobacter agglomerans*. Arch Biochem Biophys 413:139–146

Moutaouakkil A, Zeroual Y, Dzayri FZ, Talbi M, Lee K, Blaghen M (2004) Decolorization of azo dyes with Enterobacter agglomerans immobilized in different supports by by using fluidized bed bioreactor. Curr Microbiol 48:124–129

Nachiyar CV, Rajakumar GS (2005) Purification and characterization of an oxygen insensitive azoreductase from *Pseudomonas aeruginosa*. Enzyme Microbial Technol 36:503–509

Nachiyar CV, Rajkumar GS (2003) Degradation of tannery and textile dye, Navitan Fast Blue S5R by *Pseudomonas aeruginosa*. World J Microbiol Biotechnol 19:609–614

Nachiyar CV, Rajkumar GS (2004) Mechanism of Navitan fast Blue S5R degradation by Pseudomonas aeruginosa. Chemosphere 57:165–169

Nachiyar CV, Vijaylakshmi K, Muralidharan D, Rajkumar GS (2007) Mineralization of metalinic acid by *Pseudomonas aeruginosa* CLRI BL22. World J Microbiol Biotechnol 23:1733–1738

Nigam P, Banat IM, Singh D, Marchant R (1996) Microbial process for the decolorization of textile effluent containing azo, diazo and reactive dyes. Process Biochem 31:435–442

Nortemann B, Baumgarten J, Rast HG, Knackmuss H-J (1986) Bacterial communities degrading amino and naphthalene-2-sulfonates. Appl Environ Microbiol 52:1195–1202

Ohe T, Ohmoto T, Kobayashi Y, Sato A, Watanabe Y (1990) Microbial degradation of 1,6-and 2,6-naphthalene disulfonic acids by *Pseudomonas* sp. TA-2. Agricult Biol Chem 54:669–675

Ollgaard H, Frost L, Galster J, Hensen OC (1999) Survey of azo-colorants on Denmark: Milgoproject 509, Danish Environmental Protection Agency

Olukanni OD, Osuntokia AA, Kalyani DC, Gbenlea GO, Govindwar SP (2010) Decolorization and biodegradation of ReactiveBlue13 by *Proteus mirabilis* LAG. J Hazard Mater 184:290–298

O'Neill C, Hawkes FR, Hawkes DL, Lourenco ND, Pinheiro HM, Delee W (1999) Color in textile effluents sources, measurement, discharge consents and simulation: a review. J Chem Technol Biotechnol 53:249–254

Ooi T, Shibata T, Matsumoto K, Kinoshita S, Taguchi S (2009) Comparative enzymatic analysis of azoreductases from *Bacillus* sp. B29. Biosci Biotechnol Biochem 73(5):1209–1211

Oxspring DA, McMullan G, Smyth WF, Marchant R (1996) Decolourisation and metabolism of the reactive textiledye, Remazol Black B, by an immobilized microbial consortium. Biotechnol Lett 18:527–530

Öztürk A, Abdullah MI (2006) Toxicological effect of indole and its azo dye derivatives on some microorganisms under aerobic conditions. Sci Total Environ 358:137–142

Padmavathy S, Sandhya S, Swaminathan K, Subrahmanyam YV, Kaul SN (2003) Comparison of decolorization of reactive azo dyes by microorganisms isolated from various sources. J Environ Sci 15:628–633 China

Pandey A, Singh P, Iyengar L (2007) Bacterial decolorization and degradation of azo dyes. Intl Biodeter Biodegrad 59(2):73–84

Park HO, Oh S, Bade R, Shin WS (2010) Application of A2O moving-bed biofilm reactors for textile dyeing wastewater treatment. Korean J Chem Eng 27:893–899

Parvez S, Venkataraman C, Mukherji S (2005) A review on advantages of implementing luminescence inhibition test (*Vibrio fischeri*) for acute toxicity prediction of chemicals. Environ Intl 32:265–288

Pearce CI, Christie R, Boothman C, Von Canstein H, Guthrie JT, Lloyd JR (2006) Reactive azo dye reduction by Shewanella Strain J18 143. Biotechnol Bioeng 95:692–703

Pearce CI, Lloyd JR, Guthrie JT (2003) The removal of color from textile wastewater using whole bacterial cells. A review. Dyes Pigments 58:179–196

Perei K, Rákhely G, Kiss I, Polyák B, Kovács KL (2001) Biodegradation of sulfanilic acid by *Pseudomonas paucimobilis*. Appl Microbiol Biotechnol 55:101–107

Pinheiro HM, Tauraud E, Thomas O (2004) Aromatic amines from azo dye reduction: status review with emphasis on direct UV spectrophotometric detection in textile industry wastewaters. Dyes Pigments 61:121–139

Plumb JJ, Bell J, Stuckey DC (2001) Microbial populations associated with treatment of an industrial dye effluent in an anaerobic baffled reactor. Appl Environ Microbiol 67:3226–3235

Puvaneshwari N, Muthukrishnan J, Gunasekaran P (2006) Toxicity assessment and microbial degradation of azo dyes. Indian J Exp Biol 44:618–626

Rafii F, Cerniglia CE (1995) Reduction of azo dyes and nitroaromatic compounds by bacterial enzymes from the human intestinal tract. Environ Health Perspect 103(5):17–19

Rafii F, Coleman T (1999) Cloning and expression in *Escherichia coli* of an azoreductase gene from *Clostridium perfringens* and comparison with azoreductase genes from other bacteria. J Basic Microbiol 39(1):29–35

Rafii F, Franklin W, Cerniglia CE (1990) Azoreductase activity of anaerobic bacteria isolated from human intestinal microflora. Appl Environ Microbiol 56:2146–2151

Rai H, Bhattacharya M, Singh J, Bansal TK, Vats P, Banerjee UC (2005) Removal of dyes from the effluent of textile and dyestuff manufacturing industry: a review of emerging techniques with reference to biological treatment. Crit Rev Environ Sci Technol 35:219–238

Rajaguru P, Suba S, Palanivelm M, Kalaiselvi K (2003) Genotoxicity of a polluted river system measured using the alkaline comet assay on fish and earthworm tissues. Environ Mol Mutagen 41:85–91

Rajaguru P, Vidya L, Baskarasethupathi B, Kumar PA, Palanivel M, Kalaiselvi K (2002) Genotoxicity evaluation of polluted ground water in human peripheral blood lymphocytes using the comet assay. Mutat Res 517:29–37

Rajamohan N, Karthikeyan C (2006) Kinetics studies of dyes effluent degradation by *Pseudomonas stutzeri*. J Solid Waste Technol Manag 1–5. Retrieved from http://www.eco-web.com/editorial/061103.html

Rau J, Knackmuss HJ, Stolz A (2002) Effects of different quinoid redox mediators on the anaerobic reduction of azo dyes by bacteria. Environ Sci Technol 36:1497–1504

Rau J, Stolz A (2003) Oxygen-insensitive nitroreductases NfsA and NfsB of *Escherichia coli* function under anaerobic conditions as Lawsone-dependent azo reductases. Appl Environ Microbiol 69:3448–3455

Razo-Flores E, Donlon B, Field J, Lettinga G (1996) Biodegradability of N-substituted aromatics and alkylphenols under methanogenic conditions using granular sludge. Wat Sci Technol 33:47–57

Razo-Flores E, Luijten M, Donlon B, Lettinga G, Field J (1997a) Biodegradation of selected azo dyes under methanogenic conditions. Water Sci Technol 36:65–72

Razo-Flores E, Luijten M, Donlon B, Lettinga G, Field J (1997b) Complete biodegradation of the azo dye azosalicylate under anaerobic conditions. Environ Sci Technol 31:2098–2103

Robinson T, McMullan G, Marchant R, Nigam P (2001) Remediation of dyes in textile effluent: a critical review on current treatment technologies with a proposed alternative. Biores Technol 77:247–255

Rozgaj R, Glancer SM (1992) Total degradation of 6-amnionaphthalene 2-sulfonic acid by a mixed culture consisting of different bacterial genera. FEMS Microbiol Eco 86:229–236

Ruiz-Arias A, Juárez-Ramírez C, Cobos-Vasconcelos DD, Ruiz-Ordaz N, Salmerón-Alcocer A, Ahuatzi-Chacón D, Galíndez-Mayer J (2010) Aerobic biodegradation of a sulfonated phenylazonaphthol dye by a bacterial community immobilized in a multistage packed-bed BAC reactor. Appl Biochem Biotechnol 162(6):1689–1707

Russ R, Rau J, Stolz A (2000) The function of cytoplasmic flavin reductases in the reduction of azo dyes by bacteria. Appl Environ Microbiol 66:1429–1434

Ryan A, Laurieri N, Westwood I, Wang CJ, Lowe E, Sim E (2010) A novel mechanism for azoreduction. J Mol Biol 400:24–37

Sandhya S (2010) Biodegradation of azo dyes under anaerobic condition: role of azoreductase. In: Atacag Erkurt H (ed) The handbook of environmental chemistry, vol 9. Springer, Berlin, pp 39–57

Saratale RG, Saratale GD, Chang JS, Govindwar SP (2010) Decolorization and biodegradation of reactive dyes and dye wastewater by a developed bacterial consortium. Biodegradation 21:999–1015

Saratale RG, Saratale D, Chang JS, Govindwar SP (2011) Bacterial decolorization and degradation of azo dyes: a review. J Taiwan Inst Chem Eng 42:138–157

Sarayu K, Sandhya S (2010) Aerobic Biodegradation Pathway for Remazol Orange by *Pseudomonas aeruginosa*. Appl Biochem Biotechnol 160(4):1241–1253

Savelieva O, Kotova I, Roelofsenm W, Stams AJM, Netrusov A (2004) Utilization of aminoaromatic acids by a methanogenic enrichment culture and a novel *Citrobacter freundii* strain. Arch Microbiol 181:163–170

Semde R, Pierre D, Geuskens G, Devleeschouwer M, Moes AJ (1998) Study of some important factors involved in azo derivative reduction by Clostridium perfringens. Intl J Pharm 161:45–54

Senan RC, Abraham TE (2004) Bioremediation of textile azo dyes by aerobic bacterial consortium. Biodegradation 15(4):275–280

Silveira E, Marques PP, Silva SS, Lima-Filho JL, Porto ALF, Tambourgi EB (2009) Selection of Pseudomonas for industrial textile dyes decolorization. Intl Biodeter Biodegrad 63:230–235

Singh P, Birkeland NK, Iyengar L, Gurunath R (2006) Mineralization of 4-aminobenzenesulfonate (4-ABS) by *Agrobacterium* sp. strain PNS-1. Biodegradation 17:495–502

Singh S, Singh P, Awasthi SK, Pandey A, Iyengar L (2008) Mineralizationn of 2-aminobenzenesulfonate by a bacterial cosortium. World J Microbiol Biotechnol 24:841–847

Srinivasan A, Viraraghavan T (2010) Decolorization of dye wastewaters by biosorbents: a review. J Environ Manag 91:1915–1929

Stolz A (1999) Degradation of substituted naphthalenesulfonic acids by Sphingomonas xenophaga BN6. J Industrial Microbiol Biotechnol 23:391–399

Stolz A (2001) Basic and applied aspects in the microbial degradation of azo dyes. Appl Microbiol Biotechnol 56:69–80

Su Y, Zhang Y, Wang J, Zhou J, Lu X, Lu H (2009) Enhanced bio-decolorization of azo dyes by co-immobilized quinone-reducing consortium and anthraquinone. Biores Technol 100(12): 2982–2987

Sugiura W, Miyashita T, Yokoyama T, Arai M (1999) Isolation of azo-dye-degrading microorganisms and their application to white discharge printing of fabric. J Bioscien Bioeng 88(5):577–581

Suzuki Y, Yoda T, Ruhul A, Sagiura W (2001) Molecular cloning and characterization of the gene encoding azoreductase from *Bacillus* sp. OY 1–2 isolated from soil. J Biol Chem 246:9059–9065

Swaminathan K, Sandhya S, Carmalin Sophia A, Pachhade K, Subrahmanyam YV (2003) Decolorization and degradation of H-acid and other dyes using ferrous-hydrogen peroxide system. Chemosphere 50:619–625

Swati MJ, Inamdar SA, Telke AA, Tamboli DP, Govindwar SP (2010) Exploring the potential of natural bacterial consortium to degrade mixture of dyes and textile effluent. Int Biodeter Biodegrad 64:622–628

Talarposhti AM, Donnelly T, Anderson G (2001) Color removal from a simulated dye wastewater using a two-phase anaerobic packed bed reactor. Water Res 35:425–432

Tan L, Qu Y, Zhou J, Ma F, Li A (2009a) Dynamics of microbial community for X-3B wastewater decolorization coping with high-salt and metal ions conditions. Biores Technol 100:3003–3009

Tan L, Qu YY, Zhou JT, Li A, Gou M (2009b) Identification and characteristics of a novel salt-tolerant Exiguobacterium sp for azo dyes decolorization. Appl Biochem Biotechnol 159(3): 728–738

Tan NCG, Prenafeta-Boldu FX, Opsteeg JL, Lettinga G, Field JA (1999) Biodegradation of azo dyes in cocultures of anaerobic granular sludge with aerobic aromatic amine degrading enrichment cultures. Appl Microbiol Biotechnol 51:865–871

Tan NCG, van Leeuwen A, van Voorthuizen EM, Slenders P, Prenafeta-Boldu FX, Temmink H, Lettinga G, Field JA (2005) Fate and biodegradability of sulfonated aromatic amines. Biodegradation 16:527–537

Thurnheer T, Kohler T, Cook AM, Leisinger T (1986) Orthanilic acid and analogues as carbon sources for bacteria: growth physiology and enzymic desulphonation. J Gen Microbiol 132:1215–1220

Umbuzeiro GA, Freeman H, Warren SH, Oliveira DP, Terao Y, Watanabe T, Claxton LD (2005) The contribution of azo dyes to the mutagenic activity of the Cristais River. Chemosphere 60:55–64

VA Tech WABAG (2003) Drews Merane treats color by bacteria. Online at www.Vatechwabag.com

Van der Zee FP, Burwman RHM, Strik DPTB, Lettinga G, Field JA (2001a) Application of redox mediators to accelerate the transformation of reactive azo dyes in anaerobic bioreactor. Biotechnol Bioeng 75:691–701

Van der Zee FP, Bisschops IAE, Blanchard VG, Bouwman RHM, Lettinga G, Field JA (2003) The contribution of biotic and abiotic processes during azo dye reduction in anaerobic sludge. Water Res 37:3098–3109

Van der Zee FP, Cervantes FJ (2009) Impact and application of electron shuttles on the redox (Bio) transformation of contaminants: a review. Biotechnol Adv 27:256–277

Van der Zee FP, Lettinga G, Field JA (2000) The role of (auto) catalysis in the mechanism of anaerobic azo reduction. Water Sci Technol 42:301–308

Van der Zee FP, Lettinga G, Field JA (2001b) Azo dye decolorization by anaerobic granular sludge. Chemosphere 44:1169–1176

Van der Zee FP, Villaverde S (2005) Combined anaerobic-aerobic treatment of azo dyes-a short review of bioreactor studie. Water Res 39:1425–1440

Vandevivere PC, Binachi R, Verstrate W (1998) Treatment of wastewater from textile Wet processing industry: review of emerging technologies. J Chem Technol Biotechnol 72: 289–302

Walker R, Ryan AJ (1971) Some molecular parameters influencing rate of reductions of azo compounds by intestinal microflora. Xenobiotica 1:483–486

Wang A, Qu J, Liu H, Ge J (2004) Degradation of azo dye Acid Red 14 in aqueous solution by electrokinetic and electooxidation process. Chemosphere 55:1189–1196

Wang CJ, Hagemeier C, Rahman N, Lowe E, Noble M, Coughtrie M, Sim E, Westwood I (2007) Molecular cloning, characterisation and ligand-bound structure of an azoreductase from *Pseudomonas aeruginosa*. J Mol Biol 373(5):1213–1228

Wang X, Cheng X, Sun D, Qi H (2008) Biodecolorization and partial mineralization of Reactive Black 5 by a strain of *Rhodopseudomonas palustris*. J Environ Sci 20:1218–1225

Wang YQ, Zhang JS, Zhou JT, Zhang JP (2009) Biodegradation of 4-aminobenzenesulfonate by a novel *Pannonibacter* sp. W1 isolated from activated sludge. J Hazard Mater 169:1163–1167

Weber EJ (1996) Iron mediated reductive transformation: investigation of reaction mechanism. Environ Sci Techol 30:716–719

Weber EJ, Wolfe NL (1987) Kinetic studies of the reduction of aromatic azo compounds in anaerobic sediment/water systems. Environ Toxicol Chem 6:911–919

Weisburger JH (2002) Comments on the history and importance of aromatic and heterocyclic amines in public health. Mutat Res 30:506–507
Willetts JRM, Ashbolt NJ, Moosbrugger RE, Aslam MR (2000) The use of a thermophilic anaerobic system for pretreatment of textile dye wastewater. Water Sci Technol 42:309–316
Wittich RM, Rast HG, Knackmuss HJ (1988) Degradation of Naphthalene-2,6- and Naphthalene-1,6-Disulfonic Acid by a *Moraxella* sp. Appl Environ Microbiol 54(7):1842–1847
Xingzu W, Xiang C, Dezhi S, Hong Q (2008) Biodecolorization and partial mineralization of Reactive Black 5 by a strain of *Rhodopseudomonas palustris*. J Environ Sci 20:1218–1225
Xu M, Guo J, Sun G (2007) Biodegradation of textile azo dye by *Shewanella decolorationis* S12 under microaerophilic conditions. Appl Microbiol Biotechnol 76(3):719–726
Yemashova N, Telegina A, Kotova I, Netrusova A, Kalyuzhnyl S (2004) Decolorization and Partial degradation of selected azo dyes by methanogenic sludge. Appl Biochem Biotechnol 119:31–40
Yoo ES (2002) Chemical decolorization of the azo dye CI Reactive Orange 96 by various organic/inorganic compounds. J Chem Technol Biotechnol 77:481–485
Yoo ES, Libra J, Adrian L (2001) Mechanism of decolrization of azo dyes in an anaerobic mixed culture. J Environ Eng (ASCE) 127:844–849
Yoo ES, Libra J, Wiesmann U (2000) Reduction of azo dyes by *Desulfovibrio desulfuricans*. Water Sci Technol 41:15–22
Yu J, Wang X, Yue PL (2001) Optimal decolorization and kinetic modeling of synthetic dyes by *Pseudomonas strains*. Water Res 35:3579–3586
Zeroual Y, Kim BS, Yang MW, Blaghen M, Lee KM (2007) Decolorization of some Azo dyes by immobilized *Geotrichum* sp. biomass in fluidized bed reactor. Appl Biochem Biotechnol 142:307–316
Zimmermann T, Gasser F, Kulla H, Leisinger T (1984) Comparison of two bacterial azoreductases acquired during adaptation to growth on azo dyes. Arch Microbiol 138:37–43
Zimmermann T, Kulla H, Leisinger T (1982) Properties of purified orange II-azoreductase, the enzyme initiating azo dye degradation by *Pseudomonas* KF46. Eur J Biochem 129:197–203
Zollinger H (1991) Color chemistry, 2nd edn, VCH Weinheim Impact and application of electron shuttles on the redox (bio)transformation of contaminants: a review

Chapter 5
Biodegradation of the Explosives TNT, RDX and HMX

Anat Bernstein and Zeev Ronen

5.1 Environmental Significance

In the early twentieth century, more than 60 highly explosive compounds were developed and synthesized for military and civilian use. Of these, the most widely used explosives in the world are probably hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), 2,4,6-trinitrotoluene (TNT) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) (Fig. 5.1). These compounds are characterized by relatively high thermal stability, high density and high detonation velocity, all of which promote their extensive use (Yinon 1990; Cooper and Kurowski 1997). Some of their physical properties are summarized in Table 5.1.

Following the extensive production and use of explosive compounds in the twentieth century, their contamination of soil and groundwater has become a global problem, as reflected from reports from the US (Pennington and Brannon 2002), Canada (Darrach et al. 1998), Argentina (Fuchs et al. 2001), the UK (Seth-Smith et al. 2008), Germany (Steuckart et al. 1994; Lewin et al. 1996), Sweden (Wingfors et al. 2006), Spain (Van Dillewijn et al. 2007), Israel (Bernstein et al. 2008), and Australia (Martel et al. 2008). However, an account of explosives contamination worldwide is not available.

Soil and water contamination by explosives is related to their manufacture, the production and loading of munitions items, inappropriate waste-disposal practices during production or during demilitarization activities or military training, and unexploded residuals in the battlefield. The highest extent of contamination is

A. Bernstein · Z. Ronen (✉)
Department of Environmental Hydrology and Microbiology,
Zuckerberg Institute for Water Research, Blaustein Institutes
for Desert Research, Ben-Gurion University of the Negev,
Sede Boqer Campus, 84990 Sede Boqer, Israel
e-mail: zeevrone@bgu.ac.il

S. N. Singh (ed.), *Microbial Degradation of Xenobiotics*,
Environmental Science and Engineering, DOI: 10.1007/978-3-642-23789-8_5,

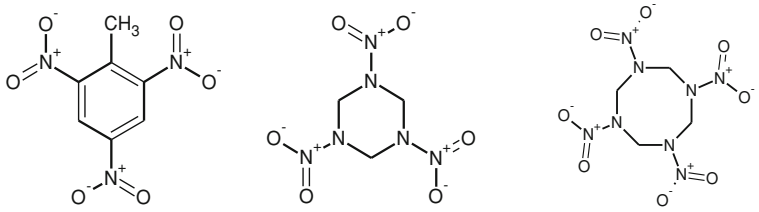


Fig. 5.1 Molecular structures of TNT, RDX and HMX

Table 5.1 Physical and chemical properties of TNT, RDX and HMX

Compound	Molecular weight (g/mol)	Melting point (°C)	Density (g/cm^3)	Vapor pressure (torr)	LogK_{ow}	Solubility (g/kg) at 20°C		
						Water	Acetone	Ethanol
TNT	227.13	80.6	1.65	5.51×10^{-6}	1.86	0.13	1,090	12.3
RDX	222.26	204	1.82	4.03×10^{-9}	0.86	0.04	83	1.5
HMX	296.20	286	1.90	3.33×10^{-14}	0.061	0.002	28	ND

ND not determined
Source Spanggord et al. (1982), Yinon and Zitrin (1993), McGrath (1995)

often associated with inappropriate wastewater handling. In particular, the discharge technique commonly used by explosives-manufacturing plants in which wastewater was discharged into unlined lagoons or streams, results in indirect contamination of soil and groundwater (Pennington and Brannon 2002). As a result, contamination often reached to high concentrations due to continuous discharge over a large surface area. Today, this method of waste disposal is prohibited throughout the western world.

In training areas where contamination by unexploded residuals is of deep concern, contamination of the upper soil layer may reach to high concentrations, but groundwater contamination may be somewhat limited since the compounds are normally spread over the soil surface in their solid phase. In this case, groundwater contamination strongly depends on precipitation, and on the compound solubility and dissolution kinetics (Morley et al. 2006).

In marine environments, contamination by explosive compounds may be caused as a result of sunken warships, military waste and navy training, as well as of dumping defective munitions. In this case, pollutant concentrations have been shown to be lower (Darrach et al. 1998).

The concentration of explosives in soil and groundwater not only depends on the polluting activities, but also on the discharge patterns, the thickness of the unsaturated zone, and natural attenuation processes which may either reduce the pollutant's point concentration (e.g., dilution) or reduce its entire mass in the environment (e.g., biodegradation). A literature review of the last few decades on soil and water pollution by explosives shows that their concentration in the

Table 5.2 Soil risk assessment screening and drinking water recommendations for explosive compounds

	Residential soil (mg/kg)	Industrial soil (mg/kg)	Drinking water (µg/l)
TNT	19	79	2
RDX	5.5	24	2
HMX	8,300	49,000	400

Source US EPA (2006, 2010)

groundwater may reach several milligrams per liter (Best et al. 1999; Charles et al. 2000; Bernstein et al. 2010), and in soils up to several or even tens of grams per kilogram (Boopathy 2000; Charles et al. 2000; Groom et al. 2001; Clark and Boopathy 2007). These concentrations exceed the recommended allowable amounts for soil and drinking water (Table 5.2).

Explosive compounds are undesirable in the environment due to their toxicity. TNT, RDX and HMX have been defined as toxic to humans and animals. Exposure to high concentrations of TNT causes anemia and abnormal liver function and is thought to promote spleen enlargement and to have other harmful effects on the blood, liver and immune system. It can also cause skin irritation after prolonged skin contact, and cataract development after long-term exposure (ATSDR 1996a). Long-term exposure to RDX can adversely affect the nervous system, and is thought to promote liver and kidney damage (ATSDR 1996b). Information on the adverse health effects of HMX is limited, but it is thought to be of lower toxicity than the other two. Nevertheless, studies in rats, mice, and rabbits indicate that HMX may be harmful to the liver and central nervous system if it is swallowed or gets on the skin (ATSDR 1997). TNT and RDX are classified by the US EPA as degree C carcinogenic, i.e., potential carcinogens in humans (US EPA 2006).

5.2 Biodegradation of Explosives

In contrast to natural attenuation processes such as dilution or sorption, which reduce the pollutant concentration, but do not reduce its overall mass in the environment, biodegradation is a natural attenuation process that promotes complete removal of the pollutant from the environment. Research on the biodegradation mechanisms of the explosive compounds that are the focus of this review has been carried out for the last four decades, starting with early works that studied degradation pathways for TNT (Won et al. 1974; McCormick et al. 1976) and RDX (McCormick et al. 1981) in sludge. These were followed by an increasing number of studies that identified additional catabolic pathways, isolated and identified increasing amounts of explosives-degrading bacteria, and studied the biochemical aspects of the processes at enzyme and genomic levels.

Research on biodegradation clearly shows the potential for reducing the concentrations of explosives in the environment via microbial activity. This can be

achieved by different degradation pathways that are strongly dictated by the redox potential of the surrounding environment and nutrient availability. The degradation pathways for the three most common explosives, RDX, HMX and TNT, are reviewed herein. The readers are also referred to some excellent review articles published in the last decade on the degradation pathways of the explosive compounds. Esteve-Núñez et al. (2001) and Stenuit et al. (2005) have focused on the biodegradation of TNT, Hawari et al. (2000a) on the biodegradation of both RDX and TNT, and Crocker et al. (2006) on the biodegradation of both RDX and HMX.

5.2.1 TNT

TNT can be biodegraded via various pathways, which mainly involve transformation of the nitro functional group, while the aromatic ring remains intact (Hawari et al. 2000a). The stability of the aromatic ring results from the strong electron-withdrawing properties of the nitro substituents which promote high electron deficiency and electrophilic characteristics on the π-electron system (Rieger and Knackmuss 1995). In addition, steric effects resulting from the symmetric position of the four functional groups protect the aromatic ring bonds from enzymatic attack (Stenuit et al. 2005).

In general, the degradation of TNT is mainly initiated by either reduction of the nitro group or C–NO_2 bond cleavage and denitration. Both can occur aerobically as well as anaerobically, yet each has unique catabolic steps. The reduction of the nitro group is often suggested to occur co-metabolically, implying that it is a non-beneficial process for the cell in terms of energy or nutrient yield. Thus, in this case, the microorganism will not derive carbon, nitrogen or energy (Stenuit et al. 2005). Nevertheless, it has been shown that TNT reduction may indeed be beneficial to the microbial cell, where it acts as a terminal electron acceptor in respiratory chains, as presented for *Pseudomonas* sp. strain JLR11 under anoxic conditions (Esteve-Núñez et al. 2000). In contrast to the presumed co-metabolic reduction pathway of the nitro group, the pathway, in which denitration of the nitro group occurs, has been shown to be clearly beneficial to the microorganism, as the nitrogen originating from the nitro group is available for further incorporation by the cell (Stenuit et al. 2005).

Normally, it is not the methyl group that plays a role in the key initial catabolic step, but the nitro groups, due to their strong electrophilic character. Nevertheless, additional possible pathways, albeit rarely documented, involve the removal or transformation of the methyl functional group at an initial transformation stage, as elaborated here.

5.2.1.1 Reduction of the Nitro Group

Anaerobic Reduction

The most common degradation pathway for TNT proceeds along the sequential reduction of the nitro groups. Following sequential steps of two-electron transfers, the corresponding mononitroso, monohydroxylamino and monoamino derivatives

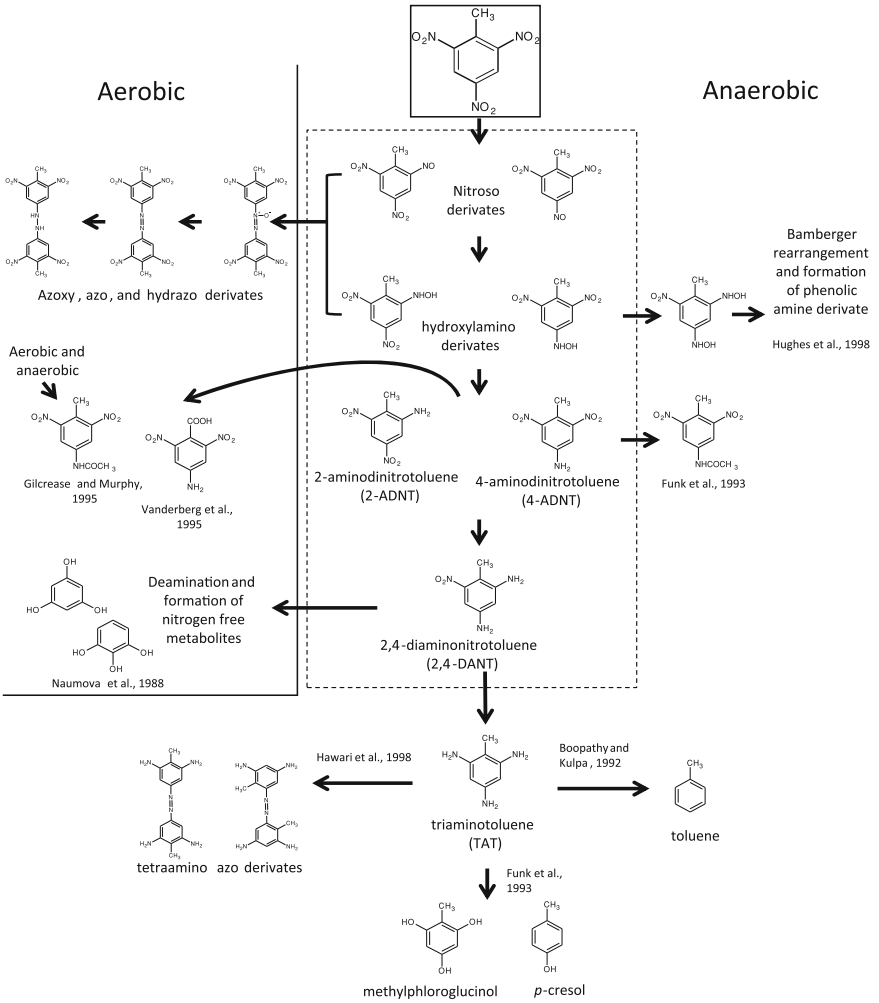


Fig. 5.2 Reduction pathway of TNT, and further transformation of the reduced derivatives under aerobic and anaerobic conditions

of TNT are sequentially formed (Fig. 5.2). Reduction of the monoamino derivatives can then proceed further to produce a diamino derivative.

The initial reduction toward the formation of diamino derivatives occurs under both aerobic and anaerobic conditions. It is performed by a large variety of microbial strains (Table 5.3) and it can be catalyzed by various enzymes, such as nitroreductase, aldehyde oxidase, dihydrolipic amide dehydrogenase, cytochrome b5 reductase, diaphorases, hydrogenases, xanthine oxidase, and carbon monoxide dehydrogenase (Esteve-Núñez et al. 2001). The reduction of the nitro group has also been shown to occur alternatively via two sequential steps of single-electron

Table 5.3 Microbial isolates of TNT degraders

Isolate	Conditions	Suggested pathway	Reference
Acinetobacter johnsonii	Aerobic	Pathway was not determined	Fuller and Manning (1997)
A. junii A8	Aerobic	TNT transforms to 2,6-DN-4-nitrosotoluene, 4-AM-2,6-dinitrotoluene, 4-AM-2,6-dinitrobenzoic acid	Soojhawon et al. (2005)
Agrobacterium sp. 2PC	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Alcaligenes eutrophus	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Anabaena sp.		Detection of azoxy-tetranitrotoluene isomers and hydroxylaminodinitrotoluene	Pavlostathis and Jackson (1999)
Arthrobacter globiformis	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Arthrobacter sp. RP17	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Bacillus cereus	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Bacillus sp.	Aerobic	(1) Release of nitrite, with final formation of toluene; (2) sequential reduction to the final formation of TAT	Kalafut et al. (1998)
B. subtilis	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Cellulomonas sp. ES6	Anaerobic	Amino and hydroxylamino derivatives	Borch et al. (2005)
Clostridium acetobutylicum	Anaerobic	Degradation via Bamberger rearrangement of 2,4-diHA-6-nitrotoluene to either 2-AM-4-HA-5-hydroxyl-6-nitrotoluene (4-AM-6-HA-3-methyl-2 nitrophenol) or 2-HA-4-AM-5-hydroxyl-6-nitrotoluene (6-AM-4-HA-3-methyl-2-nitrophenol).	Hughes et al. (1998)
C. acetobutylicum	Anaerobic	(1) Partial reduction by Bamberger rearrangement to HA intermediate; (2) TNT-TAT	Khan et al. (1997), Hughes et al. (1998)
C. bifermentans	Anaerobic	Detection of TAT, phenolic products of TAT hydrolysis, and TAT and pyruvic aldehyde condensation products	Lewis et al. (1996)
C. bifermentans	Anaerobic	Reductive TNT transformations to form TAT and phenolic products of TAT hydrolysis	Regan and Crawford (1994), Lewis et al. (1996)
C. bifermentans ATCC 638	Anaerobic	Reductive pathway	Ederer et al. (1997)
C. bifermentans KMR-1	Anaerobic	Reductive pathway	Ederer et al. (1997)

(continued)

Table 5.3 (continued)

Isolate	Conditions	Suggested pathway	Reference
C. pasteurianum DSM 525	Anaerobic	Reductive TNT transformations to form TAT and further transformation of TAT to undefined products	Preuss et al. (1993)
C. sordellii	Anaerobic	Reductive pathway	Ederer et al. (1997)
C. sporogenes	Anaerobic	Reductive pathway	Ederer et al. (1997)
C. thermoaceticum	Anaerobic		Huang et al. (2000)
Corynebacterium glutamicum	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Corynebacterium sp. Nap2	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Cytophaga pectinovora	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Desulfovibrio gigas	Anaerobic		Boopathy and Manning (1996)
D. indolicum	Anaerobic		Boopathy et al. (1997)
Desulfovibrio sp.	Anaerobic	Detection of TNT, DANT and TAT isomers	Drzyzga et al. (1999)
Desulfovibrio sp. strain B	Anaerobic	In the absence of external N sources, reductive deamination of amino derivatives to the final formation of toluene	Boopathy et al. (1993)
D. vulgaris	Anaerobic		Boopathy and Kulpa (1994)
Enterobacter cloacae PB2	Aerobic	Reduction to hydride-Meisenheimer complex with release of nitrite	French et al. (1998)
Escherichia coli	Aerobic	Pathway was not determined	Fuller and Manning (1997)
E. coli	Anaerobic	Reductive pathway	Ederer et al. (1997)
Flavobacterium odoratume	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Klebsiella sp. 1PC	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Klebsiella sp. C1	Anaerobic	Reduced to hydroxylamino dinitrotoluenes, aminodinitrotoluenes and to nitrite via denitration	Kim et al. (2002)
Lactobacillus acidophilus	Anaerobic	Reductive pathway	Ederer et al. (1997)
L. casei	Anaerobic	Reductive pathway	Ederer et al. (1997)
L. lactis	Anaerobic	Reductive pathway	Ederer et al. (1997)
Methanococcus deltae			Boopathy (1994)
Methanococcus sp. strain B	Anaerobic		Boopathy (1994)

(continued)

Table 5.3 (continued)

Isolate	Conditions	Suggested pathway	Reference
M. thermolithotrophicus			Boopathy (1994)
Methylobacterium sp. BJ001	Anaerobic	Reduced to ADNT	Van Aken et al. (2004)
Micrococcus luteus	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Mycobacterium sp. strain HL 4-NT-1	Aerobic	Formation of a hydride-Meisenheimer complex	Vorbeck et al. (1994)
M. vaccae strain JOB-5	Aerobic	(1) Detection of products as 4-AM-2,6-dinitrobenzoic acid and 2,4-diAM-6-nitrobenzyl methyl ether; (2) detection of azoxy compounds; (3) evidence of ring cleavage	Vanderberg et al. (1995)
Myxococcus xanthus	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Nocardiodes CB22-2	Aerobic	Dinitration leads to formation of Meisenheimer complex	Behrend and Heesche-Wagner (1999)
Pseudomonas	Aerobic	Production of hydride-Meisenheimer complex and transformation to DNT, NT and toluene	Duque et al. (1993)
P. aeruginosa	Aerobic	(1) Release of nitrite, with final formation of toluene; (2) sequential reduction to the final formation of TAT	Kalafut et al. (1998)
P. aeruginosa	Aerobic	Detection of HA and AM derivatives	Oh et al. (2001)
P. aeruginosa	Aerobic	Pathway was not determined	Fuller and Manning (1997)
P. aeruginosa strain MA01	Aerobic	Oxidation of ADNT isomers to N(4)-acetyl-2,4-diAM-6-nitrotoluene and tetranitroazoxytoluene isomers	Alvarez et al. (1995)
P. cepacia	Aerobic	Pathway was not determined	Fuller and Manning (1997)
P. fluorescens	Aerobic	Reduction by addition of hydride to form dihydride Meisenheimer complex to catalyze reduction of nitro group	Pak et al. (2000)
P. fluorescens	Aerobic	Pathway was not determined	Fuller and Manning (1997)
P. pseudoalcaligenes JS52	Aerobic and anaerobic	Detection of mono- and di-HADNTs, monoAM monoHA NT, and monoAM DNT	Fiorella and Spain (1997)
P. putida	Aerobic	Pathway was not determined	Fuller and Manning (1997)
P. putida HK-6	Aerobic	Reductive pathway	Cho et al. (2008)

(continued)

Table 5.3 (continued)

Isolate	Conditions	Suggested pathway	Reference
P. putida KP-T20 1	Aerobic	Amino derivatives of TNT, and denitrated products (DNT)	Park et al. (2003)
Pseudomonas sp. clone A	Aerobic	Formation of hydride-Meisenheimer complex, followed by denitration to form 2,4-DNT and an unidentified compound. AM and HA products were also detected, and condensed to azoxytoluenes	Haïdour and Ramos (1996)
Pseudomonas sp. DFC49	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Pseudomonas sp. JLR11	Anaerobic	Mineralization, via the formation of products such as 2,4,6-trinitrobenzaldehyde, 2-nitro-4-hydroxybenzoic acid, and 4-hydroxybenzaldehyde, and 4-hydroxybenzoic acid	Esteve-Núñez and Ramos (1998)
Pseudomonas sp. JS150	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Pseudomonas sp. Tol1A	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Rahnella aquitilis BFB	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Raoultella terrigena HB	Aerobic	AM derivatives and azoxy dimers	Claus et al. (2007)
Rhodococcus erythropolis	Aerobic	Dinitration leads to formation of Meisenheimer complex by F420 reductase and hydride transferase	Rieger et al. (1999)
R. erythropolis	Aerobic	Formation of hydride and dihydride TNT-Meisenheimer complexes	Vorbeck et al. (1998)
R. erythropolis	Aerobic	Pathway was not determined	Fuller and Manning (1997)
R. globerulus	Aerobic	Pathway was not determined	Fuller and Manning (1997)
R. rhodocrous	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Rhodococcus sp. TF2	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Salmonella typhimurium	Anaerobic	Reductive pathway	Ederer et al. (1997)
Serratia marcescens	Aerobic	Not defined. ADNT isomers were detected	Montpas et al. (1997)
SP1b (coryneform)	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Sphingomonas capsulata	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Staphylococcus sp.	Aerobic	(1) Release of nitrite, with final formation of toluene; (2) sequential reduction to final formation of TAT	Kalafut et al. (1998)

(continued)

Table 5.3 (continued)

Isolate	Conditions	Suggested pathway	Reference
Streptomyces albus	Aerobic	Pathway was not determined	Fuller and Manning (1997)
S. chromofuscus A11	Aerobic		Pasti-Grigsby et al. (1996)
S. griseus	Aerobic	Pathway was not determined	Fuller and Manning (1997)
Sulphate-reducing bacterium	Anaerobic	TNT reduction to TAT and further transformation to unknown products	Preuss et al. (1993)
Veillonella alkalescens			McCormick et al. (1976)
Yarrowia lipolytica AN-L15	Aerobic	Detection of eight different hydride complexes and nitrite release from the ring	Ziganshin et al. (2007)

DN dinitro, *AM* amino, *HA* hydroxylamino, *DANT* diaminonitrotoluene, *ADNT* aminodinitrotoluene, *TAT* triaminotoluene

transfers, forming first a nitroanion radical and then the corresponding nitroso derivative. This is catalyzed by the oxygen-sensitive nitroreductase enzymes, which are found in bacteria such as *Clostridium* (Angermaier and Simon 1983) and *Escherichia coli* (Peterson et al. 1979).

Because of the high electron deficiency on the nitro groups of TNT, its microbial degradation is often initiated by reductive rather than oxidative reactions, even under aerobic conditions (Vorbeck et al. 1998). Nevertheless, further transformation of the mono and diamino derivatives toward the formation of the most reduced product—triaminotoluene (TAT)—proceeds only under strictly anaerobic conditions in which redox potential values are essentially below −200 mV (Hawari et al. 2000a). Thus, under oxic conditions, diamino derivatives tend to accumulate, while the presence of TAT is indicative of strictly reduced conditions.

A few studies have aimed to identify further transformation steps of TAT under anaerobic conditions. Hawari et al. (1998) identified its further transformation to tetraaminoazo derivatives in an anaerobic sludge, which, in turn, disappeared from the solution and was suspected of polymerization (Hawari et al. 1998). Boopathy and Kulpa (1992) detected toluene as a product in TNT degradation by *Desulfovibrio* sp. strain B. They proposed that the toluene was formed by reductive elimination of the amino groups from TAT, but this pathway was never verified. Funk et al. (1993) identified the formation of para-hydroxytoluene (*p*-cresol) and methylphloroglucinol (2,4,6-trihydroxytoluene) in anaerobic mixed cultures. They suggested that these products were formed from TAT.

The reductive pathway of TNT towards the formation of amino derivatives may be desirable in contaminated environments, since the toxicity and mutagenic characteristics of the amino derivatives are found to be morally lower than those of TNT (Drzyzga et al. 1995; Lachance et al. 1999; Neuwoehner et al. 2007). In addition, the amino derivatives, and most significantly TAT, present higher sorption characteristics than TNT itself, with TAT even presenting irreversible sorption to soil particles (Daun et al. 1998; Achtnich et al. 1999). This strong affinity to soil material decreases the pollutant concentration in water. Nevertheless, it remains in the environment.

Some studies have shown that the anaerobic reductive pathway does not necessarily proceed to TAT, but to the alternative transformation of the partly reduced monohydroxylamine derivatives to dihydroxylamine derivatives which may be further transformed to phenolic amine products (Hughes et al. 1998). This was shown to occur anaerobically via Bamberger rearrangement of 2,4-dihydroxylamino-6-nitrotoluene by anaerobic *Clostridium acetobutylicum* cell extracts, as well as in whole-cell systems. The products of this mechanism were identified as either 2-amino-4-hydroxylamino-5-hydroxyl-6-nitrotoluene (4-amino-6-hydroxylamino-3-methyl-2-nitrophenol) or 2-hydroxylamino-4-amino-5-hydroxyl-6-nitrotoluene (6-amino-4-hydroxylamino-3-methyl-2-nitrophenol). Similar products were identified during incubation of TNT with the enzyme CO dehydrogenase purified from *Clostridium thermoaceticum* (Huang et al. 2000).

Aerobic Reduction

Reduced TNT derivatives are also commonly detected under aerobic conditions (Table 5.3). Nevertheless, under these conditions, the mono and diamino derivatives often accumulate in the medium without further metabolism (Esteve-Núñez et al. 2001) and the formation of TAT is halted. In the presence of oxygen, however, it has been shown that both the nitroso and the monohydroxylamino metabolites can follow an alternative abiotic transformation pathway to form tetranitroazoxytoluene dimmers, also referred as azoxytetranitrotoluene as shown in Fig. 5.2 (McCormick et al. 1976; Haïdour and Ramos 1996). These azoxy products were shown to cause a higher rate of mutations than TNT (George et al. 2001) and were thought to suppress the degradation of RDX and HMX which may coexist with TNT in the environment (Sagi-Ben Moshe et al. 2009). The accumulation of azoxy derivatives in the environment is hence clearly undesirable, but laboratory experiments have shown that these derivatives themselves may further degrade in microcosm experiments with the fungal strain *Phanerochaete chrysosporium* (Hawari et al. 1999), or in soil slurries with a mixed culture (Sagi-Ben Moshe et al. 2009), as well as in other experimental systems. In these two examples, complete disappearance of tetranitroazoxytoluene was achieved within a few weeks, indicating the non-recalcitrant nature of the compound. Its removal was followed by its further transformation to azo derivatives (tetranitroazotoluene), which, in turn, formed hydrazo derivatives (tetranitrohydrazotoluene), that also disappeared from the solution. Sorption of the azoxy metabolite to soil material in slurries was shown to occur in trace amounts (Sagi-Ben Moshe et al. 2009).

Under aerobic conditions, the amino derivatives may alternatively follow deaminization (Naumova et al. 1988), and be transformed to benzoic acid (Vanderberg et al. 1995) or *N*-acetylamino derivatives (Gilcrease and Murphy 1995). Thus, although the amino derivatives are often accumulated under aerobic conditions, they should not be treated as dead-end products.

5.2.1.2 Denitration

Aerobic Denitration

A variety of strains have been found capable of aerobic growth on TNT as sole nitrogen source. This unique phenomenon is coupled to their ability to denitrate the molecule with the subsequent release of nitrite to the medium (Table 5.3). A number of studies show that under aerobic conditions, the denitration pathway proceeds via the nucleophilic addition of a hydride ion to the aromatic ring in the presence of NAD(P)H and the formation of a hydride-Meisenheimer complex, which can be further transformed to a dihydride complex with the subsequent release of nitrite as depicted in Fig. 5.3 (Lenke and Knackmuss 1992). The formation of the Meisenheimer complex as a key metabolite that promotes denitration

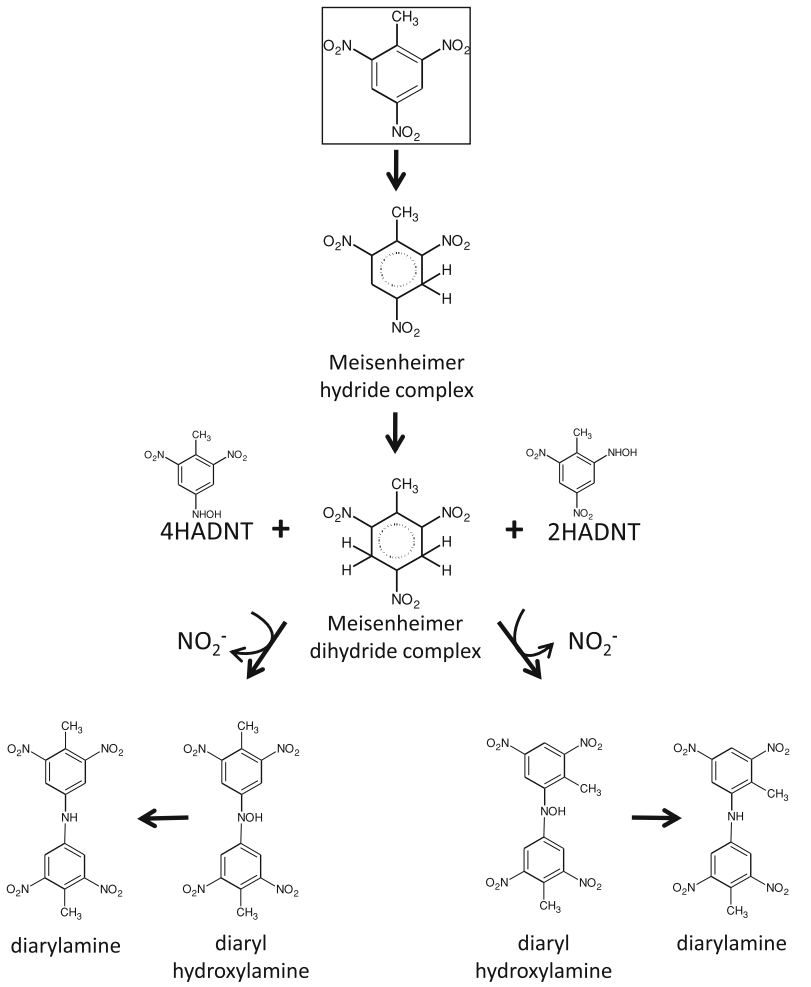


Fig. 5.3 The aerobic release of nitrite following the dimerization of dihydride complex with HADNTs

was first shown to occur with the bacterial strain *Mycobacterium* sp. strain HL 4-NT-1 (Vorbeck et al. 1994), and later proven for other strains also (Table 5.3). Several different enzymes of the type II hydride transferases have been identified capable of performing the nucleophilic addition of hydride ions (Stenuit et al. 2006): pentaerythritol tetranitrate reductase from *Enterobacter cloacae* PB2 (French et al. 1998), xenobiotic reductase B (XenB) from *Pseudomonas fluorescens* I-C (Pak et al. 2000), and N-ethylmaleimide (NEM) reductase from *E. coli* (Williams et al. 2004).

It was suggested that once the non-aromatic Meisenheimer complex is formed, aromaticity is restored upon nitrite release (Rieger and Knackmuss 1995).

This hypothesis was adopted to explain the detection of 2,4-dinitrotoluene as a metabolic product of TNT's hydride-Meisenheimer complexes during incubation with the white-rot fungus *Irpex lacteus* (Kim and Song 2000). Nevertheless, in the last few years, more and more studies have shown that the release of nitrite from the TNT molecule likely occurs following dimerization of the dihydride complex with coexisting hydroxylaminodinitrotoluenes (HADNTs) with the formation of the secondary diarylamines, accompanied by the release of stoichiometric amounts of nitrite as shown in Fig. 5.3 (Pak et al. 2000; Stenuit et al. 2006; Van Dillewijn et al. 2008; Wittich et al. 2008). The condensation of the two molecules and the release of nitrite were shown to be a potentially chemically catalyzed reaction rather than an enzymatic one, and the nitrite that is released during the condensation was shown to originate from the Meisenheimer-dihydride complex rather than from the hydroxylamine (Wittich et al. 2009).

In addition to the increasing evidence of the importance of Meisenheimer complexes in TNT denitration, it is evident that denitration can potentially occur via other routes. An example is given by Stenuit et al. (2009), in which extracellular catalysts extracted from the supernatant of *Pseudomonas aeruginosa* ESA-5, were incubated with TNT and NAD(P)H, and significant release of nitrite was observed. The release of nitrite was coupled with the formation of two polar metabolites, which had lost two nitro groups from the parent compound. It was suggested that superoxide radicals ($O_2^{\bullet-}$) and hydrogen peroxide are involved in the denitration process. This pathway, however, has not been demonstrated in living cells.

Other studies showing denitration with the release of nitrite and formation of dinitrotoluene, mononitrotoluene and even toluene were documented by Duque et al. (1993) and Kalafut et al. (1998). However, the detailed mechanisms remain still to be investigated.

Anaerobic Denitration

Under anaerobic conditions, similar to aerobic denitration, denitration of the nitro group of the aromatic ring has also been shown to occur in the absence of alternative nitrogen sources, although fewer strains have actually been shown capable of utilizing TNT as sole nitrogen source under such conditions.

One example is provided by Esteve-Núñez and Ramos (1998), who studied the metabolism of 2,4,6-trinitrotoluene by *Pseudomonas* sp. JLR11, which utilizes TNT as sole N source under anaerobic conditions. During incubation, release of nitrite was detected. This strain incorporated around 85% of the N-TNT into N-organic nitrogen. The mechanism of nitrite release by this strain remains still to be explored.

Another example is provided by Eyers et al. (2008), who identified denitration in an oxygen-depleted enrichment culture, where TNT served as sole nitrogen source. During incubation, significant release of nitrite was observed and *P. aeruginosa* ESA-5 was subsequently isolated as the denitrating strain. Reduced derivatives of

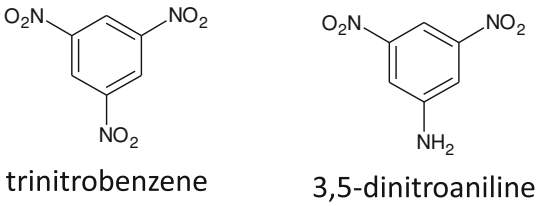


Fig. 5.4 Methyl-group transformation products detected by Esteve-Núñez and Ramos (1998)

TNT and several unidentified metabolites were detected as well. Nevertheless, the exact mechanism of the anaerobic denitration was not traced out. It should be noted that the facultative anaerobe *P. aeruginosa* had already been reported to promote TNT denitration under aerobic conditions (Kalafut et al. 1998; Oh et al. 2003), but whether the pathway in both cases was similar is still unknown. Denitration was also shown to be catalyzed by extracellular catalysts of this strain as discussed earlier (Stenuit et al. 2009), which would be governing catalysts of the denitration.

5.2.1.3 Pathways Involving the Methyl Group

The methyl group is not normally involved in the early degradation steps of TNT. Nevertheless, a few exceptional studies have shown its removal in an initial catabolic reaction. Involvement of the methyl group as an initial step in the degradation of TNT under anaerobic conditions was shown by Esteve-Núñez and Ramos (1998), who studied the metabolism of TNT by *Pseudomonas* sp. JLR11, which utilizes TNT as a sole N source under anaerobic conditions. Of the products detected, 1,3,5-trinitrobenzene and 3,5-dinitroaniline were identified (Fig. 5.4), thus indicating the potential removal of the methyl group from TNT under similar conditions.

A different pathway showing the involvement of the methyl group in TNT degradation by *Mycobacterium vaccae* was reported by Vanderberg et al. (1995), who detected the transformation of the methyl group to carboxylic acid, and the consequent formation of 4-amino-2,6-dinitrobenzoic acid (Fig. 5.2). Nevertheless, the transformation of the methyl group in this pathway was unlikely the initial transformation step, but reduction to an amino derivative was likely.

5.2.2 RDX

RDX is composed of a triazinic ring to which three nitro functional groups are perpendicularly attached. In RDX, similar to TNT, the nitro groups are the main targets of the first degradation steps by sequential reduction or denitration as key steps (Fig. 5.5, Table 5.4).

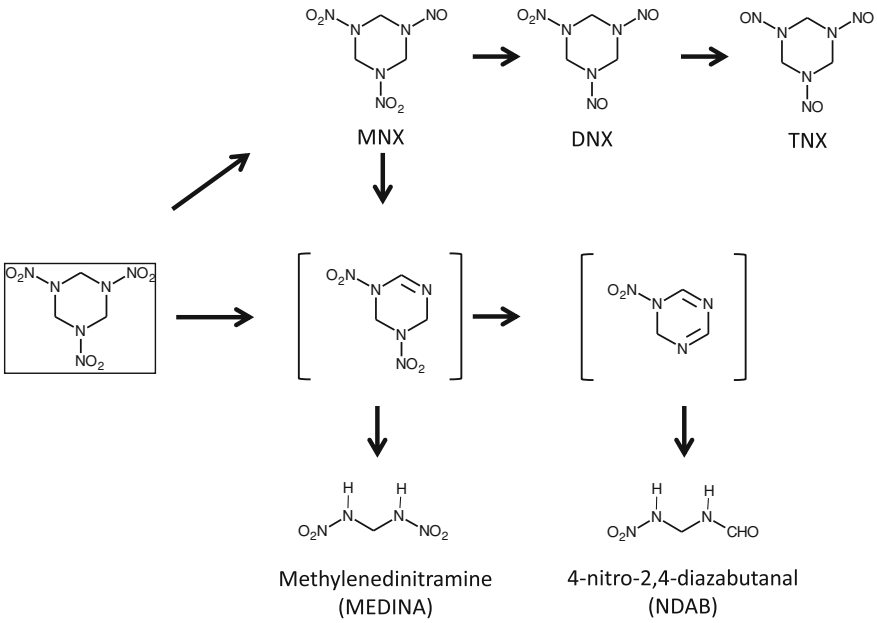


Fig. 5.5 The common degradation pathways of RDX. Compounds in *brackets* are postulated intermediates

5.2.2.1 Sequential Reduction Pathway

Anaerobic Reduction

In contrast to TNT, sequential reduction of RDX occurs mostly under anaerobic conditions, as initially presented by McCormick et al. (1981). This pathway proceeds through reduction of the nitro groups to nitroso derivatives by subsequent two-electron transfer steps, followed by accumulation of hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) derivatives. Again in contrast to TNT, the nitroso derivatives do not tend to undergo further reduction to stable detectable product. Although this degradation pathway leads to the desired decrease in RDX concentrations in the contaminated environment, the accumulation of the nitroso derivatives themselves is undesirable due to their toxicity (Zhang et al. 2006). Nevertheless, the nitroso derivatives can further degrade via ring cleavage, after being transferred to the corresponding hydroxylamine metabolites, and produce more simple compounds that can undergo complete mineralization (McCormick et al. 1981).

Table 5.4 Microbial isolates of RDX degraders

Isolate	Conditions	Suggested pathway, indicative intermediates	Reference
Bacillus strains HPB2, HPB3	Denitrifying	Pathway not defined, products not identified	Singh et al. (2009)
Acetobacterium malicum strain HAAP-1	Anaerobic	Denitration of RDX and MNX followed by ring cleavage. Detection of MEDINA	Adrian and Arnett (2004)
A. paludosum	Anaerobic	Ring cleavage of RDX. nitroso derivatives were not detected. Other intermediates were not identified	Sherburne et al. (2005)
Citrobacter freundii	Anaerobic	Sequential reduction. Detection of MNX, DNX, TNX	Kitts et al. (1994)
C. freundii NS2	Anaerobic	Sequential reduction. Detection of MNX, DNX, TNX	Kitts et al. (1994)
Clostridium acetobutylicum	Anaerobic	Reduction and formation of amino products. Detection of mononitroso-, monohydroxylamino-, mononitrosomonohydroxylamino-, monoamino-, diamino-, and triamino-compounds	Zhang and Hughes (2003)
C. bifermentans HAW-1	Anaerobic	(1) Reduction to MNX; (2) MNX denitration followed by ring cleavage	Zhao et al. (2003a, b)
Clostridium sp. EDB2	Anaerobic	Denitration pathway. Products were not reported	Bhushan et al. (2004)
Clostridium sp. HAW-1, HAW-G3, HAW-G4, HAW-E3, HAW-HC1, and HAW-ES2	Anaerobic	(1) Reduction to MNX; (2) MNX denitration followed by ring cleavage	Zhao et al. (2003b)
Clostridium sp. HAW-E3	Anaerobic	(1) Reduction to MNX; (2) MNX denitration followed by ring cleavage	Zhao et al. (2003b)
Clostridium sp. HAW-EB17	Anaerobic	Sequential reduction, denitration and ring cleavage of RDX and MNX. Detection of MNX, DNX, TNX. With resting cells, detection of NDAB and MEDINA	Zhao et al. (2004a)
Clostridium sp. HAW-G4	Anaerobic	(1) Reduction to MNX; (2) MNX denitration followed by ring cleavage	Zhao et al. (2003b)
Clostridium sp. HAW-HC1	Anaerobic	(1) Reduction to MNX; (2) MNX denitration followed by ring cleavage	Zhao et al. (2003b)

(continued)

Table 5.4 (continued)

Isolate	Conditions	Suggested pathway, indicative intermediates	Reference
Desulfovibrio sp. HAW-EB18	Anaerobic	Sequential reduction, denitration and ring cleavage of RDX and MNX. Detection of MNX, DNX, TNX. With resting cells, detection of NDAB and MEDINA	Zhao et al. (2004a)
Desulfovibrio sp.	Anaerobic	Pathway not defined. Sequential reduction or direct ring cleavage were excluded as major pathways following low nitroso derivative concentrations and no detection of MEDINA	Arnett and Adrian (2009)
Enterobacter cloacae	Anaerobic	Sequential reduction. Detection of MNX, DNX, TNX	Kitts et al. (2000), Pudge et al. (2003)
Fusobacteria isolate HAW-EB21	Anaerobic	Sequential reduction, denitration and ring cleavage of RDX and MNX. Detection of MNX, DNX, TNX. With resting cells, detection of NDAB and MEDINA	Zhao et al. (2004a, b)
Geobacter metallireducens strain *GS-15*	Anaerobic	Reduction to MNX, RDX and MNX denitration and ring cleavage. Detection of MEDINA	Kwon and Finneran (2008)
Gordonia sp. KTR9	Aerobic	Pathway not defined, products not identified	Thompson et al. (2005)
Gorodnia strain YY1	Aerobic	RDX denitration and ring cleavage. Detection of NDAB	Ronen et al. (2008)
Klebsiella pneumoniae SCZ1	Anaerobic	(1) Reduction to MNX; (2) MNX denitration followed by ring cleavage	Zhao et al. (2002)
Methylobacterium extorquens	Aerobic	Reduction to MNX followed by denitration and ring-cleavage. Detection of MNX, MEDINA	Van Aken et al. (2004)
M. organophilum	Aerobic	Reduction to MNX followed by denitration and ring-cleavage. Detection of MNX, MEDINA	Van Aken et al. (2004)
M. rhodesianum	Aerobic	Reduction to MNX followed by denitration and ring-cleavage. Detection of MNX, MEDINA	Van Aken et al. (2004)
Methylobacterium sp. BJ001	Aerobic	Reduction to MNX followed by denitration and ring-cleavage. Detection of MNX, MEDINA	Van Aken et al. (2004)
Morganella morganii B2	Anaerobic	Sequential reduction. Detection of MNX, DNX, TNX	Kitts et al. (1994)
M. morganii	Anaerobic	Sequential reduction. Detection of MNX, DNX, TNX	Kitts et al. (1994)

(continued)

Table 5.4 (continued)

Isolate	Conditions	Suggested pathway, indicative intermediates	Reference
Providencia rettgeri B1	Anaerobic	Sequential reduction. Detection of MNX, DNX, TNX	Kitts et al. (1994)
P. rettgeri	Anaerobic	Sequential reduction. Detection of MNX, DNX, TNX	Kitts et al. (1994)
Pseudomonas putida HK-6	Aerobic	Pathway not defined, products not identified	Cho et al. (2008)
Pseudomonas strain HPB1	Denitrifying	Pathway not defined, products not identified	Singh et al. (2009)
Rhodococcus rhodochrous sp. 11Y	Aerobic	Two denitration steps followed by ring cleavage. Detection of NDAB	Seth-Smith et al. (2002)
Rhodococcus sp. DN22	Aerobic	Two denitration steps followed by ring cleavage. Detection of NDAB	Coleman et al. (1998), Fournier et al. (2002), Bhushan et al. (2003b)
Rhodococcus sp. YH1	Aerobic	Two denitration steps followed by ring cleavage. Detection of NDAB	Nejidat et al. (2008)
Serratia marcescens	Anaerobic	Sequential reduction. Detection of MNX, DNX, TNX	Young et al. (1997)
Shewanella halifaxensis HAW-EB4	Anaerobic	Sequential reduction. Detection of MNX, DNX, TNX	Zhao et al. (2004a, 2006)
S. sediminis HAW-EB3	Anaerobic	Sequential reduction. Detection of MNX, DNX, TNX	Zhao et al. (2004a, 2005)
Shewanella sp. HAW-EB1, HAW-EB2, HAW-EB5	Anaerobic	Sequential reduction, denitration and ring cleavage of RDX and MNX. Detection of MNX, DNX, TNX, NDAB, MEDINA	Zhao et al. (2004a)
Stenotrophomonas maltophilia PB1	Aerobic	Pathway not determined, detection of methylene-N-(hydroxymethyl)-hydroxylamine-N'-(hydroxymethyl)nitroamine	Binks et al. (1995)
Williamsia sp. KTR4	Aerobic	Pathway not defined, products not identified	Thompson et al. (2005)

Aerobic Reduction

Under aerobic conditions, the formation of nitroso derivatives is normally not observed. This may be expected from thermodynamic considerations, where the calculated E^0 shows a decrease from trinitroaromatics to nitramine, suggesting thermodynamic control of the reduction of RDX under aerobic conditions (Uchimiya et al. 2010).

Only a few exceptions indicate formation of mononitroso derivatives under aerobic conditions: the first, reported for incubation of the white-rot fungus *P. chrysosporium* with RDX (Sheremata and Hawari 2000) which showed formation of MNX. This was followed by ring cleavage and the subsequent formation of methylene dinitramine (MEDINA). The second, presented by Van Aken et al. (2004), reported the detection of MNX during RDX biodegradation under aerobic conditions by a phytosymbiotic *Methylobacterium* sp. that was associated with poplar tissue. Latter on these authors observed a polar metabolite which was produced by the ring cleavage of MNX, and had the molecular formula of MEDINA. The detection of di- or tri-nitro derivatives under aerobic conditions has never been documented.

5.2.2.2 Denitration

Aerobic Denitration

Denitration appears to be the most important aerobic degradation pathway for RDX, and was initially demonstrated for *Rhodococcus* strain DN22 (Fournier et al. 2002; Bhushan et al. 2003b). Later, it was also shown to be the governing pathway for *Rhodococcus* strains 11Y (Seth-Smith et al. 2002) and YH1 (Nejidat et al. 2008). It was also assumed to occur in *Williamsia* sp. strain KTR4, and *Gordonia* sp. strain KTR9 (Thompson et al. 2005), with the observation of a degradation product which was thought to be 4-nitro-2,4-diazabutanal (NDAB).

In recent years, considerable effort has been made in delineating the biochemical pathway of RDX biodegradation carried out by the various *Rhodococcus* species. In contrast to denitration of TNT, which is not accompanied by ring cleavage due to the relatively high stability of the aromatic ring, denitration of RDX has been shown to be accompanied by spontaneous ring cleavage, and the denitrated RDX intermediate prior to ring cleavage is thus never observed. Under aerobic conditions, the denitration pathway has been suggested to be promoted by two subsequent single-electron transfer steps which promote the release of two nitro groups, after which ring cleavage proceeds, with the subsequent formation of NDAB as the ring-cleavage product. Recently, MEDINA was detected as well as a ring-cleavage product under aerobic conditions, in experiments conducted with *Rhodococcus* strain DN22 (Halasz et al. 2010), as well as under microaerophilic conditions by various other *Rhodococcus* strains (Fuller et al. 2010).

The denitration of RDX by *Rhodococcus* species was reported to be catalyzed by a unique form of the enzyme cytochrome P450 (Coleman et al. 2002) which was later found to be encoded by the gene *XplA* and to promote denitration with NADPH as an electron donor (Indest et al. 2007; Jackson et al. 2007; Seth-Smith et al. 2008; Roh et al. 2009; Rylott et al. 2011). The activity of cytochrome P450 in the denitration of RDX was demonstrated in vivo for the *Rhodococcus* strains DN22 (Coleman et al. 2002; Fournier et al. 2002; Bhushan et al. 2003b), 11Y (Seth-Smith et al. 2008) and YH1 (Tekoah et al. 1999; Nejidat et al. 2008).

The cytochrome P450 enzymes catalyze a vast array of chemical reactions. These enzymes are notable for both the diversity of the reactions they catalyze and the range of chemically dissimilar substrates upon which they act. Cytochrome P450s support the oxidative, peroxidative and reductive metabolism of such endogenous and xenobiotic substrates as environmental pollutants, agrochemicals, plant allelochemicals, steroids, prostaglandins and fatty acids (Danielson 2002). Encoded by *XplA/XplB*, the arrangement of the enzyme's subunits is unique: it comprises a flavodoxin domain fused to an N-terminal cytochrome P450 domain (Hlavica 2009; Indest et al. 2010; Rylott et al. 2011). *XplB* serves as a partner NADH-utilizing flavodoxin reductase (Jackson et al. 2007). Since it has been detected in RDX-degrading *Rhodoccocus* strains worldwide and since it was absent in *Rhodoccocus* species that were not exposed to RDX, it is assumed to have evolved in response to RDX exposure (Seth-Smith et al. 2008).

In most studies, RDX-degrading *Rhodococcus* strains have been isolated from explosive-contaminated soils: strain DN22 was isolated from contaminated soils in Australia (Coleman et al. 1998), strain 11Y and strains HS1-HS19 from contaminated soils in England (Seth-Smith et al. 2002, 2008), and strains YH1 and YY1 from contaminated soils in Israel (Tekoah et al. 1999; Brenner et al. 2000; Ronen et al. 2008). Recently, *Rhodococcus* strains have also been isolated from explosive-contaminated groundwater (Bernstein et al. 2011).

Anaerobic Denitration

Anaerobic denitration has been reported to be carried out by two distinct strains: *Klebsiella pneumoniae* strain SZC-1 (Zhao et al. 2002) and *Clostridium bifermentans* strain HAW-1 (Zhao et al. 2003a), both isolated from the anaerobic sludge. The RDX denitration under anaerobic conditions was accompanied by ring cleavage and the formation of MEDINA rather than the NDAB which is normally observed following denitration under aerobic conditions. This implies that during the anaerobic denitration, as postulated by Zhao et al. (2003a), only one single-electron transfer step occurs with the formation of a free-radical anion ($RDX^{\bullet -}$) and the subsequent release of a single nitro group and ring cleavage.

Anaerobic denitration has also been detected for strains isolated from cold marine sediments: *Shewanella sediminis* strain HAW-EB3 (Zhao et al. 2005) and *Shewanella halifaxensis* HAW-EB4 (Zhao et al. 2006), with the detection of both NDAB and MEDINA as ring-cleavage products. The degradation mechanism

involved a specific c-type cytochrome in the anaerobic RDX metabolism, which degraded RDX by mono-denitration (Zhao et al. 2010).

Denitration was studied at the enzyme level by Jackson et al. (2007) under anoxic conditions focusing on the activity of *XplA* which plays an important role in the aerobic degradation of RDX. With *XplA* expression under anoxic conditions, denitration was also shown to be an initial catabolic step. Nevertheless, under anaerobic conditions, following only a single denitration and a single hydration step, ring cleavage of RDX was already observed with the detection of MEDINA as a ring-cleavage product. Under aerobic conditions, on the other hand, RDX was proposed to follow two denitration and two hydration steps before ring cleavage occurs, leading to the formation of NDAB (Jackson et al. 2007). Nevertheless, in contrast to the in vitro study by Jackson et al. (2007), it was noted in in vivo studies with various *Rhodococcus* strains that denitration is actually halted under anaerobic conditions (Fuller et al. 2010).

The transformation of RDX by *Rhodococcus* strains in general and activity of the *XplA* system in particular have garnered much attention. However, RDX denitration by a different system–the xenobiotic reductase enzymes, has been studied recently (Fuller et al. 2010). Xenobiotic reductase enzymes capable of degrading TNT (Blehert et al. 1999; Pak et al. 2000) have been also shown to degrade RDX in addition to other energetic compounds. The enzymes XenA (in *Pseudomonas putida* II-B) and XenB (in *Pseudomonas fluorescens* I-C) were observed capable of transforming RDX, especially at low oxygen concentrations (and to a small extent under aerobic conditions as well), when external sources of carbon (as succinate) and nitrogen (as ammonium) are added. The primary degradation product for RDX by these studied enzymes was identified as MEDINA, but an additional minor pathway produced NDAB during transformation by whole cells of *P. putida* II-B and by purified XenA. In accordance to detected degradation products, it is likely that ring cleavage was the result of the previous denitration. The exact mechanism of the reaction, however, has not yet been studied.

5.2.2.3 Other Pathways

Ring-cleavage of RDX has also been shown to occur under anoxic conditions, via a route that is not initiated by denitration (Hawari et al. 2000b). This pathway was documented in biodegradation experiments carried out with municipal anaerobic sludge under measured Eh values of −250 to −300 mV. The anoxic ring-cleavage route was postulated to involve enzymatic hydrolysis of an inner C–N bond as the initial step. This was followed by ring cleavage, in which the triazinic ring was divided into two detectable products: MEDINA and bis(hydroxymethyl)nitramine. The ring-cleavage products were further degraded with eventual formation of simpler products. Halasz et al. (2002) suggested that during incubation of RDX with the above sludge, water is involved in the formation of MEDINA. Following experiments with deuterated water, they observed deuterated MEDINA products, but could not determine whether inclusion of water occurred through the initial

enzymatic attack on RDX with enzymatic cleavage of the inner C–N bond or was simply caused by subsequent hydrolysis of the ring-cleavage product.

Another very different RDX-transformation pathway was suggested by Zhang and Hughes (2003) who performed experiments with crude cell extract of *C. acetobutylicum* and demonstrated the transformation of RDX with H_2 as an electron donor. The degradation was accompanied by the formation of hydroxylamino compounds, analogous to the transformation of TNT. Nevertheless, this pathway was not found with whole cells, and thus not yet confirmed to be of environmental relevance.

5.2.3 HMX

HMX is less amenable to biodegradation in the environment than RDX due to its lower water solubility and its relative chemical stability (Hawari et al. 2000a). It is structurally similar to RDX, and thus follows analogous degradation pathways: both sequential reduction of the nitro group to nitroso derivatives and anaerobic denitration followed by ring cleavage have been documented as HMX-degradation pathways (Fig. 5.6). Nevertheless, the study of these degradation pathways has not been as extensive as for RDX, and limited microbial strains were found capable of degrading this compound (Table 5.5). Low documentation may be due to the more recalcitrant nature of the compound, or also because it is of less environmental significance and interest (HMX is less frequently found in the environment, and its contamination is of little concern: drinking water recommendations for HMX are more than two orders of magnitude higher than those for RDX) (Table 5.2). The degradation pathways which have been presented for HMX are summarized as below:

5.2.3.1 Sequential Reduction of the Nitro Group

Similar to the sequential reduction of RDX to its corresponding nitroso derivatives following subsequent two-electron transfer steps (McCormick et al. 1981), nitro groups of the HMX molecule can be reduced to nitroso derivatives. Nevertheless, while for RDX, all three nitroso derivatives are observed in high abundance, in HMX, only less reduced nitroso derivatives are normally detected. For example, the detection of only a mononitroso derivative was documented by Fournier et al. (2004) with the fungal strain *P. chrysosporium*. In this case, the mononitroso derivative did not continue along the sequential pathway, but underwent ring cleavage accompanied by NDAB formation. Similarly, ring cleavage of the mononitroso derivative was also observed by Zhao et al. (2007) with *C. bifermentans* strain HAW-1 isolated from an anaerobic sludge.

The formation of the first two nitroso derivatives: mono- and dinitroso HMX, was observed by Hawari et al. (2001) following incubation of HMX with

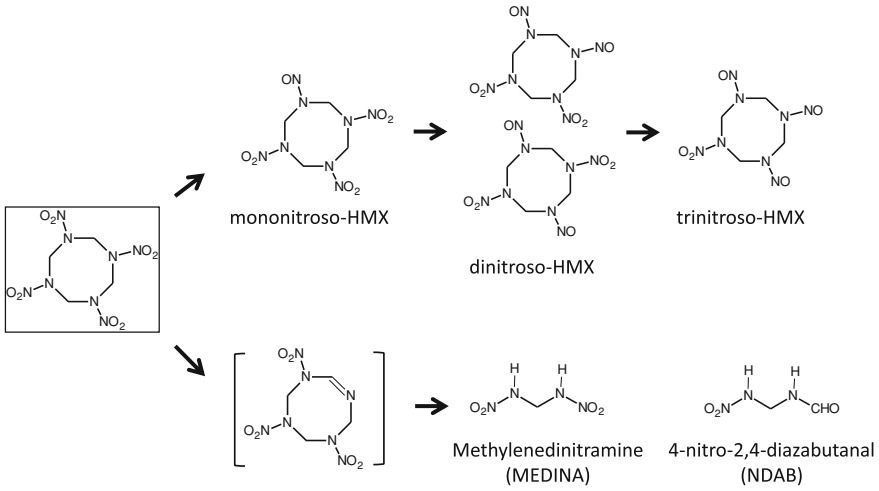


Fig. 5.6 Postulated degradation pathways of HMX. Compounds in *brackets* are postulated intermediates

anaerobic sludge. Further degradation of these nitroso derivatives was not identified in this study.

The formation of the first three nitroso derivatives: mono-, di-, and trinitroso HMX, was detected with various anaerobic strains isolated from marine sediments: strain HAW-EB21 (Zhao et al. 2004a, b), and resting-cell incubations of *Paenibacillus* strain UXO5-11 and strain UXO5-19 (which is not phylogenetically affiliated), and of *Desulfovibrion* strain midref-29 (Zhao et al. 2007).

Formation of most reduced nitroso derivative of HMX: tetranitroso-HMX has not yet been documented.

5.2.3.2 Initial Denitration Followed by Ring Cleavage

Anaerobic denitration of HMX as a result of a single-electron transfer, followed by ring cleavage and MEDINA formation, was detected by resting cells of *C. bifermentans* strains HAW-1 and HAW-EB21 (Zhao et al. 2004a, b). NDAB was not detected in this study. Bhushan et al. (2003a) studied the transformation pathway of HMX with the metallo-flavo enzyme xanthine oxidase. Based on the detected products, they proposed that HMX undergoes a single denitration step. They observed this step under anaerobic conditions, whereas under aerobic conditions, HMX competed with O_2 for binding to the enzyme's active site, and thus resulted in smaller yield. The denitrated product was found unstable and subsequent ring cleavage resulted in the formation of both NDAB and MEDINA. However, their formation was not demonstrated for a whole-cell system.

In the absence of oxygen, HMX was degraded by *P. fluorescens* I-C (Fuller et al. 2009). Microbial isolates capable of performing aerobic denitration and

Table 5.5 Microbial isolates of HMX degraders

Isolate	Conditions	Suggested pathway, indicative intermediates	Reference
Bacillus strain HPB2, strain HPB3	Denitrifying	Pathway not defined, products not identified	Singh et al. (2009)
Citrobacter freundii NS2	Anaerobic	Sequential reduction. Detection of mono- and dinitroso HMX	Kitts et al. (1994)
Clostridiale strain UXO5-19	Anaerobic	(1) Denitration followed by ring cleavage; (2) reduction of one or more nitro groups. Detection of mononitroso HMX, MEDINA	Zhao et al. (2007)
Clostridium bifermentans HAW-1	Anaerobic	(1) Sequential reduction; (2) denitration	Zhao et al. (2004b)
Clostridium sp. EDB2	Anaerobic	Pathway not defined, Products not identified	Bhushan et al. (2004)
Clostridium sp. HAW-E3	Anaerobic	(1) Sequential reduction; (2) denitration	Zhao et al. (2004b)
Clostridium sp. HAW-EB17	Anaerobic	Pathway not defined	Zhao et al. (2004b)
Clostridium sp. HAW-G4	Anaerobic	(1) Sequential reduction; (2) denitration	Zhao et al. (2004b)
Clostridium sp. HAW-HC1	Anaerobic	(1) Sequential reduction; (2) denitration. Detection of mono-, di- and trinitroso HMX	Zhao et al. (2004b)
Desulfovibrio sp. HAW-EB18	Anaerobic	Pathway not defined. Detection of mononitroso HMX	Zhao et al. (2004b)
Desulfovibrio strain Midref-29	Anaerobic	(1) Denitration followed by ring cleavage; (2) reduction of one or more nitro groups. Detection of mononitroso HMX, MEDINA	Zhao et al. (2007)
Fusobacteria isolate HAW-EB21	Anaerobic	Pathway not defined. Detection of mononitroso HMX	Zhao et al. (2004b)
Klebsiella pneumoniae SCZ1	Anaerobic	(1) Sequential reduction; (2) denitration. Detection of mononitroso HMX, MEDINA	Zhao et al. (2004b)
Methylobacterium extorquens	Aerobic	Pathway not defined, products not identified	Van Aken et al. (2004)
M. organophilum	Aerobic	Pathway not defined, products not identified	Van Aken et al. (2004)
M. rhodesianum	Aerobic	Pathway not defined, products not identified	Van Aken et al. (2004)
Methylobacterium sp. BJ001	Aerobic	Reduction	Van Aken et al. (2004)
Morganella morganii B2	Anaerobic	Sequential reduction. Detection of mono- and dinitroso HMX	Kitts et al. (1994)

(continued)

Table 5.5 (continued)

Isolate	Conditions	Suggested pathway, indicative intermediates	Reference
Paenibacillus strain UXO5-11	Anaerobic	(1) Denitration followed by ring cleavage; (2) reduction of one or more nitro groups. Detection of mononitroso HMX, MEDINA	Zhao et al. (2007)
Providencia rettgeri B1	Anaerobic	Sequential reduction. Detection of mono- and dinitroso HMX	Kitts et al. (1994)
Pseudomonas strain HPB1	Denitrifying	Pathway not defined, products not identified	Singh et al. (2009)
Pseudomonas strain HPB1	Denitrifying	Pathway not defined, products not identified	Singh et al. (2009)

ring cleavage, similar to the common aerobic pathway of RDX, have not been detected.

5.3 Influence of Chemical and Physical Properties on Biodegradation Rate

Looking at the reductive transformation of the nitro group from a purely energetic compounds, one would expect the decrease in reduction rate from TNT to RDX, and finally to HMX (Uchimiya et al. 2010). On the other hand, in complex biotic systems such as the sub-surface, factors other than energetic yield will dictate the rate at which explosives degrade.

Nutrient availability is one important factor that plays a significant role in the rate of explosives biodegradation. Compared to other common pollutants, explosives, particularly RDX and HMX, are characterized by a higher N/C ratio. Although they may theoretically serve as sources of both carbon and nitrogen, only nitrogen is used by some organisms, and hence, another carbon source must be added (Allard and Neilson 1997). Various strains have been shown to degrade TNT and RDX as sole nitrogen sources (Tables 5.3 and 5.4), but evidence for their degradation as sole carbon source is scarce. Somewhat exceptional are growths of a *Gordonia* strain and *Williamsia* spp. on RDX as sole carbon source (Thompson et al. 2005), or growth of a transconjugant *Pseudomonas* sp. on TNT as sole carbon source (Duque et al. 1993).

Since denitration is frequently carried out by strains that are capable of utilizing the explosive compound as a nitrogen source, a negative response is often observed in microcosm experiments when external nitrogen sources (either ammonium or nitrate) are added, as these nitrogen-containing compounds may compete with degradation of the explosives for nitrogen (Binks et al. 1995; Coleman et al. 1998; Nejidat et al. 2008; Ronen et al. 2008; Bernstein et al. 2011). On the other hand, a positive response can be observed with additional carbon sources, as additional carbon supports microbial growth without competing with the explosive compounds for metabolism of RDX (Speitel et al. 2001; Waisner et al. 2002; Ronen et al. 2008) and TNT (Boopathy and Manning 1996).

Nitrate may not only compete with the explosive compounds as an alternative nitrogen source, but under anaerobic conditions, it may also compete as an electron acceptor. This was observed for RDX by Wani and Davis (2003) who demonstrated a significant decrease in the first-order biotransformation rate of RDX in a column study with the addition of nitrate, or by Freedman and Sutherland (1998) who reported similar observations for microcosms. A negative effect of nitrate during wastewater treatment was also observed by Ronen et al. (1998) and Brenner et al. (2000). Similar observations have also been reported for TNT, where nitrate served as a competing electron acceptor under anoxic conditions and was reported to halt

TNT respiration by *Pseudomonas* sp. strain JLR11 (Esteve-Núñez et al. 2000). Finally, nitrogen-bearing compounds are also potential inhibitors of enzyme expression, as presented by Nejidat et al. (2008) who showed that ammonium and nitrite repress cytochrome P450 expression.

Biodegradation rates are also influenced by the type of electron acceptors and electron donors. For example, the degradation of RDX, HMX and TNT was shown to be enhanced by additional hydrogen or hydrogen-producing electron donors under anaerobic conditions (Adrian et al. 2003; Adrian and Arnett 2007), and the degradation of HMX was enhanced by adding mixed electron acceptors to anaerobic microbial consortia (Boopathy 2001). Redox potential is another important factor that not only plays an important role in dictating the mechanism of degradation (as already described), but also influences the actual biotic degradation rate. Biodegradation is generally enhanced under reduced conditions, and in saturated soils (Price et al. 2001; Speitel et al. 2001; Ringelberg et al. 2003).

Toxicity and inhibitory effects of solution components also play an important role. At high concentrations, explosive compounds may be toxic to their potentially degrading bacteria as observed in TNT-degrading bacteria (Spiker et al. 1992), although these concentrations are often not of environmental relevance. Besides inhibition of their own degrading bacteria, explosive compounds may also inhibit the degradation of other coexisting explosive compounds. For example, TNT was shown to inhibit the activity of cytochrome P450 (Jackson et al. 2007) which is responsible for a key aerobic degradation step of RDX: denitration. This inhibitory effect was found reversible and disappeared with complete elimination of TNT from the medium (Nejidat et al. 2008). Another example is the inhibitory effect of high concentrations of RDX on the degradation of HMX by the purified xenobiotic reductase XenB. No inhibition was found at lower concentrations of RDX (Fuller et al. 2009).

Inhibitory effects and toxicity of the degradation products, rather than the parent compounds, are also of importance. Sagi-Ben Moshe et al. (2009) observed an inhibitory effect of the TNT metabolite tetranitroazoxytoluene on the degradation of RDX and HMX which was later on shown to be related to the metabolite's toxicity. In the absence of toxic metabolite, RDX and HMX degradation proceeded normally. The toxic effect of the azoxy dimers was found to be more pronounced in mixed-slurry experiments than in unsaturated soils and was related to the homogeneity of the microcosm (Sagi-Ben Moshe 2011).

The factors, that influence the rates at which explosive compounds degrade, should be considered in engineered bioremediation techniques in order to optimize the performance of the system. In such systems, it is relatively simple to control and manipulate these factors. On the other hand, in the natural heterogeneous environment, defining the conditions under which explosives degrade is itself a complicated task which can never be fully achieved. Moreover, manipulation of the conditions in the sub-surface is very limited and often even impossible.

5.4 Identifying and Quantifying In situ Biodegradation of Explosives

While identifying the potential of indigenous bacteria to biodegrade a compound is relatively simple, gaining evidence that biodegradation is actually occurring and further, quantifying its extent in complex environments is intrinsically difficult. Thus, studies on degradation potential, pathway identification, and rate quantification of explosives biodegradation under controlled laboratory conditions are frequently carried out. A few studies have been also aimed at characterizing the microbial degradation of explosives in the complex, poorly defined sub-surface environment.

The most conventional technique for identifying degradation potential in the environment is laboratory slurry experiments with contaminated sediments, in which the potential of the indigenous bacteria to degrade the target compound is tested. However, a positive response, showing that indigenous bacteria can indeed degrade the compound, does not imply that they actually perform this reaction in the sub-surface. Moreover, even if the reaction does take place in the sub-surface, the degradation rates at which it occurs in the laboratory, can vary by orders of magnitudes from the actual degradation rates in the field.

Even more complicated are attempts to *quantify* the extent of in situ biodegradation. This is normally done using techniques based on monitoring the change in the contaminant's concentration in time and space and fitting degradation rates using computational modeling. However, this strategy is often not sensitive enough, as the decrease in a contaminant's concentration may not be related only to biodegradation, but also to other processes such as dispersion, sorption or volatilization, the extents of which must also be quantified. Moreover, the temporal contaminant release to the aquifer is usually unknown, variable hydrogeological conditions are often not available, and only a limited number of monitoring wells generally exist in contaminated sites, which cannot produce exact knowledge of a plume's shape. All of these factors reduce the ability to generate precise calculations (Bockelmann et al. 2003; Wilson et al. 2004). An example of this problem is demonstrated by Pennington et al. (2001) who calibrated first-order decay constants for TNT and RDX concentrations measured in the field. The values obtained by these authors were 10^{-5} per day (half life of 190 years) and 8.13×10^{-6} per day (half life of 233 years) for TNT and RDX, respectively. However, their model was reported to be only moderately sensitive to degradation rate.

Analysis of the pollutant degradation products is an alternative strategy for quantifying degradation extent along the plume. However, this strategy may not always be conclusive, as products may not be detectable, may be non-conserved or can originate from different parent compounds. Therefore, the search for degradation products may not always be appropriate to gain proof of the biodegradation activity in the field. An example of this is demonstrated by Beller and Tiemeier (2002) who detected the anaerobic nitroso transformation products of RDX in

contaminated groundwater—an important evidence that the reductive transformation of RDX occurs in situ. Nevertheless, this evidence was not sufficient to estimate the degradation extent or rates of the compound, since the nitroso products may have been further attenuated and thus their monitored concentration might not reflect the true degradation extent. For similar reasons, absence of degradation product MEDINA in the groundwater in study of Beller and Tiemeier (2002) may not indicate that the MEDINA-producing ring-cleavage pathway is not occurring in situ.

A relatively new strategy for tackling the challenge of identifying and quantifying degradation of organic pollutants in general, and explosive compounds in particular, is the use of compound-specific stable isotope analysis (CSIA) (Meckenstock et al. 2004; Schmidt et al. 2004). This strategy is based on the preferential reaction of chemical bonds of lighter isotopes, implying that pollutants become enriched in the heavier isotopes as degradation proceeds. The extent of isotope enrichment depends on the extent of degradation, as well as on the isotopic enrichment factor which is typical of the rate-limiting reaction. Thus, the extent of isotopic enrichment along a contamination plume may be used to quantify the extent of biodegradation along the flow line using appropriate enrichment factors, as demonstrated in the last decade for various organic pollutants, mostly BTEX compounds (McKelvie et al. 2005; Spence et al. 2005; Mak et al. 2006), MTBE (Kuder et al. 2005; Spence et al. 2005; McKelvie et al. 2007), PAH (Moonkoo et al. 2006), and chlorinated aliphatics such as TCE and PCE (Hunkeler et al. 2004; Morrill et al. 2005). The application of this concept to other groups of contaminants, including explosives, is emerging.

The isotopic composition and isotope fractionation accompanying explosives biodegradation has so far received only scant attention, possibly due to analytical difficulties and because explosives are of less concern than the major pollutants, such as BTEX compounds and chlorinated ethylenes.

Some publications related to the isotopic composition of various explosives can be found in the forensics literature which aims to use these compounds' isotopic fingerprints to identify their origin (Nissenbaum 1975; McGuire et al. 1995; Diegor et al. 1999; Phillips et al. 2003). Others have studied the isotopic enrichment accompanying degradation processes of explosive compounds in a laboratory environment. Hartenbach et al. (2006) and Hofstetter et al. (2008) studied $\delta^{15}N$ isotope fractionation during the abiotic reduction of different nitroaromatic compounds, such as methyl- or chloro-substituted nitrobenzenes, or dinitrotoluenes. Beller et al. (2004) studied the isotopic signatures of nitrite and nitrate formed by RDX photolysis. Hoffsommer et al. (1977) studied the $\delta^{2}H$ isotope effects following RDX alkaline hydrolysis. Of more environmental relevance to the compounds that are the focus of this review is the work of Bernstein et al. (2008) who studied $\delta^{15}N$ and $\delta^{18}O$ enrichment during aerobic and anaerobic biodegradation of RDX, where the aerobic strain used for these experiments was *Rhodococcus* strain YH1, and for the anaerobic experiments, a slurry of sediments with an indigenous consortium excavated from a contaminated site was used.

Environmental studies, that have adopted stable isotope tools to study the behavior of explosive compounds in the field, are rare. DiGnazio et al. (1998) related $\delta^{15}N$ values of nitrate in the groundwater to RDX degradation, but noted that they had no other isotopic data to confirm this correlation. Bordeleau et al. (2008) used both $\delta^{15}N$ and $\delta^{18}O$ values of nitrate to identify in situ degradation of RDX. However, the latter two studies focused on the possible RDX metabolite nitrate, rather than the parent compound itself, making the results less conclusive.

Pennington et al. (2001) explored the application of nitrogen and carbon stable isotope analysis in explosive compounds to track in situ attenuation processes. They suggested that measuring stable isotopic fractions of nitrogen in TNT is a promising strategy for monitoring the attenuation of TNT in the sub-surface.

CSIA was recently applied to RDX in a contaminated aquifer, demonstrating in situ degradation of RDX and enabling quantification of the degradation extent along the plume and an estimate of in situ degradation rates (Bernstein et al. 2010). This study revealed a decrease in degradation with depth. Stable isotope tools were also used in unsaturated soils, showing enhanced RDX degradation with increasing water content (Sagi-Ben Moshe et al. 2010). Both of these studies made use of analytical techniques which require rather laborious off-line purification procedures of the target compound, reducing the method's sensitivity.

Recently, an effort was made to improve analytical techniques for determining $\delta^{13}C$ and $\delta^{15}N$ in RDX and TNT (Gelman et al. 2011). The resultant new technique opens the way for convenient isotope analysis of compounds derived from the contaminated environments, thus shedding light on environmentally relevant processes that are difficult to detect with traditional methods. It is anticipated that the application of the new analytical method, as well as the development and optimization of other new methods, will significantly expand our knowledge of the behavior of explosives in the field.

Besides the possibility to characterize degradation products, stable isotope tools might also be useful in studying the indigenous microflora's ability to degrade explosives. A powerful new technique called stable isotope probing (SIP) has enabled researchers to identify metabolically active microorganisms in complex engineered and natural systems. Roh et al. (2009) used ^{15}N-labeled RDX to follow active RDX-degrading bacteria in a groundwater microcosm. They demonstrated that ^{15}N was incorporated into DNA sequences encoding XplA. This indicated that XplA-containing bacteria are actively taking nitrogen from the degraded RDX, an observation which enabled confirmation of activity without prior knowledge of the organisms involved.

5.5 Conclusions

In the last few years, significant efforts have been made in tracing possible degradation pathways of explosive compounds in the sub-surface. Increasing numbers of pathways have been postulated, involving mainly the nitro groups of the

aromatic or triazinic rings. While some of the documented pathways are environmentally undesirable, as they involve the accumulation of toxic products, others mineralize the compounds to simple and harmless molecules and are thus of greater interest.

Numerous microbial isolates have been identified as degraders of explosives, belonging to various families. Both facultative anaerobes (Enterobacteriaceae) as well as strict anaerobes (*Clostridia*) appear to play important roles in this process. *Pseudomonas* appears to be ubiquitous in their ability to attack explosives both aerobically as well as under anoxic conditions. A unique observation on the aerobic degradation of RDX indicates that the presence of this compound resulted in the evolution of a novel cytochrome P450 in different geographic locations. Interestingly, this evolution was restricted to a limited bacterial host range—*Rhodococcus* species from the family Nocardiaceae. Their degradation activity was shown to be controlled by various factors, including redox potential, nutrient availability, salinity and local toxicity.

Microbial strains isolated from the field imply that degradation may potentially occur in situ, even without human interference. Nevertheless, to reach remedial goals, proof must be provided of the actual occurrence of in situ degradation in a contaminated environment, and the extent of this process must be quantified. Using isotopic tools, it is anticipated that not only will these objectives will be fulfilled, but also a deeper understanding of the biodegradation processes, that actually occur in the sub-surface, will be gained, narrowing the gap between the knowledge accumulated from laboratory experiments and the uncertainties related to environmentally relevant processes.

Acknowledgments The work of A. Bernstein was supported by a generous contribution from Vera Barcza, Toronto, Canada Rosinger-Barcza Family Fund In support of Young Researchers at the Zuckerberg Institute for Water Research. This work was also supported in part by a grant 167/2008 from Israel Science Foundation.

References

Achtnich C, Sieglen U, Knackmuss H-J, Lenke H (1999) Irreversible binding of biologically reduced 2,4,6-trinitrotoluene to soil. Environ Toxicol Chem 18:2416–2423

Adrian NR, Arnett CM (2004) Anaerobic biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by Acetobacterium malicum strain HAAP-1 isolated from a methanogenic mixed culture. Curr Microbiol 48:332–340

Adrian NR, Arnett CM (2007) Anaerobic biotransformation of explosives in aquifer slurries amended with ethanol and propylene glycol. Chemosphere 66:1849–1856

Adrian NR, Arnett CM, Hickey RF (2003) Stimulating the anaerobic biodegradation of explosives by the addition of hydrogen or electron donors that produce hydrogen. Water Res 37:3499–3507

Allard A-S, Neilson AH (1997) Bioremediation of organic waste sites: a critical review of microbiological aspects. Intl Biodeter Biodegrad 39:253–285

Alvarez MA, Kitts CL, Botsford JL, Unkefer PJ (1995) Pseudomonas aeruginosa strain MA01 aerobically metabolizes the aminodinitrotoluenes produced by 2,4,6-trinitrotoluene nitro group reduction. Can J Microbiol 41:984–991

Angermaier L, Simon H (1983) On nitroaryl reductase activities in several *Clostridia*. Hoppe-Seylers Z Physiol Chem 364:1653–1663

Arnett CM, Adrian NR (2009) Cosubstrate independent mineralization of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by a Desulfovibrio species under anaerobic conditions. Biodegradation 20:15–26

ATSDR (1996a) 2,4,6-Trinitrotoluene (TNT) Fact Sheet. Agency for toxic substances and disease registry (ATSDR). Available from: http://www.atsdr.cdc.gov/toxfaqs/tfacts81.pdf

ATSDR (1996b) RDX Fact Sheet. Agency for toxic substances and disease registry (ATSDR). Available from: http://www.atsdr.cdc.gov/toxfaqs/tfacts78.pdf

ATSDR (1997) HMX Fact Sheet. Agency for toxic substances and disease registry (ATSDR). Available from: http://www.atsdr.cdc.gov/toxfaqs/tfacts98.pdf

Behrend C, Heesche-Wagner K (1999) Formation of hydride-Meisenheimer complexes of picric acid (2,4,6-trinitrophenol) and 2,4-dinitrophenol during mineralization of picric acid by Nocardioides sp. strain CB22–2. Appl Environ Microbiol 65:1372–1377

Beller HR, Tiemeier K (2002) Use of liquid chromatography/tandem mass spectrometry to detect distinctive indicators of in situ RDX transformation in contaminated groundwater. Environ Sci Technol 36:2060–2066

Beller HR, Madrid V, Hudson GB, McNab WW, Carlsen T (2004) Biogeochemistry and natural attenuation of nitrate in groundwater at an explosives test facility. Appl Geochem 19:1483–1494

Bernstein A, Ronen Z, Adar E, Nativ R, Lowag H, Stichler W, Meckenstock RU (2008) Compound-specific isotope analysis of RDX and stable isotope fractionation during aerobic and anaerobic biodegradation. Environ Sci Technol 42:7772–7777

Bernstein A, Adar E, Ronen Z, Lowag H, Stichler W, Meckenstock RU (2010) Quantifying RDX biodegradation in groundwater using δ^{15}N isotope analysis. J Contam Hydrol 111:25–35

Bernstein A, Adar E, Nejidat A, Ronen Z (2011) Isolation and characterization of RDX-degrading *Rhodococcus* species from a contaminated aquifer. Biodegradation 22:997–1005

Best EPH, Sprecher SL, Larson SL, Fredrickson HL, Bader DF (1999) Environmental behavior of explosives in groundwater from the Milan Army Ammunition Plant in aquatic and wetland plant treatments. Uptake and fate of TNT and RDX in plants. Chemosphere 39:2057–2072

Bhushan B, Paquet L, Halasz A, Spain JC, Hawari J (2003a) Mechanism of xanthine oxidase catalyzed biotransformation of HMX under anaerobic conditions. Biochem Biophys Res Commun 306:509–515

Bhushan B, Trott S, Spain JC, Halasz A, Paquet L, Hawari J (2003b) Biotransformation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by a rabbit liver cytochrome P450: insight into the mechanism of RDX biodegradation by *Rhodococcus* sp. strain DN22. Appl Environ Microbiol 69:1347–1351

Bhushan B, Halasz A, Thiboutot S, Ampleman G, Hawari J (2004) Chemotaxis-mediated biodegradation of cyclic nitramine explosives RDX, HMX, and CL-20 by *Clostridium* sp. EDB2. Biochem Biophys Res Commun 316:816–821

Binks PR, Nicklin S, Bruce NC (1995) Degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by *Stenotrophomonas maltophilia* PB1. Appl Environ Microbiol 61:1318–1322

Blehert DS, Fox BG, Chambliss GH (1999) Cloning and sequence analysis of two *Pseudomonas* flavoprotein xenobiotic reductases. J Bacteriol 181:6254–6263

Bockelmann A, Zamfirescu D, Ptak T, Grathwhol P, Teutsch G (2003) Quantification of mass fluxes and natural attenuation rates at an industrial site with a limited monitoring network: a case study. J Contam Hydrol 60:97–121

Boopathy R (1994) Transformation of nitroaromatic compounds by a methanogenic bacterium, *Methanococcus* sp (strain B). Arch Microbiol 162:167–172

Boopathy R (2000) Bioremediation of explosives contaminated soil. Intl Biodeter Biodegrad 46:29–36

Boopathy R (2001) Enhanced biodegradation of cyclotetramethylenetetranitramine (HMX) under mixed electron-acceptor condition. Biores Technol 76:241–244

Boopathy R, Kulpa CF (1992) Trinitrotoluene as a sole nitrogen source for a sulfate-reducing bacterium *Desulfovibrio* sp (B strain) isolated from an anaerobic digester. Curr Microbiol 25:235–241

Boopathy R, Kulpa CF (1994) Biotransformation of 2,4,6-trinitrotoluene (TNT) by a *Methanococcus* sp (strain B) isolated from a lake sediment. Can J Microbiol 40:273–278

Boopathy R, Manning JF (1996) Characterization of partial anaerobic metabolic pathway for 2,4,6-trinitrotoluene degradation by a sulfate-reducing bacterial consortium. Can J Microbiol 42:1203–1208

Boopathy R, Kulpa CF, Wilson M (1993) Metabolism of 2,4,6-trinitrotoluene (TNT) by *Desulfovibrio* sp (B strain). Appl Microbiol Biotechnol 39:270–275

Boopathy R, Manning J, Kulpa CF (1997) Optimization of environmental factors for the biological treatment of trinitrotoluene-contaminated soil. Arch Environ Contam Toxicol 32:94–98

Borch T, Inskeep WP, Harwood JA, Gerlach R (2005) Impact of ferrihydrite and anthraquinone-2,6-disulfonate on the reductive transformation of 2,4,6-trinitrotoluene by a gram-positive fermenting bacterium. Environ Sci Technol 39:7126–7133

Bordeleau G, Savard MM, Martel R, Ampleman G, Thiboutot S (2008) Determination of the origin of groundwater nitrate at an air weapons range using the dual isotope approach. J Contam Hydrol 98:97–105

Brenner A, Ronen Z, Harel Y, Abeliovich A (2000) Degradation of RDX during biological treatment of munitions waste. Water Environ Res 72:469–475

Charles PT, Gauger PR, Patterson CH Jr, Kusterbeck AW (2000) On-site immunoanalysis of nitrate and nitroaromatic compounds in groundwater. Environ Sci Technol 34:4641–4650

Cho Y-S, Lee B-U, Oh K-H (2008) Simultaneous degradation of nitroaromatic compounds TNT, RDX, atrazine, and simazine by *Pseudomonas putida* HK-6 in bench-scale bioreactors. J Chem Technol Biotechnol 83:1211–1217

Clark B, Boopathy R (2007) Evaluation of bioremediation methods for the treatment of soil contaminated with explosives in Louisiana Army Ammunition Plant, Minden, Louisiana. J Hazard Mater 143:643–648

Claus H, Bausinger T, Lehmler I, Perret N, Fels G, Dehner U, Preuß J, König H (2007) Transformation of 2,4,6-trinitrotoluene (TNT) by Raoultella terrigena. Earth Environ Sci 18:27–35

Coleman NV, Nelson DR, Duxbury T (1998) Aerobic biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) as a nitrogen source by a *Rhodococcus* sp., strain DN22. Soil Biol Biochem 30:1159–1167

Coleman NV, Spain JC, Duxbury T (2002) Evidence that RDX biodegradation by *Rhodococcus* strain DN22 is plasmid-borne and involves a cytochrome p-450. J Appl Microbiol 93:463–472

Cooper PW, Kurowski SR (1997) Chemistry of explosives. In: Introduction to the Technology of Explosives. Wiley-VCH Inc, New York, pp 1–38

Crocker FH, Indest KJ, Fredrickson HL (2006) Biodegradation of the cyclic nitramine explosives RDX, HMX, and CL-20. Appl Microbiol Biotechnol 73:274–290

Danielson PB (2002) The cytochrome P450 superfamily: biochemistry, evolution and drug metabolism in humans. Curr Drug Metab 37:561–597

Darrach MR, Chutjian A, Plett GA (1998) Trace explosives signatures from World War II unexploded undersea ordnance. Environ Sci Technol 32:1354–1358

Daun G, Lenke H, Reuss M, Knackmuss H-J (1998) Biological treatment of TNT-contaminated soil. 1. Anaerobic cometabolic reduction and interaction of TNT and metabolites with soil components. Environ Sci Technol 32:1956–1963

Diegor EJM, Abrajano T, Stehmeier L, Patel T, Winsor L (1999) In: Proceedings of the 19th international meeting on organic geochemistry, Istanbul, Turkey, pp 29

DiGnazio FJ, Krothe NC, Baedke SJ, Spalding RF (1998) δ^{15}N of nitrate derived from explosive sources in karst aquifer beneath the ammunition burning ground. J Hydrol 206:164–175

Drzyzga O, Gorontzy T, Schmidt A, Blotevogel KH (1995) Toxicity of explosives and related compounds to the luminescent bacterium *Vibrio fischeri* NRRL-B-11177. Arch Environ Contam Toxicol 28:229–235

Drzyzga O, Bruns-Nagel D, Gorontzy T, Blotevogel K-H, von Löw E (1999) Anaerobic incorporation of the radiolabeled explosive TNT and metabolites into the organic soil matrix of contaminated soil after different treatment procedures. Chemosphere 38:2081–2095

Duque E, Haıdour A, Godoy F, Ramos J-L (1993) Construction of a Pseudomonas hybrid strain that mineralizes 2,4,6-trinitrotoluene. J Bacteriol 175:2278–2283

Ederer MM, Lewis TA, Crawford RL (1997) 2,4,6-Trinitrotoluene (TNT) transformation by Clostridia isolated from a munition-fed bioreactor: comparison with non-adapted bacteria. J Ind Microbiol Biotechnol 18:82–88

Esteve-Núñez A, Ramos JL (1998) Metabolism of 2,4,6-trinitrotoluene by *Pseudomonas* sp. JLR11. Environ Sci Technol 32:3802–3808

Esteve-Nuñez A, Lucchesi G, Philipp B, Schink B, Ramos JL (2000) Respiration of 2,4,6-trinitrotoluene by *Pseudomonas* sp. strain JLR11. J Bacteriol 182:1352–1355

Esteve-Núñez A, Caballero A, Ramos JL (2001) Biological degradation of 2,4,6trinitrotoluene. Microbiol Mol Biol Rev 65:335–352

Eyers L, Stenuit L, Agathos SN (2008) Denitration of 2,4,6-trinitrotoluene by Pseudomonas aeruginosa ESA-5 in the presence of ferrihydrite. Appl Microbiol Biotechnol 79:489–497

Fiorella PD, Spain JC (1997) Transformation of 2,4,6-trinitrotoluene by Pseudomonas pseudoalcaligenes JS52. Appl Environ Microbiol 63:2007–2015

Fournier D, Halasz A, Spain J, Fiurasek P, Hawari J (2002) Determination of key metabolites during biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine with *Rhodococcus* sp. strain DN22. Appl Environ Microbiol 68:166–172

Fournier D, Halasz A, Thiboutot S, Ampleman G, Manno D, Hawari J (2004) Biodegradation of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) by Phanerochaete chrysosporium: new insight into the degradation pathway. Environ Sci Technol 38:4130–4133

Freedman DL, Sutherland KW (1998) Biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) under nitrate-reducing conditions. Water Sci Technol 38:33–40

French CE, Nicklin S, Bruce NC (1998) Aerobic degradation of 2,4,6-trinitrotoluene by *Enterobacter cloacae* PB2 and by pentaerythritol tetranitrate reductase. Appl Environ Microbiol 64:2864–2868

Fuchs JS, Oneto ML, Casabé NB, Gómez Segura O, Tarulla R, Vaccarezza M, Sánchez-Rivas C, Kesten EM, Wood EJ (2001) Ecotoxicological characterization of a disposal lagoon from a munition plant. Bull Environ Contam Toxicol 67:696–703

Fuller ME, Manning JF (1997) Aerobic gram-positive and gram-negative bacteria exhibit differential sensitivity to and transformation of 2,4,6-trinitrotoluene (TNT). Curr Microbiol 35:77–83

Fuller M, McClay K, Hawari J, Paquet L, Malone T, Fox B, Steffan R (2009) Transformation of RDX and other energetic compounds by xenobiotic reductases XenA and XenB. Appl Microbiol Biotechnol 84:535–544

Fuller ME, Perreault N, Hawari J (2010) Microaerophilic degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by three Rhodococcus strains. Lett Appl Microbiol 51:313–318

Funk SB, Roberts DJ, Crawford DL, Crawford RL (1993) Initial-phase optimization for bioremediation of munition compound-contaminated soils. Appl Environ Microbiol 59:2171–2177

Gelman F, Kotlyar A, Chiguala D, Ronen Z (2011) Precise and accurate compound-specific carbon and nitrogen isotope analysis of RDX by GC-IRMS. Intl J Environ Anal Chem (in press)

George SE, Huggins-Clark G, Brooks LR (2001) Use of a *Salmonella* microsuspension bioassay to detect the mutagenicity of munitions compounds at low concentrations. Mutation Res 490:45–56

Gilcrease CP, Murphy VG (1995) Bioconversion of 2,4-diamino-6-nitrotoluene to a novel metabolite under anoxic and aerobic conditions. Appl Environ Microbiol 61:4209–4214

Groom CA, Beaudet S, Halasz A, Paquet L, Hawari J (2001) Detection of the cyclic nitramine explosives hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazine (HMX) and their degradation products in soil environments. J Chromatogr A 909:53–60

Haïdour A, Ramos JL (1996) Identification of products resulting from the biological reduction of 2,4,6-trinitrotoluene, 2,4-dinitrotoluene and 2,6-dinitrotoluene by *Pseudomonas* sp. Environ Sci Technol 30:2365–2370

Halasz A, Spain J, Paquet L, Beaulieu C, Hawari J (2002) Insights into the formation and degradation mechanisms of methylenedinitramine during the incubation of RDX with anaerobic sludge. Environ Sci Technol 36:633–638

Halasz A, Manno D, Strand SE, Bruce NC, Hawari J (2010) Biodegradation of RDX and MNX with *Rhodococcus* sp. strain DN22: new insights into the degradation pathway. Environ Sci Technol 44:9330–9336

Hartenbach A, Hofstetter TB, Berg M, Bolotin J, Schwarzenbach RP (2006) Using nitrogen isotope fractionation to assess abiotic reduction of nitroaromatic compounds. Environ Sci Technol 40:7710–7716

Hawari J, Halasz A, Paquet L, Zhou E, Spencer B, Ampleman G, Thiboutot S (1998) Characterization of metabolites in the biotransformacion of 2,4,6-trinitrotoluene with anaerobic sludge: role of triaminotoluene. Appl Environ Microbiol 64:2200–2206

Hawari J, Halasz A, Beaudet S, Paquet L, Ampleman G, Thiboutot S (1999) Biotransformation of 2,4,6-trinitrotoluene with Phanerochaete chrysosporium in agitated cultures at pH 4.5. Appl Environ Microbiol 65:2977–2986

Hawari J, Beaudet S, Halasz A, Thiboutot S, Ampleman G (2000a) Microbial degradation of explosives: biotransformation versus mineralization. Appl Microbiol Biotechnol 54:605–618

Hawari J, Halasz A, Sheremata T, Beaudet S, Groom C, Paquet L, Rhofir C, Ampleman G, Thiboutot S (2000b) Characterization of metabolites during biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) with municipal anaerobic sludge. Appl Environ Microbiol 66:2652–2657

Hawari J, Halasz A, Beaudet S, Paquet L, Ampleman G, Thiboutot S (2001) Biotransformation routes of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine by municipal anaerobic sludge. Environ Sci Technol 35:70–75

Hlavica P (2009) Assembly of non-natural electron transfer conduits in the cytochrome P450 system: a critical assessment and update of artificial redox constructs amenable to exploitation in biotechnological areas. Biotechnol Adv 27:103–121

Hoffsommer JC, Kubose DA, Glover DJ (1977) Kinetic isotope effects and intermediate formation for the aqueous alkaline homogeneous hydrolysis of 1,3,5-triaza-1,3,5-trinitrocyclohexane (RDX). J Phys Chem 81:380–385

Hofstetter TB, Neumann A, Arnold WA, Bolotin J, Cramer CJ, Schwarzenbach RP (2008) Substituent effects on nitrogen isotope fractionation during abiotic reduction of nitroaromatic compounds. Environ Sci Technol 42:1997–2003

Huang S, Lindahl PA, Wang C, Bennett GN, Rudolph FB, Hughes JB (2000) 2,4,6-Trinitrotoluene reduction by carbon monoxide dehydrogenase from *Clostridium thermoaceticum*. Appl Environ Microbiol 66:1474–1478

Hughes JB, Wang C, Yesland K, Richardson A, Bhadra R, Bennet G, Rudolph F (1998) Bamberger rearrangement during TNT metabolism by *Clostridium acetobutylicum*. Environ Sci Technol 32:494–500

Hunkeler D, Chollet N, Pittet X, Aravena R, Cherry JA, Parker BL (2004) Effect of source variability and transport processes on carbon isotope ratio of TCE and PCE in two sandy aquifers. J Contam Hydrol 74:265–282

Indest KJ, Crocker FH, Athow R (2007) A TaqMan polymerase chain reaction method for monitoring RDX-degrading bacteria based on the xplA functional gene. J Microbiol Methods 68:267–274

Indest KJ, Jung CM, Chen H-P, Hancock D, Florizone C, Eltis LD, Crocker FH (2010) Functional characterization of pGKT2, a 182-kilobase plasmid containing the xplAB genes, which are

involved in the degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine by *Gordonia* sp. strain KTR9. Appl Environ Microbiol 76:6329–6337

Jackson RJ, Rylott EL, Fournier D, Hawari J, Bruce NC (2007) Exploring the biochemical properties and remediation applications of the unusual explosive-degrading P450 system XplA/B. Proc Natl Acad Sci U S A 104:16822–16827

Kalafut T, Wales ME, Rastogi VK, Naumova RP, Zaripova SK, Wild JR (1998) Biotransformation patterns of 2,4,6-trinitrotoluene by aerobic bacteria. Curr Microbiol 36:45–54

Khan TA, Bhadra R, Hughes J (1997) Anaerobic transformation of 2,4,6-TNT and related nitroaromatic compounds by *Clostridium acetobutylicum*. J Ind Microbiol Biotechnol 18:198–203

Kim HY, Song HG (2000) Comparison of 2,4,6-trinitrotoluene degradation by seven strains of white rot fungi. Curr Microbiol 41:317–320

Kim H-Y, Bennett GN, Song H-G (2002) Degradation of 2,4,6-trinitrotoluene by *Klebsiella* sp isolated from activated sludge. Biotechnol Lett 24:2023–2028

Kitts CL, Cunningham DP, Unkefer PJ (1994) Isolation of three hexahydro-1,3,5-trinitro-1,3,5-triazine-degrading species of the family Enterobacteriaceae from nitramine explosive-contaminated soil. Appl Environ Microbiol 60:4608–4711

Kitts CL, Green CE, Otley RA, Alvarez MA, Unkefer PJ (2000) Type 1 nitroreductases in soil enterobacteria reduce TNT (2,4,6-trinitrotoluene) and RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine). Can J Microbiol 26:278–282

Kuder T, Wilson JT, Kaiser P, Kolhatkar R, Philp P, Allen J (2005) Enrichment of stable carbon and hydrogen isotopes during anaerobic biodegradation of MTBE: microcosm and field evidence. Environ Sci Technol 39:213–220

Kwon MJ, Finneran KT (2008) Biotransformation products and mineralization potential for hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in abiotic versus biological degradation pathways with anthraquinone-2,6-disulfonate (AQDS) and Geobacter metallireducens. Biodegradation 19:705–715

Lachance B, Robidoux PY, Hawari J, Ampleman G, Thiboutot S, Sunahara GI (1999) Cytotoxic and genotoxic effects of energetic compounds on bacterial and mammalian cells in vitro. Mutat Res 444:25–39

Lenke H, Knackmuss H-J (1992) Initial hydrogenation during catabolism of picric acid by Rhodococcus erythropolis HL 24–2. Appl Environ Microbiol 58:2933–2937

Lewin U, Efer J, Engewald W (1996) High-performance liquid chromatographic analysis with electrochemical detection for residues of explosives in water samples around a former ammunition plant. J Chromatogr A 730:161–167

Lewis TA, Goszczynski S, Crawford RL, Korus RA, Admassu W (1996) Products of anaerobic 2,4,6-trinitrotoluene (TNT) transformation by *Clostridium* bifermentans. Appl Environ Microbiol 62:4669–4674

Mak KS, Griebler C, Meckenstock RU, Liedl R, Peter A (2006) Combined application of conservative transport modelling and compound-specific carbon isotope analyses to assess in situ attenuation of benzene, toluene, and *o*-xylene. J Contam Hydrol 88:306–320

Martel R, Robertson TJ, Doan MQ, Thiboutot S, Ampleman G, Provatas A, Jenkins T (2008) 2,4,6-Trinitrotoluene in soil and groundwater under a waste lagoon at the former explosives factory Maribyrnong (EFM), Environ Geol 53:1249–1259

McCormick NG, Feeherry FE, Levinson HS (1976) Microbial transformation of 2,4,6-TNT and other nitroaromatic compounds. Appl Environ Microbiol 31:949–958

McCormick NG, Cornell JH, Kaplan AM (1981) Biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine. Appl Environ Microbiol 42:817–823

McGrath CJ (1995) Review of formulations for processes affecting the subsurface transport of explosives. Technical Report IRRP-95-2. US Army Corps of Engineers, Waterways Experiment Station, Vicksburg, MS

McGuire RR, Lee CG, Velsko CA, Raber E (1995) Application of stable isotope ratios to the analysis of explosive residues. In: Proceedings of the fifth international symposium on the analysis and detection of explosives, Washington DC, Dec 4–8

McKelvie JR, Lindstrom JE, Beller HR, Richmond SA, Sherwood Lollar B (2005) Analysis of anaerobic BTX biodegradation in a subarctic aquifer using isotopes and benzylsuccinates. J Contam Hydrol 81:167–186

McKelvie JR, Mackay DM, de Sieyes NR, Lacrampe-Couloume G, Sherwood Lollar B (2007) Quantifying MTBE biodegradation in the Vandenberg Air Force Base ethanol release study using stable carbon isotopes. J Contam Hydrol 94:157–165

Meckenstock RU, Morasch B, Griebler C, Richnow HH (2004) Stable isotope fractionation analysis as a tool to monitor biodegradation in contaminated aquifers. J Contam Hydrol 75:215–255

Montpas S, Samson J, Langlois E, Lei J, Piche Y, Chêvenert R (1997) Degradation of 2,4,6-trinitrotoluene by *Serratia marcescens*. Biotechnol Lett 19:291–294

Moonkoo K, Mahlon CK, Yaorong Q (2006) Molecular and stable carbon isotopic characterization of PAH contaminants at McMurdo Station, Antarctica. Marine Poll Bull 52:1585–1590

Morley MC, Yamamoto H, Speitel GE, Clausen J (2006) Dissolution kinetics of high explosives particles in a saturated sandy soil. J Contam Hydrol 85:141–158

Morrill PL, Lacrampe-Couloume G, Slater GF, Sleep BE, Edwards EA, McMaster ML, Major DW, Sherwood Lollar B (2005) Quantifying chlorinated ethene degradation during reductive dechlorination at Kelly AFB using stable carbon isotopes. J Contam Hydrol 76:279–293

Naumova RP, Selivanovskaya SLU, Mingatina FA (1988) Possibilities for the deep bacterial destruction of 2,4,6-trinitrotoluene. Mikrobiologia 57:218–222

Nejidat A, Kafka L, Tekoah Y, Ronen Z (2008) Effect of organic and inorganic nitrogenous compounds on RDX degradation and cytochrome P-450 expression in *Rhodococcus* strain YH1. Biodegradation 19:313–320

Neuwoehner J, Schofer A, Erlenkaemper B, Steinbach K, Hund-Rinke K, Eisentraeger A (2007) Toxicological characterization of 2,4,6-trinitrotoluene, its transformation products, and two nitramine explosives. Environ Toxicol Chem 26:1090–1099

Nissenbaum A (1975) The distribution of natural stable isotopes of carbon as a possible tool for the differentiation of samples of TNT. J Forensic Sci 20:455–459

Oh B-T, Sarath G, Shea PJ (2001) TNT nitroreductase from a *Pseudomonas aeruginosa* strain isolated from TNT-contaminated soil. Soil Biol Biochem 33:875–881

Oh B-T, Shea PJ, Drijber RA, Vasilyeva GK, Sarath G (2003) TNT biotransformation and detoxification by a *Pseudomonas aeruginosa* strain. Biodegradation 14:309–319

Pak JW, Knoke KL, Noguera DR, Fox BG, Chambliss GH (2000) Transformation of 2,4,6-trinitrotoluene by purified xenobiotic reductase B from Pseudomonas fluorescens I-C. Appl Environ Microbiol 66:4742–4750

Park C, Kim T-H, Kim S, Kim S-W, Lee J, Kim S-H (2003) Optimization for biodegradation of 2,4,6-trinitrotoluene (TNT) by Pseudomonas putida. J Biosci Bioeng 95:567–571

Pasti-Grigsby MB, Lewis TA, Crawford DL, Crawford RL (1996) Transformation of 2,4,6-trinitrotoluene (TNT) by Actinomycetes isolated from TNT-contaminated and uncontaminated environments. Appl Environ Microbiol 62:1120–1123

Pavlostathis SG, Jackson GH (1999) Biotransformation of 2,4,6-trinitrotoluene in Anabaena sp cultures. Environ Toxicol Chem 18:412–419

Pennington JC, Brannon JM (2002) Environmental fate of explosives. Thermochim Acta 384:163–172

Pennington JC, Brannon JM, Gunnison D, Harrelson DW, Zakikhani M, Miyares P, Jenkins TF, Clarke J, Hayes C, Ringleberg D, Perkins E, Fredrickson H (2001) Monitored natural attenuation of explosives. Soil Sediment Contam 10:45–70

Peterson FJ, Mason RP, Horspian J, Holtzman JL (1979) Oxygen-sensitive and insensitive nitroreduction by *Escherichia coli* and rat hepatic microcosomes. J Biol Chem 254:4009–4014

Phillips SA, Doyle S, Philp L, Coleman M (2003) Proceedings: network developing forensic applications of stable isotope ratio mass spectrometry conference. Sci Justice 43:153–160

Preuss A, Fimpel J, Dickert G (1993) Anaerobic transformation of 2,4,6-trinitrotoluene (TNT). Arch Microbiol 159:345–353

Price CB, Brannon JM, Yost SL, Hayes CA (2001) Relationship between redox potential and pH on RDX transformation in soil–water slurries. J Environ Eng 127:26–31

Pudge IB, Daugulis AJ, Dubois C (2003) The use of Enterobacter cloacae ATCC 43560 in the development of a two-phase partitioning bioreactor for the destruction of hexahydro-1,3,5-trinitro-1,3,5-s-triazine (RDX). J Biotechnol 100:65–75

Regan KM, Crawford RL (1994) Characterization of *Clostridium bifermentans* and its biotransformation of 2,4,6-trinitrotoluene (TNT) and 1,3,5-triaza-1,3,5-trinitrocyclohexane (RDX). Biotechnol Lett 16:1081–1086

Rieger P-G, Knackmuss HJ (1995) Basic knowledge and perspectives on biodegradation of 2,4,6-trinitrotoluene and related nitroaromatic compounds in contaminated soil. In: Spain JC (ed) Biodegradation of Nitroaromatic Compounds. Plenum Press, New York, pp 1–18

Rieger P-G, Sinnwell V, Preuss A, Franke W, Knackmuss H-J (1999) Hydride-Meisenheimer complex formation and protonation as key reactions of 2,4,6-trinitrophenol biodegradation by *Rhodococcus erythropolis*. J Bacteriol 181:1189–1195

Ringelberg DB, Reynolds CM, Walsh ME, Jenkins TF (2003) RDX loss in a surface soil under saturated and well drained conditions. J Environ Qual 32:1244–1249

Roh H, Yu C, Fuller M, Chu K-H (2009) Identification of hexahydro-1,3,5-trinitro-1,3,5-triazine-degrading microorganisms via 15N-stable isotope probing. Environ Sci Technol 43:2505–2511

Ronen Z, Brenner A, Abeliovich A (1998) Biodegradation of RDX-contaminated wastes in a nitrogen-deficient environment. Water Sci Technol 38:219–224

Ronen Z, Yanovich Y, Goldin R, Adar E (2008) Metabolism of the explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in a contaminated vadose zone. Chemosphere 73:1492–1498

Rylott EL, Jackson RG, Sabbadin F, Seth-Smith HMB, Edwards J, Chong CS, Strand SE, Grogan G, Bruce NC (2011) The explosive-degrading cytochrome P450 XplA: biochemistry, structural features and prospects for bioremediation. Biochim Biophys Acta 1:230–236

Sagi-Ben Moshe S (2011) Biodegradation and transport of explosives in sandy unsaturated zone. PhD Thesis, The Hebrew University of Jerusalem, pp 119

Sagi-Ben Moshe S, Ronen Z, Dahan O, Weisbrod N, Groisman L, Adar E, Nativ R (2009) Sequential biodegradation of TNT, RDX and HMX in a mixture. Environ Pollut 157:2231–2238

Sagi-Ben Moshe S, Ronen Z, Dahan O, Bernstein A, Weisbrod N, Gelman F, Adar E (2010) Isotopic evidence and quantification assessment of in situ RDX biodegradation in the deep unsaturated zone. Soil Biol Biochem 42:1253–1262

Schmidt TC, Zwank L, Elsner M, Berg M, Meckenstock RU, Haderlein SB (2004) Compound-specific stable isotope analysis of organic contaminants in natural environments: a critical review of the state of the art, prospects, and future challenges. Anal Bioanal Chem 378:283–300

Seth-Smith HMB, Rosser SJ, Basran A, Travis ER, Dabbs ER, Nicklin S, Bruce NC (2002) Cloning, sequencing, and characterization of the hexahydro-1,3,5-trinitro-1,3,5-triazine degradation gene cluster from Rhodococcus rhodochrous. Appl Environ Microbiol 68:4764–4771

Seth-Smith HMB, Edwards J, Rosser SJ, Rathbone DA, Bruce NC (2008) The explosive-degrading cytochrome P450 system is highly conserved among strains of *Rhodococcus* spp. Appl Environ Microbiol 74:4550–4552

Sherburne LA, Shrout JD, Alvarez PJJ (2005) Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) degradation by *Acetobacterium paludosum*. Biodegradation 16:539–547

Sheremata TW, Hawari J (2000) Mineralization of RDX by the white rot fungus *Phanerochaete chrysosporium* to carbon dioxide and nitrous oxide. Environ Sci Technol 34:3384–3388

Singh R, Soni P, Kumar P, Purohit S, Singh A (2009) Biodegradation of high explosive production effluent containing RDX and HMX by denitrifying bacteria. World J Microbiol Biotechnol 25:269–275

Soojhawon I, Lokhande PD, Kodam KM, Gawai KR (2005) Biotransformation of nitroaromatics and their effects on mixed function oxidase system. Enzyme Microb Technol 37:527–533

Spanggord RJ, Mabey WR, Chuo T, Haynes DL, Alferness PL, Tee DS, Mill T (1982) Environmental fate studies of HMX. Phase 1, screening studies, final report. SRI International, Menlo Park, CA

Speitel G, Engels T, McKinney D (2001) Biodegradation of RDX in unsaturated soil. Bioremediation J 5:1–11

Spence MJ, Bottrell SH, Thornton SF, Richnow HH, Spence KH (2005) Hydrochemical and isotopic effects associated with petroleum fuel biodegradation pathways in a chalk aquifer. J Contam Hydrol 79:67–88

Spiker JK, Crawford DL, Crawford RL (1992) Influence of 2,4,6-trinitrotoluene (TNT) concentration on the degradation of TNT in explosive-contaminated soils by the white rot fungus Phanerochaete chrysosporium. Appl Environ Microbiol 58:3199–3202

Stenuit B, Eyers L, El Fantroussi S, Agathos SN (2005) Promising strategies for the mineralisation of 2,4,6-trinitrotoluene. Rev Environ Sci Biotechnol 4:39–60

Stenuit B, Eyers L, Rozenberg R, Habib-Jiwan J-L, Agathos SN (2006) Aerobic growth of Escherichia coli with 2,4,6-trinitrotoluene (TNT) as the sole nitrogen source and evidence of TNT denitration by whole cells and cell-free extracts. Appl Environ Microbiol 72:7945–7948

Stenuit B, Eyers L, Rozenberg R, Habib-Jiwan J-L, Matthijs S, Cornelis P, Agathos SN (2009) Denitration of 2,4,6-trinitrotoluene in aqueous solutions using small-molecular-weight catalyst(s) secreted by *Pseudomonas aeruginosa* ESA-5. Environ Sci Technol 43:2011–2017

Steuckart C, Berger-Prelss E, Levsen K (1994) Determination of explosives and their biodegradation products in contaminated soil and water from former ammunition plants by automated multiple development high-performance thin-layer chromatography. Anal Chem 66:2570–2577

Tekoah Y, Abeliovich A, Nejidat A (1999) Participation of cytochrome P450 in the biodegradation of RDX by a *Rhodococcus* strain. In: 2nd *international symposium, biodegradation of nitroaromatic compounds and explosives*, Leesburg, VA, pp 7

Thompson KT, Crocker FH, Fredrickson HL (2005) Mineralization of the cyclic nitramine explosive hexahydro-1,3,5-trinitro-1,3,5-triazine by *Gordonia* and *Williamsia* spp. Appl Environ Microbiol 71:8265–8272

Uchimiya M, Gorb L, Isayev O, Qasim MM, Leszczynski J (2010) One-electron standard reduction potentials of nitroaromatic and cyclic nitramine explosives. Environ Pollut 158:3048–3053

US EPA (2006) 2006 Edition of the Drinking Water Standards and Health Advisories. Office of Water, EPA 822-R-06-013, Washington, DC

US EPA (2010) Risk-Based Concentration Table [online]. Available from: http://www.epa.gov/reg3hwmd/risk/human/rb-concentration_table/Generic_Tables/pdf/master_sl_table_run_MAY2010.pdf

Van Aken B, Yoon JM, Schnoor JL (2004) Biodegradation of nitro-substituted explosives 2,4,6-trinitrotoluene, hexahydro-1,3,5-trinitro-1,3,5-triazine, and octahydro-1,3,5, 7-tetranitro-1,3,5-tetrazocine by a phytosymbiotic Methylobacterium sp. associated with poplar tissues (Populus deltoides x nigra DN34). Appl Environ Microbiol 70:508–517

Van Dillewijn P, Caballero A, Paz JA, González-Pérez MM, Oliva JM, Ramos JL (2007) Bioremediation of 2,4,6-trinitrotoluene under field conditions. Environ Sci Technol 41:1378–1383

Van Dillewijn P, Wittich R-M, Caballero A, Ramos J-L (2008) Type II hydride transferases from different microorganisms yield nitrite and diarylamines from polynitroaromatic compounds. Appl Environ Microbiol 74:6820–6823

Vanderberg LA, Perry JJ, Unkefer PJ (1995) Catabolism of 2,4,6-trinitrotoluene by *Mycobacterium vaccae*. Appl Microbiol Biotechnol 43:937–945

Vorbeck C, Lenke H, Fischer P, Knackmuss H-J (1994) Identification of a hydride-Meisenheimer complex as a metabolite of 2,4,6-trinitrotoluene by a *Mycobacterium* strain. J Bacteriol 176:932–934

Vorbeck C, Lenke H, Fischer P, Spain JC, Knackmuss H-J (1998) Initial reductive reactions in aerobic microbial metabolism of 2,4,6-trinitrotoluene. Appl Environ Microbiol 64:246–252

Waisner S, Hansen L, Fredrickson H, Nestler C, Zappi M, Banerji S, Bajpai R (2002) Biodegradation of RDX within soil-water slurries using a combination of differing redox incubation conditions. J Hazard Mater B95:91–106

Wani AH, Davis JL (2003) RDX biodegradation column study: influence of ubiquitous electron acceptors on anaerobic biotransformation of RDX. J Chem Technol Biotechnol 78:1082–1092

Williams RE, Rathbone DA, Scrutton NS, Bruce NC (2004) Biotransformation of explosives by the old yellow enzyme family of flavoproteins. Appl Environ Microbiol 70:3566–3574

Wilson RD, Thornton SF, Mackay DM (2004) Challenges in monitoring the natural attenuation of spatially variable plumes. Biodegradation 15:359–369

Wingfors H, Edlund C, Hägglund L, Waleij A, Sjöström J, Karlsson R-M, Leffler P, Qvarfort U, Ahlberg M, Thiboutot S, Ampelman G, Martel R, Duvalois W, Creemers A, Van Ham N (2006) Evaluation of the Contamination by Explosives and Metals in Soils at the Älvdalen Shooting Range. Part II: Results and Discussion. NBC Defence Scientific report, FOI-R-1877-SE

Wittich R-M, Haïdour A, Van Dillewijn P, Ramos J-L (2008) OYE flavoprotein reductases initiate the condensation of TNT-derived intermediates to secondary diarylamines and nitrite. Environ Sci Technol 42:734–739

Wittich R-M, Ramos J-L, Van Dillewijn P (2009) Microorganisms and explosives: mechanisms of nitrogen release from TNT for use as an N-source for growth. Environ Sci Technol 43:2773–2776

Won WD, Heckly RJ, Glover DJ, Hoffsommer JC (1974) Metabolic disposition of 2,4,6-trinitrotoluene. Appl Microbiol 27:513–516

Yinon J (1990) Toxicity and metabolism of explosives. CRC Press Inc., Boca Raton, FL

Yinon J, Zitrin S (1993) Modern methods and applications in analysis of explosives. Wiley, Chichester

Young DM, Unkefer PJ, Ogden KL (1997) Biotransformation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by a prospective consortium and its most effective isolate Serratia marcescens. Biotechnol Bioeng 53:515–522

Zhang C, Hughes JB (2003) Biodegradation pathways of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by *Clostridium acetobutylicum* cell-free extract. Chemosphere 50:665–671

Zhang B, Kendall RJ, Anderson TA (2006) Toxicity of the explosive metabolites hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) and hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) to the earthworm Eisenia fetida. Chemosphere 64:86–95

Zhao J-S, Halasz A, Paquet L, Beaulieu C, Hawari J (2002) Biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine and its mononitroso derivative hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine by *Klebsiella pneumoniae* strain SCZ-1 isolated from an anaerobic sludge. Appl Environ Microbiol 68:5336–5341

Zhao J-S, Paquet L, Halasz A, Hawari J (2003a) Metabolism of hexahydro-1,3-5-trinitro-1,3,5-triazine through initial reduction to hexahydro-1-nitroso-3, 5-dinitro-1,3,5-triazine followed by denitration in *Clostridium bifermentans* HAW-1. Appl Microbiol Biotechnol 63:187–193

Zhao J-S, Spain J, Hawari J (2003b) Phylogenetic and metabolic diversity of hexahydro-1,3,5-trintitro-1,3,5-triazine (RDX) transforming bacteria in strictly anaerobic mixed cultures enriched on RDX as nitrogen source. FEMS Microbiol Ecol 46:189–196

Zhao J-S, Paquet L, Halasz A, Manno D, Hawari J (2004a) Metabolism of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine by *Clostridium* bifermentans strain HAW-1 and several other H2-producing fermentative anaerobic bacteria. FEMS Microbiol Lett 237:65–72

Zhao J-S, Spain J, Thiboutot S, Ampleman G, Greer C, Hawari J (2004b) Phylogeny of cyclic nitramine-degrading psychrophilic bacteria in marine sediment and their potential role in the natural attenuation of explosives. FEMS Microbiol Ecol 49:349–357

Zhao J-S, Manno D, Beaulieu C, Paquet L, Hawari J (2005) *Shewanella sediminis* sp. nov., a novel Na+-requiring and hexahydro-1,3,5-trinitro-1,3,5-triazine-degrading bacterium from marine sediment. Intl J Syst Evol Microbiol 55:1511–1520

Zhao J-S, Manno D, Leggiadro C, O'Neil D, Hawari J (2006) *Shewanella halifaxensis* sp nov., a novel obligately respiratory and denitrifying psychrophile. Intl J Syst Evol Microbiol 56:205–212

Zhao J-S, Manno D, Hawari J (2007) Abundance and diversityofoctahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX)-metabolizing bacteria in UXO-contaminated marine sediments. FEMS Microbiol Ecol 59:706–717

Zhao J-S, Den Y, Manno D, Hawari J (2010) *Shewanella* spp. genomic evolution for a cold marine lifestyle and in situ explosive biodegradation. PLoS One 5:e9109

Ziganshin AM, Gerlach R, Borch T, Naumov AV, Naumova RP (2007) Production of eight different hydride complexes and nitrite release from 2,4,6-trinitrotoluene by *Yarrowia lipolytica*. Appl Environ Microbiol 73:7898–7905

Chapter 6
Biodegradation of Mono-Aromatic Hydrocarbons by Fungi

Christian Kennes and María C. Veiga

6.1 Fungal Biodegradation of Pollutants

The biodegradation of organic compounds by fungal strains has been studied for several decades. Fungi have proven to be able to degrade a wide range of different pollutants, including aliphatic, aromatic and polycyclic aromatic compounds, but not necessarily as sole carbon and energy source.

Extensive and detailed studies on fungal assimilation of non-oxygenated mono-aromatic compounds are, however, quite recent. A clear evidence on the use of such substrates as sole carbon and energy source was found for the first time in the early 1990s. Considering the high volatility of such compounds and their relatively common presence in polluted air, much of the research published on this topic is often related to biotechniques for air pollution control (Kennes and Veiga 2004; Kennes et al. 2009).

6.2 Fungal Biodegradation of Aliphatic Compounds

Fungal biodegradation of aliphatic compounds has been studied and reported in the literature for several decades and will, therefore, not be reviewed here extensively. Many different fungal species have been described for their growth on aliphatic substrates including alcohols, alkanes, and aldehydes, among others. Some examples are given in Table 6.1. Often these substrates can be used as sole carbon and energy source, although there are some exceptions, such as, the co-metabolic degradation

C. Kennes (✉) · M. C. Veiga
Campus da Zapateira, University of La Coruña,
Rua da Fraga 10, 15008 La Coruña, Spain
e-mail: kennes@udc.es

S. N. Singh (ed.), *Microbial Degradation of Xenobiotics*,
Environmental Science and Engineering, DOI: 10.1007/978-3-642-23789-8_6,

Table 6.1 Examples of fungal strains degrading aliphatic compounds

Fungal species	Substrate	Reference
Paecilomyces variotii	Methanol	Sakaguchi et al. (1975)
Gliocladium deliquescens	Methanol	Sakaguchi et al. (1975)
Paecilomyces variotii	Formaldehyde	Sakaguchi et al. (1975)
Gliocladium deliquescens	Formaldehyde	Sakaguchi et al. (1975)
Acremonium sp.	C_2–C_4 Alkanes	Davies et al. (1973)
Cladosporium resinae	C_6–C_{19} Alkanes	Cofone et al. (1973); Lindley and Heydeman (1983)
Scedosporium sp.	C_1–C_9 Alkanes	Onodera et al. (1989)
Graphium sp.	*n*-Butane	Hardison et al. (1997)
Trichosporon veenshuisii	*n*-Hexadecane	Middelhoven et al. (2000)
Graphium sp.	Di-ethyl-ether	Hardison et al. (1997)
Graphium sp.	Methyl-*tert*-butyl-ether	Hardison et al. (1997)

of the gasoline oxygenate methyl-*tert*-butyl ether (MTBE) by a *Graphium* sp., ATCC 58,400, in the presence of some *n*-alkanes (Hardison et al. 1997).

When dealing with mixtures of hydrocarbons, as present in oil, many yeasts and filamentous fungi easily degrade short-chain hydrocarbons (mainly alkanes), while long-chain hydrocarbons are generally degraded more slowly and aromatic hydrocarbons may even remain undegraded (Lindley and Heydeman 1986a, Lindley et al. 1986b).

Although most studies have been done with mesophilic organisms, some psychrophilic and psychrotolerant alkane-degrading fungi have also been described (Hughes et al. 2007). In these fungi, in the presence of glucose as an additional carbon source, growth rates generally increased if aliphatic hydrocarbons were available but decreased when a mixture of glucose and aromatic compounds was provided.

6.3 Fungal Biodegradation of Polycyclic Compounds

Polycyclic aromatic compounds are hydrophobic pollutants characterized by the presence of more than one benzene ring and found in oil derived products or creosotes together with some other pollutants (Kennes and Lema 1994a). Extensive studies were done on the biodegradation of polycyclic aromatic hydrocarbons (PAHs) in the late 1990s mainly with white-rot ligninolytic fungi, such as *Phanerochaete chrysosporium* or *Trametes versicolor*. Some non-white-rot fungi were also shown to be able to biodegrade and use some PAHs as sole carbon source. This was the case of the fungus *Fusarium solani* degrading benzo[A]pyrene as sole carbon source and partly converting it to carbon dioxide (Rafin et al. 2000). In this organism, the amount CO_2 released decreased in the presence of cytochrome P-450 inhibitors. Fungal strains are generally more efficient than bacteria in degrading polycyclic compounds. It is now known that they can use either the cytochrome P-450 system (Yadav et al. 2006) or extracellular ligninolytic enzymes (Tortella and Diez 2005) such as

lignin- and manganese- peroxidases (known as MnP and LiP) (Hammel 1992) and laccases (Majcherczyk et al. 1998). One of the main roles of lignin-degrading enzymes is to attack lignin. The latter is a complex molecule containing different structures, rings and links between the rings. Ligninolytic enzymes are, therefore, non-specific and highly versatile in their biodegradation capabilities, which allow them to biodegrade many different molecules, such as PAHs. On the other hand, the cytochrome P-450 system is also found in mammals, where PAH degradation follows similar pathways as in fungi. The biodegradation products, with the cytochrome P-450 system, are mainly epoxides and dihydrodiols which may show carcinogenic properties. In the perodixase-linked pathway, quinones are formed (Kennes and Lema 1994a), which are much less harmful. In many cases, these metabolites tend to accumulate as dead-end products.

Reports on the biodegradation of some other compounds with more than one ring, such as biphenyls, by other fungi are also available. Fungal biodegradation and accumulation of soluble metabolites was observed in biphenyl degradation by lygninolytic fungi as well as yeasts, such as *Cunninghamella elegans* (Dodge et al. 1979), *Candida lipolytica* (Cerniglia and Crow 1981), *Saccharomyces cerevisiae* (Wiseman et al. 1975), or *Trichosporon mucoides* (Sietmann et al. 2001). Schauer et al. (1995) reported the ability of the yeast *Trichosporon beigelii* to metabolize and partly degrade diphenyl ether.

6.4 Fungal Biodegradation of Non-Oxygenated Mono-Aromatic Compounds

6.4.1 Biodegradation of Oxygenated Mono-Aromatic Compounds

Although studies on the biodegradation of oxygenated aromatic compounds by fungi were already published more than 40 years ago (Neujahr and Varga 1970), much less research was done at that time on the non-oxygenated mono-aromatic pollutants, such as the benzene-compounds. Most of the research on oxygenated substrates was done with compounds such as phenol and cresols. Some of the researches published over the past 40 years have been summarized in Table 6.2. This is obviously not an exhaustive list, but it illustrates the typical pollutants and fungal species that have been studied. Fungal growth on some other oxygenated benzene compounds, such as benzoate or hydroxybenzoate, is also possible, although these substrates have been less studied (Middelhoven 1993). Again, these pollutants can be degraded either as sole carbon and energy source or co-metabolically. For example, a *Penicillium* strain Bi 7/2 was shown to use phenol as sole source of carbon and energy (Hofrichter et al. 1993). The same was true for *Trichosporon guehoae* CBS 8521 using either phenol or *m*–cresol (Middelhoven et al. 1999). Conversely, *Penicillium* strain Bi 7/2 degraded substituted phenols, such as chlorophenols or cresols, only co-metabolically (Hofrichter et al. 1993, 1995).

Table 6.2 Examples of fungal strains degrading oxygenated mono-aromatic compounds

Fungal species	Substrate	Reference
Trichosporon cutaneum	Phenol	Neujahr and Varga (1970)
Aspergillus japonicus	Phenol	Milstein et al. (1983)
Penicillum spp	Phenol	Scow et al. (1990); Hofrichter et al. (1993)
Phanerochaete chrysosporium	Phenol	Kennes and Lema (1994b)
Trichosporon guehoae	Phenol	Middelhoven et al. (1999)
Trichosporon veenhuisii	Phenol	Middlehoven et al. (2000)
Trichosporon cutaneum	Fluorinated phenols	Peelen et al. (1995)
Trichosporon cutaneum	*o*-, *m*-, *p*-cresols	Hasegawa et al. (1990)
Aspergillus fumigatus	*p*-cresol	Jones et al. (1993)
Phanerochaete chrysosporium	*p*-cresol	Kennes and Lema (1994b)
Penicillium frequentans	*o*-cresol	Hofrichter et al. (1995)
Trichosporon guehoae	*m*-cresol	Middelhoven et al. (1999)
Trichosporon veenhuisii	*m*-cresol	Middlehoven et al. (2000)

6.4.2 Biodegradation of Non-Oxygenated Mono-Aromatic Compounds

Until the end of the past century, the biodegradation of non-oxygenated mono-aromatic compounds seemed to be an unusual capability of fungi (Kennes and Veiga 2004). The first isolated report suggesting minimal growth of a fungal strain on benzene compounds appeared in 1973 (Cofone et al. 1973). However, not enough evidence was obtained at that time to conclude that growth of the *Cladosporium resinae* strain was possible on benzene. Another report appeared several years later (Fedorak and Westlake 1986) showing slow growth of different fungal strains on *n*-alkylbenzenes. The experiments were performed with *Penicillium*, *Beauveria*, *Paecilomyces* and *Verticillium* spp. Compounds with relatively short side chain length, i.e. toluene, ethylbenzene or even benzene, were not degraded. Minimum length of the side chain for the substrates to be degraded, was C_4 or even C_8 or C_9, depending on the strain. Partial removal of those pollutants was detected after four weeks, i.e. 28 days, with transient accumulation of intermediate compounds, mainly aromatic acids. Evolution of end products, such as carbon dioxide, was not reported. It was only in the mid-1990s that a clear evidence for the use of alkylbenzenes as sole carbon and energy source and their complete biodegradation to end products was found in fungi. The use of toluene, as sole carbon and energy source, was reported in 1995 in the fungal strain *Cladosporium sphaerospermum* (Weber et al. 1995), with complete removal of the substrate at relatively high rate and complete conversion to carbon dioxide, water and biomass. According to the following stoichiometric equation, complete substrate biodegradation should yield 6.35 mol of CO_2 per mole toluene degraded (Estévez et al. 2005a) taking biomass growth into account, and with ammonium chloride as nitrogen source:

$$1.55\ C_7H_8 + 12\ O_2 + 0.2\ NH_4Cl \rightarrow CH_{1.8}N_{0.2}O_{0.5} + 9.85\ CO_2 + 5.6\ H_2O + 0.2\ HCl$$

Weber et al. (1995) reported that about 220 μmoles CO_2 was generated from 60 μmoles toluene after ten days, corresponding to a carbon dioxide to substrate ratio of about 3.7, showing that at least 66% of CO_2 was recovered experimentally, compared to the theoretical equation. However, at the end of the ten-day experiment, carbon dioxide release was still increasing exponentially, suggesting that higher product concentrations were presumably released. Toluene biodegradation by *Cladosporium sphaerospermum* seemed to involve Cytochrome P-450, starting initial attack on the side chain (Weber et al. 1995). Earlier, experiments were performed with another benzene-compound, namely styrene (Cox et al. 1993). The black yeast *Exophiala jeanselmei* showed slow growth on styrene, utilizing it as sole carbon and energy source, with an average specific removal rate around 30 nmol/min.mg protein. Potential metabolic intermediates of the biodegradation were suggested to be styrene oxide, phenylacetic acid, 1-phenyl-ethanol, 2-phenyl-ethanol, acetophenone, benzoic acid and phenol, which all supported yeast growth. Around that same period, it was also found that the white-rot fungus *Phanerochaete chrysosporium* was able to biodegrade benzene, toluene, ethylbenzene and xylenes (Yadav and Reddy 1993). However, only a part of the original alkylbenzenes concentration disappeared in the batch culture media. Besides, mass balance calculations also showed that the alkylbenzenes were only partly converted to carbon dioxide.

Non-substituted benzene (benzene as such) is often much more difficult to be degraded by fungal strains (Prenafeta-Boldú et al. 2002), suggesting the importance of the side-chains. Bacteria are known to attack first either the alkyl side chain or the aromatic ring of alkylbenzenes. However, in zygomycetous fungi, both pathways have been observed in case of co-metabolic degradation. Otherwise, only the oxidation of the side chain has been reported, when the removal is not through co-metabolism (Prenafeta-Boldú et al. 2001b). This also confirms the earlier results of Fedorak and Westlake (1986), where benzene compounds with longer side chains were generally more degraded than organic pollutants with shorter side chains. Conversely, the presence of several side-chains, such as in the xylene isomers, does not favour fungal biodegradation. It was shown in one study that in case of mixtures of benzene-compounds, such as benzene, toluene, ethylbenzene and xylene isomers; both toluene and ethylbenzene were used by *Cladophialophora* sp., as sole carbon and energy source, while most of the xylenes were only degraded co-metabolically in presence of other alkylbenzenes (Prenafeta-Boldú et al. 2002). Benzene was also not used by this fungus as carbon source. In some cases, however, benzene was shown to be used as sole carbon source for fungal growth, such as in *Cladosporium sphaerospermim*, *Exophialalecanii-corni*, *Phanerochaete chrysosporium*, or *Paecilomyces variotii* (Qi et al. 2002; García-Peña

Table 6.3 Fungal strains degrading non-oxygenated mono-aromatic compounds

Fungal species	Substrate	Reference
Cladosporium sphaerospermum	Benzene	Qi et al. (2002)
Exophiala lecanii-corni	Benzene	Qi et al. (2002)
Phanerochaete chrysosporium	Benzene	Qi et al. (2002)
Cladosporium resinae	Ethylbenzene	Qi et al. (2002)
Cladosporium sphaerospermum	Ethylbenzene	Qi et al. (2002)
Exophiala lecanii-corni	Ethylbenzene	Qi et al. (2002)
Mucor rouxii	Ethylbenzene	Qi et al. (2002)
Cladophialophora sp.	Ethylbenzene	Prenafeta-Boldú et al. (2002)
Ophiostoma stenoceras	α-Pinene	Jin et al. (2006)
Exophiala jeanselmei	Styrene	Cox et al. (1993)
Cladosporium sphaerospermum	Styrene	Qi et al. (2002)
Exophiala lecanii-corni	Styrene	Qi et al. (2002)
Sporothrix variecibatus	Styrene	Rene et al. (2010a, b)
Cladosporium sphaerospermum	Toluene	Weber et al. (1995)
Cladosporium sphaerospermum	Toluene	Prenafeta-Boldú et al. (2001b)
Cladophialophora sp.	Toluene	Prenafeta-Boldú et al. (2001b)
Pseudeurotium zonatum	Toluene	Prenafeta-Boldú et al. (2001b)
Exophiala sp.	Toluene	Prenafeta-Boldú et al. (2001b)
Leptodontium sp.	Toluene	Prenafeta-Boldú et al. (2001b)
Exophiala oligosperma	Toluene	Estévez et al. (2005b)
Paecilomyces variotii	Toluene	Estévez et al. (2005b)
Cladophialophora sp.	*o*-Xylene	Nikolova and Nenov (2005)
Cladophialophora sp.	*m*-Xylene	Nikolova and Nenov (2005)

et al. 2008). Conversely, the presence of easily metabolized sugars, such as glucose, were shown to inhibit the biodegradation of benzene-compounds, such as styrene, by black yeasts like *Exophiala jeanselmei* (Cox et al. 1993) and white-rot fungi (Braun-Lüllemann et al. 1997).

Many new fungal strains growing on such non-oxygenated mono-aromatic compounds were isolated over the past ten years as reflected in Table 6.3. Studies with these organisms also indicated the effect of physical parameters, such as pH, temperature and the relative humidity on pollutant biodegradation. It is worth mentioning that Table 6.3 only lists results obtained from the use of pure cultures. It is important to mention that many results have been published based on bioreactors inoculated with pure fungal cultures, but operated under open conditions. This does not rule out the possibility of having other microbial strains present in the system. Table 6.3 only summarizes information on fungi or yeast-like fungi using benzene-compounds as sole carbon and energy source. Most of the fungi quoted in Table 6.3 are characterized by quite close taxonomic affinities, suggesting that there may be only a limited number of fungal strains which are able to use these organic pollutants. Other fungi degrade these

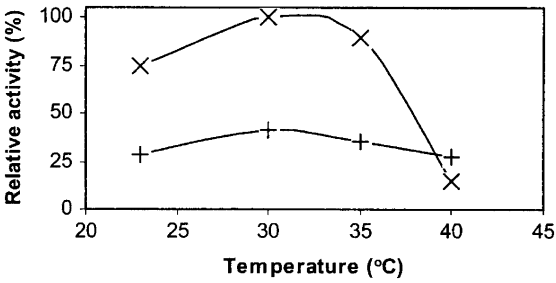


Fig. 6.1 Influence of temperature on the relative activity, in terms of toluene biodegradation rate, in *Pacilomyces variotii* (+) and *Exophiala oligosperma* (×) separately (Estévez et al. 2005b)

compounds co-metabolically, for example, *Aspergillus niger* CBS126.48 and *Cunninghamella echinulata* CBS596.68 (Prenafeta-Boldú et al. 2001b)

6.4.3 *Influence of Environmental Conditions on the Fungal Biodegradation of Non-Oxygenated Mono-Aromatic Compounds*

For most microorganisms, environmental conditions, such as pH, temperature, relative humidity, moisture content of the ecosystem, play a key role on the activity of fungal species degrading mono-aromatic compounds.

6.4.3.1 Temperature

Interestingly, all fungi isolated so far on alkylbenzenes and other related non-oxygenated benzene-compounds are mesophilic organisms. Their optimal temperature is generally around 30°C. Toluene biodegradation by *Exophiala oligosperma* and *Paecilomyces variotii* at different temperatures is reflected in Fig. 6.1 (Estévez et al. 2005b). Another study was done on toluene biodegradation to carbon dioxide by different fungi including *Cladosporium*, *Cladophialophora*, *Pseudeurotium*, *Exophiala*, and *Leptodontium* strains (Prenafeta-Boldú et al. 2001a). For a tested temperature range of 20–37°C, most of the strains exhibited their optimal activity around 30°C, but in two of the strains, namely *Pseudeurotium* and *Leptodontium*, the maximum activity for degradation was recorded at 20°C. In some of our own studies on thermophilic ecosystems, isolations done from mixed cultures, with alkylbenzenes or α-pinene, resulted in the overgrowth of bacteria on plates. However, scanty information is available on thermophilic fungi as compared to thermophilic bacteria, suggesting that fungi tolerating high temperatures are not so common (Maheshwari et al. 2000).

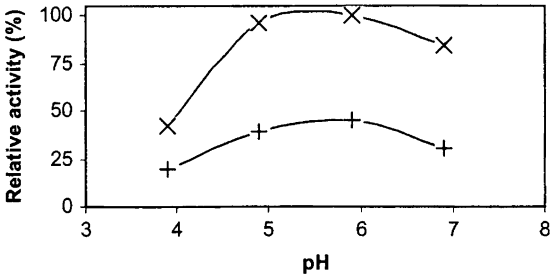


Fig. 6.2 Influence of pH on the relative activity, in terms of toluene biodegradation rate, in *Pacilomyces variotii* (+) and *Exophiala oligosperma* (×) (Estévez et al. 2005b)

6.4.3.2 pH

Compared to bacteria, fungi are generally more tolerant to low pH conditions. The optimal pH of fungi isolated so far on mono-aromatic compounds is often around 5.5–6.0, although this has only been checked for some strains. The effect of pH on toluene biodegradation by *Exophiala oligosperma* and *Paecilomyces variotii* is shown in Fig. 6.2. It appears that biodegradation was possible over all the pH range tested, between 3.9 and 6.9, with optimal conditions around pH 5.9, for cultures grown at a constant temperature of 30°C (Estévez et al. 2005b). Other researchers checked the fungal growth on plates at pH 3.5, 5.0, and 6.5, with different substrates as sole carbon source, including different mono-aromatic benzene compounds (Qi et al. 2002). Although the level of growth was estimated visually, it appeared that optimal conditions were almost in all cases close to pH 5.0, and occasionally pH 6.5, for strains of *Cladosporium*, *Exophiala lecanii-corni*, *Mucor rouxii*, and *Phanerochaete chrysosporium*. For white-rot fungi, there was not much difference between pH 5.0 and 6.5. It is worth mentioning that for white-rot fungi, biodegradation studies are in general performed near pH 4.5 considered as optimal, in the case of BTEX (i.e. benzene, toluene, ethylbenzene, and xylenes) compounds (Yadav and Reddy 1993), but also for other oxygenated mono-aromatic phenolic compounds (Kennes and Lema 1994b).

Fungi have often been inoculated in gas-phase biofilters for the treatment of air polluted with volatile benzene-related compounds (Kennes and Veiga 2004). Such packed-bed bioreactors are open systems and a mixed microbial community may often develop in the long-run. In all cases, simple microscopic observations showed that fungi remained highly dominant species. In such experiments, it was proven that in biofilters in which fungi are dominant, the system can much better withstand pH drops and acidification of the packing material than reactors in which bacteria are dominant. However, in many cases, a higher performance is still observed near neutral or in slightly acidic conditions (Estévez et al. 2005a). Occasionally, it was reported that performance did even improve at lower pH in studies performed at either pH 4.0 or at higher pH 8.0 (van Groenestijn et al. 2001). SEM pictures of some fungi growing on the surface of packing materials in gas-phase biofilters are shown in Fig. 6.3.

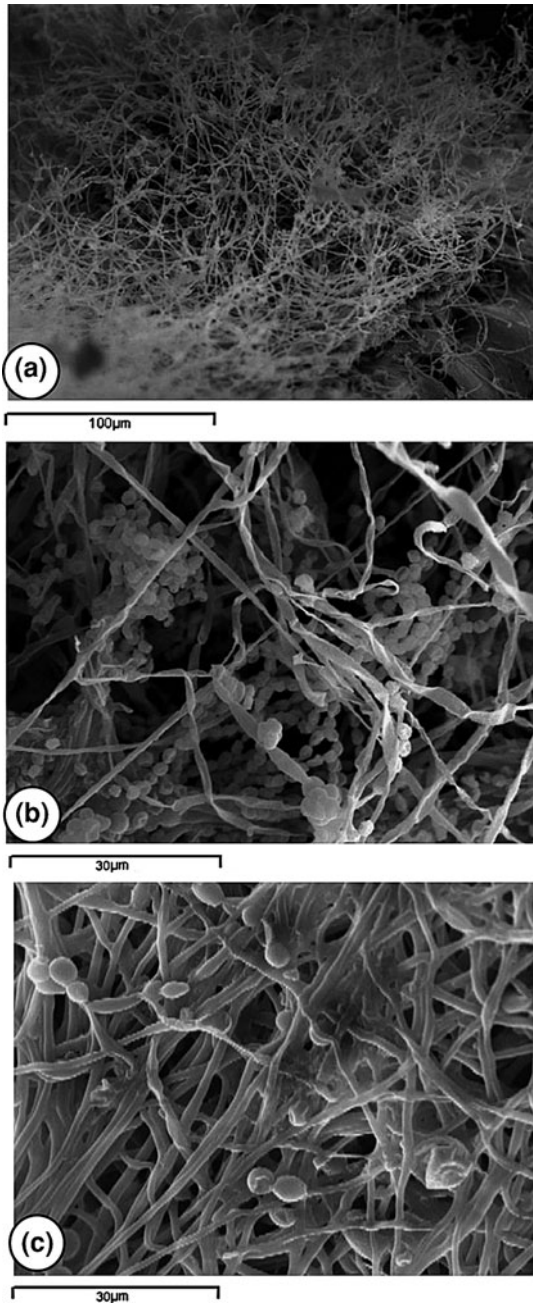


Fig. 6.3 Growth of fungal strains on packing materials, i.e. perlite, in biofilters. **a** *Ophiostoma* sp. growing on α-pinene. **b** *Paecilomyces* sp. growing on toluene. **c** *Exophiala* sp. growing on toluene

6.4.3.3 Relative Humidity and Water Content

As mentioned earlier, mono-aromatic hydrocarbons are commonly found in polluted air. Microorganisms need some moisture to be active and biodegrade pollutants. Compared to bacteria, filamentous fungi usually tolerate better somewhat dryer environments. When polluted air is fed to a packed-bed bioreactor, the air needs a minimum level of relative humidity and the packed-bed needs at least some minimum water content. Both with bacteria as well as with fungi, a relatively high moisture content of the reactor's packing material seems to be favourable in order to reach optimal removal of gas-phase pollutants. Thus, although fungi are more tolerant to dry conditions, their activity will gradually decrease, unless the air's relative humidity is at least 85% (Estévez et al. 2005a), and the packed bed's moisture content should preferably be at least 40–60% with a water activity, a_w, close to one (Cox et al. 1996).

6.5 Conclusions

The biodegradability of aliphatic and polycyclic aromatic pollutants as well as oxygenated mono-aromatic compounds by fungal strains has been studied for several decades. However, a clear evidence of the biodegradability of non-oxygenated mono-aromatic benzene-compounds and their use as sole carbon and energy source by fungi, was only reported for the first time in the 1990s. To the best of our knowledge, all strains isolated so far are mesophilic organisms. In most cases, their optimal pH and temperature are close to 6 and 30°C, respectively. Most fungi tolerate acidic environments, although they exhibit lower growth rates and biodegradation activities at low pH. Several fungal strains have been used in the treatment of air polluted with mono-aromatic benzene-compounds. In packed bed bioreactors, these fungi are less sensitive than bacteria to conditions of low moisture content, although relatively dry environments will decrease reactor performance.

References

Braun-Lüllemann A, Majcherczyk A, Hüttermann A (1997) Degradation of styrene by white-rot fungi. Appl Microbiol Biotechnol 47:150–155

Cerniglia CE, Crow SA (1981) Metabolism of aromatic hydrocarbons by yeast. Arch Microbiol 129:9–13

Cofone L, Walker JD, Cooney JJ (1973) Utilization of hydrocarbons by *Cladosporium resinae*. J Gen Microbiol 76:243–246

Cox HHJ, Houtman JHM, Doddema HJ, Harder W (1993) Growth of the black yeast *Exophiala jeanselmei* on styrene and styrene-related compounds. Appl Microbiol Biotechnol 39:372–376

Cox HHJ, Magielsen FJ, Doddema HJ, Harder W (1996) Influence of the water content and water activity on styrene degradation by *Exophiala jeanselmei* in biofilters. Appl Microbiol Biotechnol 45:851–856

Davies JS, Wellman AM, Zajic JE (1973) Hyphomycetes utilizing natural gas. Can J Microbiol 19:81–85

Dodge RH, Cerniglia CE, Gibson DT (1979) Fungal metabolism of biphenyl. Biochem J 178:223–230

Estévez E, Veiga MC, Kennes C (2005a) Biofiltration of waste gases with the fungi *Exophiala oligosperma* and *Paecilomyces variotii*. Appl Microbiol Biotechnol 67:563–568

Estévez E, Veiga MC, Kennes C (2005b) Biodegradation of toluene by the new fungal isolates *Paecilomyces variotii* and *Exophiala oligosperma*. J Ind Microbiol Biotechnol 32:33–37

Fedorak PM, Westlake DWS (1986) Fungal metabolism of n-alkylbezenes. Appl Environ Microbiol 51:435–437

García-Peña I, Ortiz I, Hernández S, Revah S (2008) Biofiltration of BTEX by the fungus *Paecilomyces variotii*. Intl Biodeter Biodegrad 62:442–447

Hammel KE (1992) Oxidation of aromatic pollutants by lignin degrading fungi and their extracellular peroxidises. Met Ions Biol Syst 28:41–60

Hardison LK, Curry SS, Ciuffetti LM, Hyman MR (1997) Metabolism of diethyl ether and cometabolism of methyl *tert*-butyl ether by a filamentous fungus, a *Graphium* sp. Appl Environ Microbiol 63:3059–3067

Hasegawa Y, Okamoto T, Obata H, Tokuyama T (1990) Utilization of aromatic compounds by *Trichosporon cutaneum* KUY-6A. J Ferment Bioeng 69(2):122–124

Hofrichter M, Günther T, Fritsche W (1993) Metabolism of phenol, chloro and nitrophenols by the *Penicillium* strain Bi 7/2 isolated from a contaminated soil. Biodegrad 3:415–421

Hofrichter M, Bublitz F, Fritsche W (1995) Cometabolic degradation of o-cresol and 2, 6-dimethylphenol by *Penicillium frequentans* Bi 7/2. J Basic Microbiol 35:303–313

Hughes KA, Bridge P, Clark MS (2007) Tolerance of antarctic soil fungi to hydrocarbons. Sci Total Environ 372:39–548

Jin Y, Veiga MC, Kennes C (2006) Performance optimization of the fungal biodegradation of α-pinene in gas-phase biofilter. Proc Biochem 41:172–1728

Jones KH, Trudgill PW, Hopper DJ (1993) Metabolism of *p*-cresol by the fungus *Aspergillus fumigatus*. Appl Environ Microbiol 59:1125–1130

Kennes C, Lema JM (1994a) Degradation of major compounds of creosotes (PAH and phenols) by *Phanerochaete chrysosporium*. Biotechnol Lett 16:759–764

Kennes C, Lema JM (1994b) Simultaneous biodegradation of *p*-cresol and phenol by the basydiomycete *Phanerochaete chrysosporium*. J Indust Microbiol 13:311–314

Kennes C, Veiga MC (2004) Fungal biocatalysts in the biofiltration of VOC-polluted air. J Biotechnol 113:305–319

Kennes C, Rene ER, Veiga MC (2009) Bioprocesses for air pollution control. J Chem Technol Biotechnol 84:1419–1436

Lindley ND, Heydeman MT (1983) Uptake of vapour phase [^{14}C]dodecane by whole mycelia of *Cladosporium resinae*. J Gen Microbiol 129:2301–2305

Lindley ND, Heydeman MT (1986) The uptake on *n*-alkanes from alkane mixtures during growth of the hudrocarbon-utilizing fungus *Cladosporium resinae*. Appl Microbiol Biotechnol 23:384–388

Lindley ND, Pedley JF, Kay SP, Heydeman MT (1986) The metabolism of yeasts and filamentous fungi which degrade hydrocarbon fuels. Intl Biodeter 22:281–287

Maheshwari R, Bharadwaj G, Bhat MK (2000) Thermophilic fungi: their physiology and enzymes. Microb Mol Biol Rev 64:461–488

Majcherczyk A, Johannes C, Hüttermann A (1998) Oxidation of polycyclic aromatic hydrocarbons (PAH) by laccase of *Trametes versicolor*. Enzyme Microb Technol 22:335–341

Middelhoven WJ (1993) Catabolism of benzene compounds by ascomycetous and basidiomycetous yeast and yeastlike fungi. Antonie van Leeuwenhoek 63:125–144

Middelhoven WJ, Scorzetti G, Fell JW (1999) *Trichosporon guehoae* sp nov., an anamorphic basidiomycetous yeast. Can J Microbiol 45:686–690

Middlehoven WJ, Scorzetti G, Fell JW (2000) *Trichosporon veenshuisii* sp nov., an alkane-assimilating anamorphic basidiomycetous yeast. Intl J Syst Evol Microbiol 50:381–187

Milstein O, Vered Y, Shragina L, Gressel J, Flowers HM, Hüttermann A (1983) Metabolism of lignin related aromatic compounds by *Aspergillus japonicus*. Arch Microbiol 135:147–154

Neujahr HY, Varga JM (1970) Degradation of phenols by intact cells and cell-free preparations of *Trichosporon cutaneum*. Eur J Biochem 13:37–44

Nikolova N, Nenov V (2005) BTEX degradation by fungi. Water Sci Technol 51:87–93

Onodera M, Endo Y, Ogasawara N (1989) Utilization of short-chain hydrocarbons and accumulation of methylketones by a gaseous hydrocarbon assimilating mold, *Scedosporium* sp. A-4. Agric Biol Chem 53:1431–1432

Peelen S, Rietjens IMCM, Boersma MG, Vervoort J (1995) Conversion of phenol derivatives to hydroxylated products by phenol hydroxylase from *Trichosporon cutaneum*. Eur J Biochem 227:284–291

Prenafeta-Boldú FX, Kuhn A, Luykx DMAM, Anke H, van Groenestijn JW, de Bont JAM (2001a) Isolation and characterisation of fungi growing on volatile aromatic hydrocarbons as their sole carbon and energy source. Mycol Res 105:477–484

Prenafeta-Boldú FX, Luykx DMAM, Vervoort J, de Bont JAM (2001b) Fungal metabolism of toluene: monitoring of fluorinated analogs by ^{19}F nuclear magnetic resonance spectroscopy. Appl Environ Microbiol 67:1030–1034

Prenafeta-Boldú FX, Vervoort J, Grotenhuis JTC, van Groenestijn JW (2002) Substrate interactions during the biodegradation of benzene, toluene, ethylbenzene, and xylene (BTEX) hydrocarbons by the fungus *Cladophialophora* sp. strain T1. Appl Environ Microbiol 68:2660–2665

Qi B, Moe WM, Kinney KA (2002) Biodegradation of volatile organic compounds by five fungal species. Appl Microbiol Biotechnol 58:684–689

Rafin C, Potin O, Veignie E, Lounes-Hadj Sahraoui A, Sancholle M (2000) Degradation of benzo[A]pyrene as sole carbon source by a non white rot fungus, *Fusarium solani*. Polycyclic Arom Hydroc 21:311–329

Rene ER, Špačková R, Veiga MC, Kennes C (2010a) Biofiltration of mixtures of gas-phase styrene and acetone with the fungus *Sporothrix variecibatus*. J Hazard Mater 184:204–214

Rene ER, Veiga MC, Kennes C (2010b) Biodegradation of gas-phase styrene using *Sporothrix variecibatus*: Impact of pollutant load and transient operations. Chemosphere 79:221–227

Sakaguchi K, Kurane R, Murata M (1975) Assimilation of formaldehyde and other C1-compounds by *Gliocladium deliquescens* and *Paecilomyces variotii*. Agric Biol Chem 39:1605–1702

Schauer F, Henning K, Pscheidl H, Wittich RM, Fortnagel P, Wilkes H, Sinnwell V, Francke W (1995) Biotransformation of diphenyl ether by the yeast *Trichosporon beigelii* SBUG 752. Biodegrad 6:173–180

Scow KM, Li D, Manilal VB, Alexander M (1990) Mineralization of organic compounds at low concentrations by filamentous fungi. Mycol Res 94:793–398

Sietmann R, Hammer E, Specht M, Cerniglia CE, Schuer F (2001) Novel ring cleavage products in the biotransformation of biphenyl by the yeast *Trichosporon mucoides*. Appl Environ Microbiol 67:4158–4165

Tortella GR, Diez MC (2005) Fungal diversity and use in decomposition of environmental pollutants. Crit Rev Microbiol 31:197–212

Van Groenestijn JW, Heiningen WNM, Kraakman NJR (2001) Biofilters based on the action of fungi. Water Sci Technol 44:227–232

Weber FJ, Hage KC, de Bont JAM (1995) Growth of the fungus *Cladosporium sphaerospermum* with toluene as the sole carbon and energy source. Appl Environ Microbiol 61:3562–3566

Wiseman A, Gondal J, Sims P (1975) 4-Hydroxylation of biphenyl by yeasts containing cytochrome P-450. Biochem Soc Trans 3:278–285

Yadav JS, Reddy CA (1993) Degradation of benzene, toluene, ethylbenzene, and xylenes (BTEX) by the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Appl Environ Microbiol 59:756–762

Yadav JS, Doddapaneni H, Subramanian V (2006) P450ome of the white rot fungus *Phanerochaete chrysosporium*: structure, evolution and regulation of expression of genomic P450 clusters. Biochem Soc Trans 34:1165–1169

Chapter 7
Bacterial Degradation of High Molecular Weight Polynuclear Aromatic Hydrocarbons

Suparna Mukherji and Indrani Ghosh

7.1 Introduction

7.1.1 Sources of PAHs in the Environment

Polycyclic aromatic hydrocarbons (PAHs) are a group of organic compounds consisting of two and more than two fused benzene rings. PAHs are naturally present in the fossil fuel. However, the increased level of PAHs in the environment over the last few decades is due to the huge increment in production and use of petroleum and petroleum products. Point sources of PAHs originate from accidental discharges during production, transportation and disposal of petroleum and its products and industrial processes such as, liquefaction and gasification of coal and waste incineration. Creosote and coal tar, which are by-products of coking, are rich source of PAHs containing 85–90% of it (Cerniglia 1992).

At contaminated sites, PAHs are often present along with other contaminants, such as, BTEX (benzene, toluene, ethyl-benzene and xylene) compounds, aliphatic hydrocarbons and heavy metals. Depending on the source of contamination, the level of PAHs in soil has been found to range from 1 to 300 μg/kg. Air-borne PAHs originating from incomplete combustion of organic materials can give rise to high PAH levels in the atmosphere (60 $\mu g/m^3$–3 mg/m^3 of air) (Bamforth and Singleton 2005). PAHs as well as numerous PAH derivatives, i.e., alkylated PAHs, nitro-PAHs, oxygenated PAHs, quinones, hydroxy and hydroxynitro compounds, are formed during incomplete combustion of various fuels such as, coal, gasoline, diesel and biofuels (Mukherji et al. 2002). Natural processes, such as volcanic

S. Mukherji (✉) · I. Ghosh
Centre for Environmental Science and Engineering,
Indian Institute of Technology, Powai,
Mumbai 400076, India
e-mail: mitras@iitb.ac.in

S. N. Singh (ed.), *Microbial Degradation of Xenobiotics*,
Environmental Science and Engineering, DOI: 10.1007/978-3-642-23789-8_7,

eruption and forest fires are other sources of PAHs in the environment. PAHs are also formed through geochemical processes, such as, exposure of sediments to high temperature during sediment diagenesis.

7.1.2 Physical and Chemical Properties of PAHs

PAHs are a group of compounds composed of two or more fused aromatic rings in linear, angular, and cluster arrangements as shown in Fig. 7.1. Based on structural diversity, PAHs can be broadly categorized into alternant and non-alternant classes. Alternant PAHs contain only fused benzenoid rings (e.g., anthracene, phenanthrene and pyrene), whereas non-alternant PAHs contain four or five carbon aromatic rings in addition to benzene ring (e.g., fluorene and fluoranthene) (Harvey 1991). PAHs can also be classified as low molecular weight (LMW) and high molecular weight (HMW) PAHs depending upon the number of aromatic rings. PAHs containing three or less than three aromatic rings are known as LMW PAHs (e.g., naphthalene and phenanthrene) and PAHs having greater than three aromatic rings are known as HMW PAHs (e.g., pyrene, fluoranthene, chrysene). Most of the PAHs have melting points above room temperature and boiling points above 100°C and are characterized by relatively low aqueous solubility.

Greater thermodynamic stability of PAHs arises from the delocalization of π-electron density. Thermodynamic stability and low aqueous solubility make them recalcitrant in the environment. The impact of PAH structure on its chemical behavior can generally be categorized as follows: PAHs with a linear structure are more unstable compared to their angular counterparts. Increased size and angularity of the PAH structure increase hydrophobicity and electrochemical stability which, in turn, affect their chemical and photochemical reactivity, as well as their ionization potential, vapor pressure, solubility and adsorption characteristics. The environmental fate of a PAH molecule depends on the number of aromatic rings and also on the pattern of ring linkage (Peters et al. 1999). The persistence of PAHs increases with an increase in the number of benzene rings and this is inversely correlated with the environmental biodegradation rates of PAHs.

Some PAHs contain a “bay-region” and a “K-region” (Mrozik et al. 2003). The bay- and K-region epoxides are reported to be highly reactive. Both these regions are present in phenanthrene as illustrated in Fig. 7.2. The bay-region of phenanthrene is between carbon atoms 4 and 5 whereas the K-region is between the carbon atoms 9, 10. The bay-region is a sterically hindered area. According to the Schmidt-Pullman electronic theory, K-region epoxides are likely to be more carcinogenic than the parent hydrocarbon.

LMW PAHs are characterized by higher volatility, higher solubility and greater ease of degradation than the higher molecular weight PAHs. HMW PAHs strongly sorb on to soil and sediments due to their high hydrophobicity and are therefore less available to indigenous microorganisms for degradation. The association of a contaminant with organic matter in soil (expressed as the organic matter

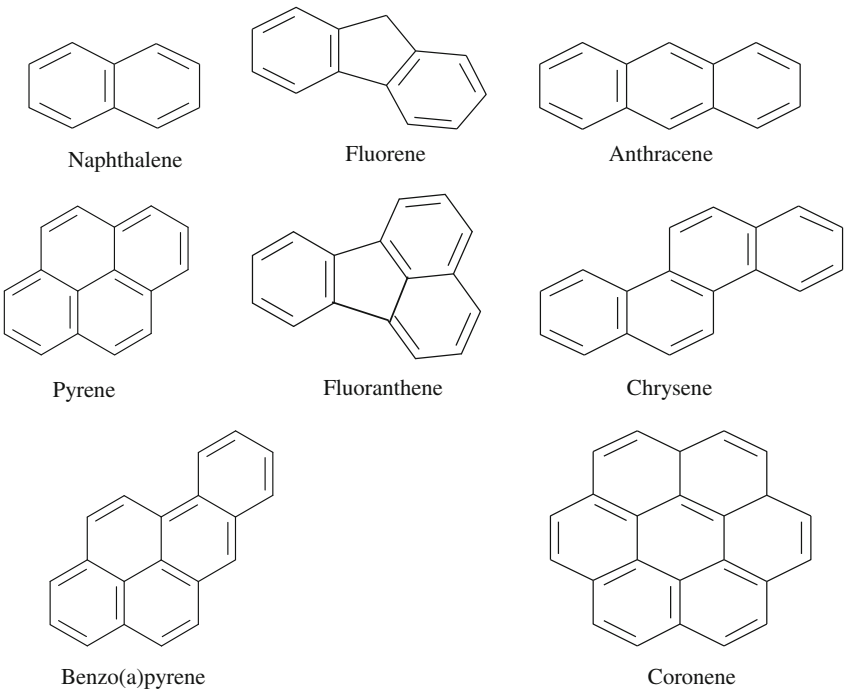


Fig. 7.1 Chemical structure of some polynuclear aromatic hydrocarbons

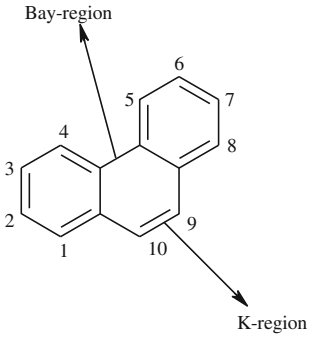


Fig. 7.2 Structure of phenanthrene showing bay-region and K-region

normalized partition coefficient K_{oc}) is a function of hydrophobicity of the compound which is expressed in terms of the octanol–water partitioning coefficient K_{ow}. Some properties of selected PAHs are presented in Table 7.1.

7.1.3 Toxicity of PAHs

Removal of PAHs from the environment is a major concern since they can exert acute as well as chronic toxicity (Juhasz et al. 2000). The highly hydrophobic and ipophilic

Table 7.1 Physical and chemical properties of selected PAHs

PAHs	Phenanthrene	Fluorene	Fluoranthene	Pyrene
Molecular formula	$C_{14}H_{10}$	$C_{13}H_{10}$	$C_{16}H_{10}$	$C_{16}H_{10}$
Molecular weight	178.22	166.21	202.26	202.26
Boiling point (°C)	340	295	375	404
Melting point (°C)	101	116.5	105–110	151.2
Density (g/cc)	1.179	1.202	1.252	1.271
No. of aromatic rings	3	3	4	4
Aq. solubility* (mg/L)	1.18	1.89	0.24	0.13

*Aqueous solubility is reported at 25°C
Source Yaws (1999) and Material Safety Data Sheets

nature of PAHs is responsible for their accumulation in the fatty tissue. Thus, PAHs are biomagnified through the food chain. Lower molecular weight PAHs impose greater acute toxicity because of their higher aqueous solubility compared to the higher molecular weight PAHs. PAHs can enter the human body through dermal exposure and food intake. It has been found that PAHs, especially, pyrene can get absorbed through dermal administration (75%). Acute toxicity is often reported based on lethal dose required to kill 50% of the test animals ($LD_{50,}$ mg/Kg-Body weight) in animal assays. LD_{50} value of naphthalene based on mice bioassay with oral administration is found to lie in the range of 533–710 mg/Kg while that for phenanthrene is 750 mg/Kg. The LD_{50} value for anthracene, fluoranthene, pyrene and benzo(a)pyrene (BaP) are $>$ 450, 100, 514 and 232 mg/Kg, respectively, based on mice bioassays with intraperitonial exposure (Bamforth and Singleton 2005). Exposure through inhalation may occur near combustion sources since PAHs are formed during incomplete combustion of carbonaceous fuels. Bacterial assays, human cell mutagenicity assays, animal assays and epidemiological studies have demonstrated the mutagenicity and carcinogenicity of PAHs associated with combustion and ambient aerosols. Although unsubstituted PAHs are not directly genotoxic to the mammalian system, a cytochrome P450 monooxygenase enzyme mediated reaction activates them to reactive epoxides that are genotoxic (Mukherji et al. 2002). However, various substituted PAHs are directly genotoxic. USEPA has listed 17 PAHs as priority pollutants for their toxic, mutagenic and carcinogenic properties. BaP is recognized as a potent human carcinogen while several other PAHs, such as, chrysene, are also classified as possible human carcinogens (IARC 1983, 1998).

7.2 Bioremediation of PAHs

Over the past two decades, numerous researchers have demonstrated the ability of microorganisms, such as, bacteria, fungi and algae to degrade PAHs (Cerniglia 1992). Such PAH-degrading microorganisms are commonly found in soil and in the aquatic environment. The first step in bacterial degradation of PAHs involves an initial oxidative attack in the presence of dioxygenase enzyme to form

cis dihydrodiol. The introduction of two hydroxy groups facilitates further degradation. Cleavage of the aromatic ring also occurs in the presence of molecular oxygen. Microbial degradation can potentially transform toxic PAHs, into benign compounds at a relatively low cost. Hence, biodegradation has gained attention for the clean-up of PAH contaminated sites. Bioremediation was adopted at numerous PAH contaminated Superfund sites in United States. Biodegradation of PAHs composed of three rings is well established and the degradation of HMW PAHs (composed of more than three rings) by bacteria has also been reported. Several bacterial genera have been identified for their ability to degrade PAHs, including *Pseudomonas*, *Alcaligenes, Mycobacterium*, *Rhodococcus*, *Sphingomonas* and *Cycloclasticus*. However, the microbial degradation of PAHs containing four or more aromatic rings is energetically less favorable compared to degradation of LMW PAHs (Mueller et al. 1997). BaP has been the subject of comprehensive studies on biodegradation due to its carcinogenicity (Kanaly and Harayama 2000).

7.2.1 Degradation of Four Ring PAHs

Of the four ring PAHs, degradation of fluoranthene, pyrene, chrysene, and benzo(*a*)anthracene has been investigated to varying degree. Fluoranthene, a non-alternant PAH containing a five carbon aromatic ring, is observed to be metabolized by a variety of bacteria, and pathways describing its biodegradation have been proposed.

7.2.1.1 Degradation of Fluoranthene by Bacteria

In 1990, two independent groups of researcher reported the isolation of a single organism capable of utilizing fluoranthene as a sole source of carbon and energy. Weissenfels et al. (1990) demonstrated the isolation of the soil microorganism *Alcaligenes denitrificans* strain WW1, which biodegraded fluoranthene at a rate of 0.3 mg/ml per day for an initial concentration of 1 mg/ml. It could also utilize pyrene and benzo(*a*)anthracene by cometabolism. A dioxygenase pathway was involved as confirmed by identification of some metabolites. Three metabolites of fluoranthene biodegradation by strain WW1, 7-hydroxyacenaphthylene, 7-acenaphthenone, and 3-hydroxymethyl-4,5-benzo-coumarine, were identified by UV, mass spectroscopy and NMR spectroscopic methods (Weissenfels et al. 1991).

The bacterium *Sphingomonas paucimobilis* EPA505 reported by Mueller et al. (1990) was isolated from a seven member bacterial community. This culture was capable of utilizing fluoranthene as a sole source of carbon and energy. Use of a non-ionic surfactant Tween 80 (200 mg/L) increased the aqueous solubility of fluoranthene from 1 to 28 μM and decreased the generation time of the strain from 21 to 11 h. A resting cell suspension of EPA505 grown on fluoranthene was also capable of utilizing other four ring and five ring PAHs, such as, benzo(*b*)fluorene, benzo(*a*)anthracene, chrysene and pyrene.

In batch cultures with 1.66 mg/L fluoranthene, Kelley and Cerniglia (1991) reported that *Mycobacterium* sp. strain PYR-1 was capable of degrading greater than 78% fluoranthene within 5 days. When added to soil and water microcosms, *Mycobacterium* strain caused seven-fold increase in mineralization of fluoranthene over the indigenous microorganisms. Although mineralization of fluoranthene to CO_2 was rapid, some metabolites were identified: 8-hydroxy-7-methoxyfluoranthene, 9-hydroxyfluorene, 9-fluorenone, 9-fluorenone-1-carboxylic acid, 1-acenaphthenone, 9-hydroxy-1-fluorene carboxylic acid, phthalic acid, 2-carboxybenzaldehyde, benzoic acid, phenyl acetic acid, and adipic acid.

7.2.1.2 Degradation of Pyrene by Bacteria

The first report of pyrene degradation as sole source of carbon and energy was demonstrated for a *Rhodococcus* sp. strain UW1 (Walter et al. 1991). Within 2 weeks, 72% (initial concentration 500 mg/L) was mineralized to CO_2. A metabolite of molecular formula $C_{16}H_{10}O_4$ was identified. It was formed as a result of recyclization of the direct *meta*- fission product of pyrene. This strain could also utilize chrysene as sole source of carbon and energy.

Various *Mycobacterium* species have been reported for their pyrene degrading ability and the pathways have also been hypothesized based on identification of metabolites. Heitkamp and Cerniglia (1988) isolated a *Mycobacterium* sp. from sediments near a hydrocarbon source. This culture could mineralize pyrene when grown in mineral salts medium supplemented with organic nutrients. Pyrene induced culture mineralized over 60% of radiolabeled pyrene within 96 h. Enzymes responsible for pyrene catabolism seemed to be inducible, since a long lag phase in pyrene mineralization was observed in cultures grown in the absence of pyrene. In contrast, no pyrene mineralization was observed in non-induced culture. Seven metabolites of pyrene metabolism were detected by high-performance liquid chromatography. This included three ring oxidation products, *cis*-4,5-pyrene dihydrodiol, *trans*-4,5-pyrene dihydrodiol, and pyrenol. Four ring fission products were also observed, i.e., 4-hydroxyperinaphthenone, 4-phenanthroic acid, phthalic acid, and cinnamic acid. 4-phenanthroic acid was the major metabolite. Pyrenol was possibly formed by non-enzymatic dehydration of pyrene dihydrodiols or by oxidative metabolism of pyrene by the *Mycobacterium*. Multiple pathways for initial oxidative attack on pyrene was suggested since both *cis*- and *trans*-4,5-dihydrodiols were identified as metabolites. Further studies confirmed that both dioxygenase and monooxygenase enzymes were secreted by this microorganism. Sediment microcosms from where this strain was isolated showed enhanced mineralization of various PAHs, including pyrene and BaP when inoculated with the *Mycobacterium* strain (Heitkamp and Cerniglia 1989). Although this strain could utilize pyrene as primary substrate, pyrene degradation was inhibited by increasing the organic nutrients in the microcosm. Nutrient depletion was possibly caused by the utilization of organic nutrients by the rapidly growing indigenous microorganisms.

Later, other *Mycobacterium* species, i.e., *Mycobacterium* sp. strain RJGII-145 (Grosser et al. 1991), *Mycobacterium flavescens* (Dean-Ross and Cerniglia 1996) and *Mycobacterium* sp. strain KR2 (Rehmann et al. 1998) have been reported to utilize pyrene as a sole source of carbon and energy. *Mycobacterium* sp. strain RJGII-135 was isolated by Grosser et al. (1991) from soil near a coal gasification plant. It utilized pyrene as sole source of carbon and energy. When the pyrene induced culture was reintroduced in the soil containing pyrene, enhanced mineralization of pyrene was observed. Pyrene mineralization reached 55% within 2 days, compared to 1% for the indigenous population. Metabolites of pyrene degradation were also identified through further studies (Schneider et al. 1996). Three stable intermediates, 4-phenanthrene carboxylic acid, 4,5-pyrene dihydrodiol, 4,5-phenanthrene dicarboxylic acid were formed within 4–8 h after the start of the experiment.

Mycobacterium sp. strain BB1 was isolated from a former coal gasification site (Boldrin et al. 1993). Fluoranthene and pyrene were used as sole source of carbon and energy by this culture. It exhibited a maximum growth rate of 1.2 mg/L/h for large crystals of pyrene and 5.6 mg/L/h for small crystals during exponential growth in a 1.6 L fermenter with an initial concentration of 0.5 g/L of pyrene. This strain was used to examine the effects of various culture conditions, such as degradation of pyrene at low dissolved oxygen concentrations (Fritzsche 1994), utilization of PAHs in mixtures (Tiehm and Fritzsche 1995) and degradation of pyrene in presence of non-ionic surfactants. Jimenez and Bartha (1996) used a *Mycobacterium* sp. in solvent-augmented mineralization of pyrene. The cells, which physically adhered to solvent droplets, showed 8.5 times faster rate of pyrene degradation compared to cells in suspension. Another *Mycobacterium* sp. strain CH1 isolated from PAH contaminated freshwater sediments could mineralize pyrene and fluoranthene as the sole carbon and energy source. This culture was also capable of growth on a wide range of branched alkanes and n-alkanes (Churchill et al. 1999). *Gordona* sp. strain BP9 and *Mycobacterium* sp. strain VF1 were isolated from hydrocarbon-contaminated soil and each was capable of utilizing fluoranthene and pyrene as sole carbon and energy sources (Kastner et al. 1994). Reintroduction of BP9 into soil after growth on pyrene (200 mg/L) as sole source of carbon and energy exhibited six-fold increase in pyrene metabolism compared to native un-inoculated soil microorganisms (Kastner et al. 1998). The rate and extent of pyrene degradation by various pure cultures are listed in Table 7.2.

7.2.2 Limitations in Degradation of PAHs in Soil

Many microorganisms are metabolically capable of mineralizing or partially transforming PAHs in the environment. Some fungi can produce extracellular enzymes, but for most of the bacteria, the pollutants must cross the cell membrane to get direct access to the enzymes or at least attach to the membrane bound enzymes. With the exception of some bacterial species that can attach directly to the interface between the organic and aqueous phase, in general for degradation to

Table 7.2 Rate and extent of pyrene degradation by pure cultures

Bacterial strains	μ_{max} (1/h)	K_s	Y	Maximum biodegradation rate (mg/ml d)	% Degradation (Initial conc., mg/L)	Time (day)	Reference
Rhodococcus sp. UW1	ND	ND	ND	0.08	72% (500 mg/L)	14	Walter et al. (1991)
M. flavescens ATCC 700033	ND	ND	ND	0.806	38.8% (50 mg/L)	14	Dean-Ross and Cerniglia (1996)
Mycobacterium sp. KR2	ND	ND	ND	0.037	60% (500 mg/L)	8	Rehmann et al. (1998)
Pseudomonas sp. strain LP1	0.018	ND	ND	0.159	68% (100 mg/L)	30	Obayori et al. (2008)
P. aeruginosa LP5	0.024	ND	ND	0.118	67% (100 mg/L)	30	Obayori et al. (2008)
P. aeruginosa LP5	0.017	ND	ND	0.096	47% (100 mg/L)	30	Obayori et al. (2008)

take place, the contaminants must be solubilized in the aqueous phase. The two major mass transfer limitations are caused by: (a) compound availability and (b) crossing of the membrane.

7.2.2.1 Compound Availability

Bioavailability of a compound depends on its physico-chemical properties. Aqueous solubility plays the most important role in determining the potential for bioremediation. Solubility of PAHs is decreased with increase in the number of rings. Very low aqueous solubility and high hydrophobicity (high K_{ow}) are the two governing factors responsible for the recalcitrance of four and greater than four ring PAHs. Higher ring PAHs often exist in a separate phase, i.e., as components of non-aqueous phase liquids (NAPLs) or remain strongly sorbed on to soil. Mukherji and Weber (1998, 2001) demonstrated that mass transfer of naphthalene from a NAPL affected its biodegradation rate. However, some cultures are reported to overcome bioavailability limitations by virtue of enhanced cell surface hydrophobicity (CSH) as discussed later.

7.2.2.2 Crossing of Membrane

Another challenge is for PAHs to cross the cell membrane of the microorganism for gaining access to the enzymes present inside the cell. Crossing the membrane is required even for membrane bound enzymes since they are located on the inner side of the cell membrane. Both sides of the membrane are considered fairly polar due to

the presence of polar head groups on the phospholipids that comprise the membrane while the center region is non-polar in nature due to the fatty acid moieties of the phospholipids. In addition to the polarity obstacles, transport through the cell membrane is also affected by the membrane associated proteins and peptidoglycan. In gram-negative bacteria, an additional outer membrane needs to be crossed. For a contaminant to overcome all the cell barriers and access the enzymes inside the cell, it must either be selectively taken into the cell, or it must have the right combination of polarity, size, and functional groups to get across the cell membrane. Bressler and Gray (2003) surveyed the literature and determined the maximum biodegradation rate of various PAHs under aerobic condition. The maximum biodegradation rate was correlated with log K_{ow}. PAHs with log K_{ow} values above three demonstrated reduced biodegradation. This limitation is mainly due to the poor aqueous solubility and adsorption properties of these compounds, however, the repulsion of these highly non-polar compounds by the polar regions of the cell membrane cannot be ignored. Compounds with log K_{ow} values below one also demonstrated reduced rates of biodegradation possibly due to reduced ability of these extremely polar compounds to diffuse across the non-polar regions of the cell membrane.

Bressler and Gray (2003) derived an expression for maximum flux through the membrane (F_{max}) as a function of the octanol water partition coefficient (K_{ow}), molecular weight (MW) and aqueous solubility (C_{aq}) of a compound (Eq. 7.1) and computed the values for some known environmental contaminants. They assumed the membrane crossing step as the rate limiting step in biodegradation assuming that the intracellular concentration of a compound is maintained as zero by the active enzyme and demonstrated a linear relationship between the maximum reported biodegradation rate and the maximum membrane flux for various environmental contaminants and fitted a regression line using the data set.

$$F_{max} = 0.003\left(K_{ow}C_{aq}/\,MW^{0.5}\right) \tag{7.1}$$

They concluded that compounds with biodegradation rate falling significantly above the regression line (such as, toluene and phenol) are not transported by diffusion through the lipid bilayer. In contrast, those compounds having biodegradation rate falling significantly below the regression line are limited by the rate of enzymatic conversion rather than by membrane permeation. Biodegradation of fluorene, chrysene and several other PAHs falling on the regression line are limited by the membrane permeation. In contrast, the maximum biodegradation rate of the four ring PAHs, fluoranthene and pyrene was well below the predicted line. Thus, biodegradation of pyrene and fluoranthene is limited by the synthesis of key enzymes.

7.2.3 Surfactant Enhanced Biodegradation of HMW PAHs

Contradictory and inconclusive results have been observed regarding the effects of surfactants on the biodegradation of PAHs at surfactant concentration below and above the critical micelle concentration (CMC) (Margesin

and Schinner 1999). Some studies showed an enhanced effect in which not only LMW PAHs were degraded successfully, but even compounds containing four and more than four rings were also degraded effectively. In contrast, in other studies, degradation was found to be inhibited. While some researchers believe that PAHs solubilized within surfactant micelles are completely bioavailable, yet others believe that they are only partially bioavailable or not at all bioavailable (Guha and Jaffe 1996; Luning Prak and Pritchard 2002). Volkering et al. (1995) reported a strong inhibition in biodegradation of polynuclear aromatic hydrocarbons (PAHs) in the presence of surfactants above their CMC. Surfactants may also directly interact with the bacterial cell surfaces, the interaction being dependent both on the type of surfactant and the type of microorganisms. Efroymson and Alexander (1991) reported that Triton X-100 prevented the adherence of cells to an organic-aqueous interface, however, this had a beneficial effect on naphthalene biodegradation by *Arthrobacter* sp. Since naphthalene has relatively high aqueous solubility, it was available both from the aqueous phase as well as from the organic-water interface. Hindered adherence may have adversely affected the uptake of HMW PAHs with very low aqueous solubility. However, Triton X-100 is also reported to promote the attachment of *Burkholderia* cultures to the NAPL-water interface (Mohanty 2010). Some surfactants are toxic to bacterial cultures while enhanced solubilization of toxic components from a multi-component NAPL phase such as petroleum caused by the surfactants, may adversely affect bacterial cultures.

Thibault et al. (1996) used two pyrene degrading soil *Pseudomonas* species in soil reinoculation experiments to test the effects of four surfactants, namely, Simple Green, Biosolve, Witconol SN70 and Sodium dodecyl sulphate (SDS). Witconol SN70 caused maximum enhancement in solubilization and mineralization of pyrene under unsaturated conditions. However, when pyrene degraders were inoculated into soil slurries, degradation of pyrene was highest in the absence of surfactants. It was concluded that increased solubilization of pyrene in the soil slurry, caused toxicity to the microorganisms.

Boonchan et al. (1998) evaluated various surfactants for their impact in improving the biodegradation of four, five and seven ring PAHs by *Strenotrophomonas maltophilia* VUN 10,010, isolated from manufactured gas plant site soil. All the cationic and anionic surfactants tested were found to be highly toxic for the bacterial strains while the Tween series was utilized as growth substrate. Five non-ionic surfactants (Brij 35, Igepal CA-630, Triton X-100, Tergitol NP-10, and Tyloxapol) increased the apparent solubility of fluoranthene, pyrene and B(a)P at least 250 fold at 10 g/L surfactant concentration. They were less toxic and were not used as growth substrates. Pyrene and also the five and seven ring PAHs were utilized by the culture as sole source of carbon and energy. Not only the solubility, but also the rate of pyrene degradation by strain VUN 10,010 was enhanced by the addition of the four non-ionic surfactants (5–10 g/L). However, an inhibition in degradation rate was observed with Igepal CA-630 (5 g/L). The specific growth rate of VUN 10,010 on pyrene was increased by 67% in the presence of 10 g/L

Brij 35 or Tergitol NP-10. The addition of Brij 35 and Tergitol NP-10 to media containing a single HMW PAH (four and five benzene rings) as the sole carbon source increased the maximum specific PAH degradation rate and decreased the lag period normally seen for PAH degradation. The addition of Tergitol NP-10 to VUN 10,010 cultures which contained a PAH mixture (three to seven benzene rings) substantially improved the overall degradation rate of each PAH and increased the specific growth rate of VUN 10,010 by 30%. Their results suggested that besides the improvement in dissolution rate, surfactants may also facilitate the transport of PAHs through the cell membrane.

Doong and Lei (2003) observed a correlation between the polyoxyethylene chain length and the solubilization efficiency of surfactants. Four non-ionic surfactants and one anionic surfactant were found to enhance the bioavailability of naphthalene, phenanthrene and pyrene with efficiencies ranging from 21.1 to 60.6, 33.3 to 62.8 and 26.8 to 70.9%, respectively. The trend in degradation efficiency was as follows: Brij 30 > Triton X-100 > Tween 80 > Brij 35. The HLB values of Brij 30, Triton X-100, Tween 80 and Brij 35 were 9.7, 13.5, 15.0 and 16.9, respectively. With increase in HLB value, the degradation potential of the surfactant was decreased. Triton X-100 and Brij 30 were utilized as sole source of carbon and energy by *Pseudomonas putida*. Although the dissolution of PAHs was increased, Brij 35 and Tween 80 inhibited the growth of *P. putida*. Increase in surfactant concentration also decreased the mineralization rate of pyrene possibly due to mass transfer limitation from the micelle. Mass transfer processes are proportional to concentration gradient of PAHs inside the micelle so that when the PAHs are diluted in a larger micellar mass, transfer to the cells may decrease. Another possibility may be catabolic repression due to preferential utilization of the surfactant.

Willumsen and Arvin (1999) studied the impact of Triton X-100 on fluoranthene degradation by *S. paucimobilis* EPA505. Although the surfactant enhanced mineralization of fluoranthene, it was much lower compared to the increase in solubilization. A model which described the degradation kinetics of Triton X-100 solubilized fluoranthene by *S. paucimobilis* EPA505 cells was also developed after accounting for micellar solubilization, metabolite accumulation and its bioavailability considerations. In the presence of Triton X-100 and calcium, the mineralization rate of fluoranthene by *S. paucimobilis* EPA505 was almost doubled (Willumsen and Karlson 1998). In contrast, absence of calcium inhibited fluoranthene mineralization possibly due to the adverse effect of Triton X-100 on the cytoplasmic membrane.

In a study involving the Tween series surfactants (Tween 20, Tween 40, Tween 60 and Tween 80), Kim and Weber (2003) reported enhanced solubilization of phenanthrene at different doses of surfactant above their CMC. However, the micelle solubilized phenanthrene was not bioavailable to *S. paucimobilis* EPA 505. Preferential microbial uptake of the hydrophobic part of the chemical surfactant caused destabilization of the micelles due to an imbalance in the amphiphilic molecule. Thus, phenanthrene was released from the micellar core such that its biodegradation was increased.

7.3 Bacterial Mechanisms for Overcoming Bioavailability Limitation

It is generally believed that most bacteria can access and degrade pollutants only when they are dissolved in water. However, some PAH-degrading bacteria are reported to overcome the bioavailability limitations for enhancing uptake through (1) direct interfacial uptake and (2) uptake facilitated by secretion of biosurfactants.

7.3.1 Direct Interfacial Uptake

In direct interfacial uptake, bacteria attach itself to the surface of the NAPL droplets which are larger in size compared to the bacterial cell. Substrate uptake takes place at the point of contact through diffusion. CSH plays an important role in direct uptake. Microorganisms capable of direct uptake can modify their cell surface outer membrane to enhance CSH. In this case, substrate uptake does not depend on the dissolution rate. Some microorganisms have surface structures such as, fimbriae and capsules which help them to attach to the NAPL-water interface such that they can directly uptake substrate from a separate phase.

CSH is an important parameter affecting the adhesion behavior of bacterial cells to surfaces and interfaces. CSH is commonly measured by determining the contact angle (Busscher et al. 1984) of a uniform layer of cells. Some other assays for hydrophobicity include hydrophobic interaction chromatography (Dillon et al. 1986), salt aggregation test (Lindahl et al. 1981), polystyrene microsphere attachment (Rosenberg 1981) and microbial attachment to microsphere (Zita and Hermansson 1997). The bacterial adhesion to hydrocarbons (BATH) assay (Rosenberg et al. 1980) is also used for determining the potential of a culture for direct interfacial uptake.

BATH assay is a simple and quantitative method for determining the degree of adherence of bacterial cells to a specific liquid hydrocarbon, i.e., n-hexadecane when an aqueous suspension of bacterial cells is mixed with the hydrocarbon. Adherence is directly related to change in absorbance of the aqueous suspension with respect to the control. However, BATH assay results are affected by solution phase interaction between the NAPL and bacterial culture, hence it is not a true measure of CSH. Bacterial cell surface charge is reported to vary with pH, growth medium, culture age and ionic strength. At a certain pH, the maximum adherence occurs when either the bacterial culture or the NAPL droplet is uncharged. Busscher et al. (1995) reported the zeta potential of various solvents such as, n-hexadecane, toluene and xylene ranges from -63 to -18 mV at pH 7. Chakraborty et al. (2010) demonstrated the effect of NAPLs of varying composition serving as growth substrate on adherence measured in BATH assay and surface charge of three bacterial strains *Burkholderia cepacia* (ES1) and

Burkholderia multivorans (NG1 and HN1). All the cultures used depicted growth on the NAPLs composed of n-hexadecane, naphthalene, phenanthrene and pyrene in varying proportion. Change in NAPL used as growth substrate affected both the zeta potential and CSH of the cultures (based on contact angle measurements), which in turn affected their adherence to the NAPL-water interface determined using the BATH assay. Zeta potential was found to be close to zero at pH 2 for all the cultures. For each culture a strong inverse correlation was observed between zeta potential and adherence to n-hexadecane. Thus, BATH assay results are the outcome of both hydrophobic and electrostatic interaction between the bacterial culture and NAPL phase.

Some bacterial cultures are capable of inducing high CSH when only hydrocarbon/oil is provided as sole substrate. This enhancement in hydrophobicity is reported to facilitate the direct interfacial uptake of hydrocarbons from NAPLs and the uptake of solid hydrocarbons. Most PAHs other than naphthalene and substituted naphthalenes have low aqueous solubility of the order of 1 mg/L or less so that direct interfacial uptake may provide a distinct advantage to the microorganism utilizing it as a substrate. Induction of CSH has been demonstrated by various researchers by growing cultures both in the presence of a readily degradable soluble substrate and in the presence of hydrocarbons/oil. Subsequently, the CSH is determined through contact angle measurements or BATH assay. *Pseudomonas* sp. strain PP2 grown on phenanthrene depicted greater adherence to aliphatic (hexane and heptane) and aromatic hydrocarbons (benzene and xylene) compared to the dextrose grown cells (Prabhu and Phale 2003). These changes were attributed to growth associated with production of an extracellular biosurfactant. *Mycobacterium* sp. LB501T is reported to form a biofilm on solid anthracene provided as sole substrate (Wick et al. 2002). Such a biofilm formation was not observed when glucose was provided as a co-substrate along with anthracene. This was attributed to surface modifications that caused enhancement in hydrophobicity of anthracene grown cells. In adhesion experiments, anthracene grown cells demonstrated 1.5–8.0 fold greater adherence to Teflon and up to 70 times greater adherence to anthracene surfaces compared to glucose grown cells (Wick et al. 2002). This activity was linked to specific changes in the phospholipid fatty acid (PLFA) and glycolipid fatty acid (GLFA) patterns (Wick et al. 2003). In another study, mineralization of fluoranthene by a *Mycobacterium* strain was facilitated by direct attachment to fluoranthene provided as sole substrate (Willumsen and Karlson 2001). High CSH and adherence to emulsified solvent droplets containing dissolved pyrene facilitated its mass transfer to the degrading bacteria, during solvent-augmented biodegradation by a *Mycobacterium* sp. (Jimenez and Bartha 1996).

Similar enhancement in hydrophobicity and attachment to NAPLs has been reported for uptake of aliphatic hydrocarbons from NAPLs, such as n-hexadecane, diesel and petroleum (Amin et al. 1996; Mohanty and Mukherji 2008). Thus, cultures can actively regulate their CSH as a strategy for optimizing the uptake of substrates characterized by low aqueous solubility. In addition to extracellular biosurfactants, the extracellular polymeric substances (EPS) associated with

bacterial cells may play a role in bioavailability enhancement and enhanced attachment to NAPLs. The role of EPS in affecting attachment to NAPLs was implicated for an alkane degrading *Rhodococcus* strain Q15 (Whyte et al. 1999). For *Rhodococcus* sp. strain 094, a good correlation was observed between CSH, alkane oxidation and EPS activity (Berdholt et al. 2002). EPS release in the stationary phase was correlated with decrease in hydrophobicity and emulsification of the culture broth. Membrane bound vesicles with some emulsifying activity were responsible for CSH in *Acinetobacter venetianus* RAG-1 and *Acinetobacter* sp. strain H01-N (Leahy et al. 2003). Mohanty and Mukherji (2008) demonstrated the effect of growth substrates by conducting the BATH assay using two oil degrading bacterial cultures *B. cepacia* ES1 and *Exiguobacterium aurantiacum* AS1 with two different NAPL types, i.e., diesel and n-hexadecane. For both the cultures, adherence to a NAPL phase was highest when the corresponding NAPL was used for culture growth. For dextrose grown cultures of *B. cepacia* and *E. aurantiacum*, negligible adherence to NAPL phase was observed irrespective of the NAPL phase used in the assay.

7.3.2 Production of Biosurfactants and PAH Uptake

Production of biosurfactant is another mechanism adopted by certain bacterial species to degrade complex petroleum hydrocarbons. Like chemical surfactants, biosurfactants are amphiphilic molecules having both hydrophilic and hydrophobic regions which help them in attachment to different surfaces. Biosurfactants are categorized into groups based on their MW (Ron and Rosenberg 2002). LMW surfactants are typically glycolipids where the carbohydrate group is attached to long chain alkyl acids or lipoproteins. Rhamnolipids, trehalolipids and sophorolipids are a few known biosurfactants that come under this category. Several species of *Pseudomonas* are reported to produce rhamnolipids. External addition of rhamnolipids was reported to have varying impacts on CSH of bacterial cultures degrading phenanthrene, i.e., *Bacillus subtilis* BUM and *P. aeruginosa* P-CG3, which, in turn, was found to alter the relative contribution of the two cultures in phenanthrene biodegradation (Zhao et al. 2011). External addition of a sophorolipid mixture produced by *Candida bombicola* has been shown to enhance phenanthrene biodegradation by *Pseudomonas yanoikuyae* through enhanced solubilization (Schippers et al. 2000).

HMW biosurfactants are composed of polysaccharides, proteins, lipopolysaccharides, lipoproteins or a mixture of these polymers. They are less effective in reducing interfacial tension compared to LMW biosurfactants. However, they can create a hydrophilic coat around the oil droplets which inhibits coalescence. The most studied are surfactants produced by different species of the genera *Acienetobacter*. Emulsan produced by *Acinetobacter* RAG-1 and alasan produced by *Acinetobacter radioresistens* are a complex mixture of heteropolysaccharides and proteins. One of the protein moieties of 45 kDa in alasan has

been studied extensively and reported to be the most important structural component determining the activity of the complex. It contributed to the formation of stable oil–water emulsion (Navon-Venezia et al. 1998; Toren et al. 2001). Only 500 μg/ml of alasan increased the aqueous solubility of phenanthrene, fluoranthene and pyrene by 6–25 times. The presence of alasan more than doubled the mineralization rate of the PAHs by *S. paucimobilis* EPA 505 (Barkay et al. 1999).

Biosurfactants are usually produced during the stationary growth phase and this is induced by molecular signals involved in quorum sensing (Ron and Rosenberg 2002). Emulsification of oil by biosurfactants increases bioavailability by increasing the surface area. Biosurfactants are also reported to cause adhesion/detachment of bacteria by regulating their CSH. Biosurfactants may be cell bound or may be released into the medium. Cell bound biosurfactants may be bound to the hydrophobic region in the outer layer of the cell surface thereby exposing the hydrophilic part to the outside and thus the bacteria will be able to interact with hydrophilic surfaces but not hydrophobic surfaces (Nue 1996). The reverse orientation is also possible, so that the surfactant is bound through the hydrophilic part to the cell surface thereby exposing the hydrophobic part to the environment. This would allow the bacteria to interact with hydrophobic surfaces only. CSH of *Pseudomonas aeruginosa* was increased by the presence of cell bound biosurfactant rhamnolipids whereas, for *Acinetobacter* strain, reduced CSH has been reported due to presence of cell bound bioemulsifier (Nue 1996).

Deziel et al. (1996) first reported the production of rhamnolipid biosurfactant by *P. aeruginosa* 19 SJ for facilitating growth on the PAHs, naphthalene and phenanthrene. Maximum extracellular biosurfactant production was observed at the onset of the stationary phase when high cell density limited the availability of the substrate. Production of the biosurfactant enhanced solubilization of the substrate. Prabhu and Phale (2003) also indicated the role of extracellular biosurfactants in phenanthrene biodegradation. Bordoloi and Konwar (2009) reported biosurfactant production by various strains of *P. aeruginosa* isolated from petroleum contaminated soil in Assam, India. All the strains were capable of degrading the PAHs, fluorene, phenanthrene and pyrene as sole substrate. The biosurfactants produced by the various strains lowered the surface tension up to 30–32 mN/m and was characterized by CMC in the range 100–110 mg/L. Differences were observed in the nature of the various biosurfactants. The lipopeptide biosurfactant produced by strains MTCC 7815 and 7812 depicted the best pyrene solubilizing activity. Strain MTCC 8163 produced a biosurfactant that was proteino-starchy in nature, while strains MTCC 8165 and MTCC 7814 produced structurally complex biosurfactants comprising of protein, carbohydrates and lipids. Enhanced solubilization by the biosurfactants was linked to sustained growth on the PAHs. *B. subtilis* DM04 and a muccoidal (M) and non-muccoidal (NM) strain of *P. aeruginosa* isolated from petroleum contaminated soil in north-eastern India were reported to degrade pyrene as sole carbon and energy source with the help of biosurfactants that enhanced solubilization (Das and Mukherjee 2007).

7.4 Degradation of HMW PAHs and Substrate Interaction Effects

In the environment, PAHs exist as complex mixtures along with a multitude of other compounds, such as, in creosote and coal tar. In this scenario, one compound may affect the rate and extent of degradation of another compound, e.g., through increasing the biomass growth, competitive inhibition and co metabolism. These effects are referred as substrate interaction effects. As observed by various researchers, the net result could be an increase in the rate of biodegradation as a result of enhanced biomass growth (Beckles et al. 1998), a decrease in biodegradation rate due to competitive inhibition or hindrance in enzyme induction (Guha et al. 1999; Molina et al. 1999), or no noticeable effect because the opposing effects cancel out (Beckles et al. 1998; Guha et al. 1999). Degradation of a difficult to degrade substance may be enhanced due to enhancement in the biomass population caused by an easily degradable substrate. The interactions and effects encountered in a multi-substrate system are a function of the microbial community, the type of culture (mixed versus pure) and the physiological state of the community. In general, the biodegradation rates of the more degradable and abundant compounds are reduced due to competitive inhibition, while enhanced biodegradation occurs for the recalcitrant PAHs due to simultaneous increase in biomass growth on multiple substrates. Studies by Guha et al. (1999), using naphthalene, phenanthrene and pyrene as sole substrate, and in binary and ternary mixtures revealed that in ternary mixture, biodegradation of naphthalene was inhibited while the biodegradation rates of phenanthrene and pyrene were enhanced. Knightes and Peters (2006) studied the interactions in binary mixtures and in a complex mixture using nine PAHs, i.e., naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, 2-ethylnaphthalene, phenanthrene, anthracene, pyrene, fluorene and fluoranthene. They reported both positive and negative interactions between the substrates in these multi-substrate systems.

Competitive inhibition, i.e., retardation in rates of degradation of a substrate may be caused as a result of competition for the same enzyme by other substrates. For example, *cis*-naphthalene dihydrodiol dehydrogenase has a broad substrate specificity and oxidizes *cis*-dihydrodiols of many PAHs beside naphthalene so that a competitive inhibition is observed in naphthalene biodegradation. Such negative effects due to competitive inhibition may be observed where at least one step in the multistep transformation of a PAH occurs through a common enzyme. Biodegradation pathways for a wide range of PAHs are similar, and a dioxygenase enzyme is involved in the initial step of aerobic biodegradation. Thus, competitive inhibition is quite prevalent. The degradation of phenanthrene by *Pseudomonas stutzeri* P-16 and *P. saccharophila* P-15 isolated from a creosote contaminated soil was inhibited by naphthalene, 1-methylnaphthalene, 2-methylnaphthalene and fluorene. These cultures could also utilize naphthalene, 1-methylnaphthalene and 2-methylnaphthalene as sole substrate but were unable to utilize fluorene (Stringfellow and Aitken 1995). Bouchez et al. (1995)

observed that a *Pseudomonas* sp. strain S Ant Mu5 degraded anthracene to a greater extent when it is supplied alone compared to when it is supplied in a mixture with fluorene.

Positive effects and enhanced degradation of substrate may occur as a result of proliferation of biomass on multiple substrates. Since degradation rate is a function of the biomass concentration, the biodegradation rate for each compound in a mixture may be increased if common bacterial cultures are responsible for their utilization. The presence of a suitable PAH substrate or pre-exposure to suitable PAH can facilitate the degradation of other PAHs through enzyme induction. Bauer and Capone (1988) observed enhanced degradation of PAHs as a result of pre-exposure to other aromatic hydrocarbons. Naphthalene and phenanthrene enhanced the degradation of various other PAHs through cross acclimation (Bauer and Capone 1988). Exposure to certain compounds may block enzyme induction. Such malefic association has been reported for a pyrene degrading *Rhodococcus* strain S Pyr Na 1 in the presence of either phenanthrene or fluoranthene (Bouchez et al. 1995). In another study, phenanthrene degradation by several strains was found to decrease due to lack of pre-exposure to specific PAHs (Molina et al. 1999). McLellan et al. (2002) reported that the addition of phenanthrene as a second substrate could increase benzo[a]pyrene metabolism by *Mycobacterium* sp. strain RGJII-135 up to six-fold. Pyrene and phenanthrene were potent inducers of benzo[a]pyrene degradation activity.

Co-metabolism of a non-growth substrate can occur in the presence of a growth substrate, however, the products of co-metabolic transformation may hinder degradation of the growth substrate. Partial co-metabolic transformation of HMW PAHs can yield more water soluble intermediates which may be degraded by other bacterial strains. PAH-degrading bacteria, *Burkholderia* sp. VUN10013 was found to utilize and degrade low MW PAHs, such as phenanthrene and anthracene, but not the HMW PAHs, pyrene, fluoranthene, chrysene and benzo[a]pyrene (Somtrakoon et al. 2008). However, when present in a mixture with phenanthrene or anthracene, the HMW PAHs, pyrene and fluoranthene could be co metabolically degraded by this strain. Phenanthrene was better at enhancing the transformation of the non-growth substrates compared to anthracene. Presence of the HMW PAHs adversely affected the degradation of anthracene, but not that of phenanthrene. Bouchez et al. (1995) investigated the degradation of PAHs in binary mixtures using six bacterial strains, including two *Rhodococcus* spp. capable of growth on pyrene and fluoranthene. All individual strains were capable of transforming the PAHs co-metabolically and both inhibition and synergistic interactions were observed. The ability of *Pseudomonas* sp. strain S Phe Na l to degrade phenanthrene was partially inhibited by the transformation products formed by fluorene co metabolism. Moreover, the presence of fluoranthene as a second PAH slightly decreased (92%) the degradation of phenanthrene by *Pseudomonas* sp. strain S Phe Na l. Inhibition was most commonly observed when the added PAH was more water soluble than the PAH added originally. It was also observed that mineralization yields were higher and biomass yields were lower for HMW PAH-degrading bacteria than for LMW PAH-degrading bacteria.

Luning Prak and Pritchard (2002) reported the degradation of mixtures of PAHs, pyrene, fluoranthene and phenanthrene by *S. paucimobilis* EPA 505 in the presence of the surfactant, Tween 80. This strain could utilize both phenanthrene and fluoranthene as growth substrates, but could not utilize pyrene. In mixtures, phenanthrene was preferentially degraded over fluoranthene, and preference for pyrene was the least. Competing substrates hindered degradation, however, after degradation of the competing substrate, the other PAHs were degraded at rate higher than or comparable to the single-substrate system.

7.5 Degradation of PAHs in Presence of Other Hydrocarbons

The effect of the presence of aliphatics on the rate and extent of degradation of PAHs has not been studied extensively. Ghosh et al. (2010) demonstrated negligible degradation of pyrene present in a NAPL along with other constituents, i.e., n-alkanes and LMW PAHs although the *Sphingomonas* sp. used was grown on pyrene provided as sole source of carbon and energy. Zytner et al. (2006) used a synthetic diesel containing the aliphatics: dodecane, tetradecane, heptadecane, pristane, octadecane and eicosane together contributing to 90%. The remaining 10% was composed of naphthalene and phenanthrene. Biodegradation rate was studied in 1-L bioreactors containing soil spiked with the synthetic diesel/individual compounds. Degradation by heterotrophic microorganisms indigenous to the Elora silt loam soil was studied and the fraction of contaminants remaining was analyzed over time by GC-FID over a period of 30 days. The degradation trend observed for the synthetic diesel fuel was: C12 > naphthalene > C17 > C19 > C20 > phenanthrene. The order changed when the compounds were degraded individually: C17, C19, C20, C12 > phenanthrene > naphthalene. The amount degraded was also changed in the mixture compared to individual compound. For example, phenanthrene degradation decreased to 9% in the synthetic diesel mixture compared to 26% when present individually. This difference may be due to the competitive inhibition.

7.6 Conclusions

Bacterial degradation of HMW PAHs with more than four rings has been conclusively demonstrated in-spite of their structural complexity and low aqueous solubility. Pyrene, fluoranthene and other HMW PAHs can serve as sole source of carbon and energy for naturally occurring microorganisms. The degradation pathways have been inferred for various organisms through identification of metabolites. The degradation rates are often found to be low due to either bioavailability limitations or membrane flux limitations. In engineered systems, the bioavailability limitations may be overcome by the addition of surfactants, however, chemical surfactants are also reported to cause adverse effects on

biodegradation. Bacteria degrading HMW PAHs are often found to induce high CSH that promotes adherence to solid PAHs, NAPLs or sorbed PAHs. Alternatively, some bacteria gain a selective advantage by secretion of biosurfactants. Biosurfactants are reported to increase uptake through enhancement in solubilization, emulsification and also by inducing changes in CSH. The presence of HMW PAHs along with other easily degradable PAHs and other hydrocarbons (i.e., n-alkanes) may adversely affect their degradation due to competitive inhibition. However, beneficial effects such as, cometabolic degradation of HMW PAHs are also reported. Bioavailability limitations together with adverse substrate interaction effects may be largely responsible for the persistence of HMW PAHs in the environment.

Acknowledgements The authors will like to acknowledge Department of Science and Technology, New Delhi, India for providing funding for the project "Surfactant aided biodegradation of model NAPLs" which has given us practical insights on PAH biodegradation.

References

Amin M, El-Dien A, Hosny MS, El-Mohammady Y (1996) Involvement of bacterial cell surface hydrophobicity in the biodegradation of petroleum hydrocarbons. J Microbiol 31:1–12

Bamforth SM, Singleton I (2005) Bioremediation of polycyclic aromatic hydrocarbons: current knowledge and future directions. J Chem Technol Biotechnol 80:723–736

Barkay T, Navon-Venezia S, Ron EZ, Rosenberg E (1999) Enhancement of solubilization and biodegradation of polyaromatic hydrocarbons by the bioemulsifier alasan. Appl Environ Microbiol 65(6):2697–2702

Bauer JE, Capone DG (1988) Effects of co-occurring aromatic hydrocarbons on degradation of individual aromatic hydrocarbons in marine sediment slurries. Appl Environ Microbiol 54:1649–1655

Beckles DM, Ward CH, Hughes JB (1998) Effects of mixtures of PAHs and sediments on fluoranthene biodegradation patterns. Environ Toxicol Chem 17:1246–1251

Berdholt H, Bruheim P, Potocky M, Eimhjellen K (2002) Hydrophobicity development, alkane oxidation, and crude-oil emulsification in a *Rhodococcus* species. Can J Microbiol 48:295–304

Boldrin B, Tiehm A, Fritzche C (1993) Degradation of phenanthrene, fluorene, fluoranthene, and pyrene by a *Mycobacterium* sp. Appl Environ Microbiol 59:1927–1930

Boonchan S, Britz MS, Stanley GL (1998) Surfactant-enhanced biodegradation of high molecular weight polycyclic aromatic hydrocarbons by *Stenotrophomonas maltophilia*. Biotechnol Bioeng 59(4):482–494

Bordoloi NK, Konwar BK (2009) Bacterial biosurfactant in enhancing solubility and metabolism of petroleum hydrocarbons. J Hazard Mater 170(1):495–505

Bouchez MD, Blanchet VP, Vandecasteele (1995) Degradation of polycyclic aromatic hydrocarbons by pure strains and by defined strain associations: inhibition phenomena and cometabolism. Appl Microbiol Biotechnol 43:156–164

Bressler DC, Gray MR (2003) Transport and reaction processes in bioremediation of organic contaminants. Int J Chem React Eng 1:1–16

Busscher HJ, Weerkamp AH, van der Mei HC, van Pilt AWJ, de Jong HP, Arends J (1984) Measurement of the surface free energy of bacterial cell surfaces and its relevance for adhesion. Appl Environ Microbiol 48:980–993

Busscher HJ, van der Belt-Gritter B, van der Mei HC (1995) Implications of microbial adhesion to hydrocarbons for evaluating cell surface hydrophobicity 1. Zeta potential of hydrocarbon droplets. Colloids Surf B 5:111–116

Cerniglia CE (1992) Biodegradation of PAHs. Biodegradation 3:351–368
Chakraborty S, Mukherji S, Mukherji S (2010) Surface hydrophobicity of petroleum hydrocarbon degrading *Burkholderia* strains and their interactions with NAPLs and surfaces. Colloids Surf B 78:101–108
Churchill SA, Harper JP, Churchill PF (1999) Isolation and characterization of a *Mycobacterium* species capable of degrading three and four-ring aromatic and aliphatic hydrocarbons. Appl Environ Microbiol 65:549–552
Das K, Mukherjee AK (2007) Differential utilization of pyrene as the sole source of carbon by *Bacillus subtilis* and *Pseudomonas aeruginosa* strains: role of biosurfactants in enhancing bioavailability. J Appl Microbiol 102(1):195–203
Dean-Ross D, Cerniglia CE (1996) Degradation of pyrene by *Mycobacterium flavescens*. Appl Microbiol Biotechnol 46:307–312
Deziel E, Paquette G, Villemur R, Lepine F, Bisaillon J (1996) Biosurfactant production by a soil *Pseudomonas* strain growing on polycyclic aromatic hydrocarbons. Appl Environ Microbiol 62(6):1908–1912
Dillon JK, Fuerst JA, Hayward AC, Davis GHG (1986) A comparison of five methods for assaying bacterial hydrophobicity. J Microbiol Methods 6:13–19
Doong R, Lei W (2003) Solubilization and mineralization of polycyclic aromatic hydrocarbons by *Pseudomonas putida* in the presence of surfactant. J Hazard Mater B96:15–27
Efroymson RA, Alexander M (1991) Biodegradation by an *Arthrobacter* species of hydrocarbons partitioned into an organic solvent. Appl Environ Microbiol 57(5):1441–1447
Fritzsche C (1994) Degradation of pyrene at low defined oxygen concentrations by a *Mycobacterium* sp. Appl Environ Microbiol 60:1687–1689
Ghosh I, Mohanty S, Mukherji S (2010) Comparative study on degradation of a model NAPL by *Sphingomonas* and *Burkholderia* sp. and impact of triton-X 100. In: Proceedings of 9th international conference on hydroscience and engineering (ICHE 2010), IIT, Madras, 2–5 Aug 2010
Grosser RJ, Warshawsky D, Vestal JR (1991) Indigenous and enhanced mineralization of pyrene, benzo[*a*]pyrene, and carbazole in soils. Appl Environ Microbiol 57:3462–3469
Guha S, Jaffe PR (1996) Bioavailability of hydrophobic compounds partitioned into the micellar phase of nonionic surfactants. Environ Sci Technol 30:1382–1391
Guha S, Peters CA, Jaffe PR (1999) Multisubstrate biodegradation kinetics of naphthalene, phenanthrene, and pyrene mixtures. Biotechnol Bioeng 65:491–499
Harvey RG (1991) Polycyclic aromatic hydrocarbons: chemistry and carcinogenicity. Cambridge University Press, Cambridge
Heitkamp MA, Cerniglia CE (1988) Mineralization of polycyclic aromatic hydrocarbons by a bacterium isolated from sediment below an oil field. Appl Environ Microbiol 54: 1612–1614
Heitkamp MA, Cerniglia CE (1989) Polycyclic aromatic hydrocarbon degradation by a *Mycobacterium* sp. in microcosms containing sediment and water from a pristine ecosystem. Appl Environ Microbiol 55:1968–1973
IARC Monograph (1983, 1998) Evaluation of the carcinogenic risk of chemicals to humans: Polynuclear aromatic compounds, Part I, Vol 32. http://monographs.iarc.fr/ENG/Monographs/allmonos49.php
Jimenez I, Bartha R (1996) Solvent-augmented mineralization of pyrene by a *Mycobacterium* sp. Appl Environ Microbiol 62:2311–2316
Juhasz AL, Britz ML, Stanley GA (2000) Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: a review of the microbial degradation of benzo[a]pyrene. Int Biodeter Biodegrad 45:57–88
Kanaly RA, Harayama S (2000) Biodegradation of high molecular-weight PAHs by bacteria. J Bacteriol 182:2059–2067
Kastner M, Breuer-Jammali M, Mahro B (1994) Enumeration and characterization of the soil microflora from hydrocarbon-contaminated soil sites able to mineralize polycyclic aromatic hydrocarbons (PAH). Appl Microbiol Biotechnol 41:267–273

Kastner M, Breuer-Jammali M, Mahro B (1998) Impact of inoculation protocols, salinity, and pH on the degradation of polycyclic aromatic hydrocarbons (PAHs) and survival of PAH-degrading bacteria introduced into soil. Appl Microbiol Biotechnol 64:359–362

Kelley I, Cerniglia CE (1991) The metabolism of fluoranthene by a species of *Mycobacterium*. J Ind Microbiol 7:19–26

Kim HS, Weber WJ Jr (2003) Preferential surfactant utilization by a PAH-degrading strain: effects on micellar solubilization phenomena. Environ Sci Technol 37:3574–3580

Knightes CD, Peters CA (2006) Multisubstrate biodegradation kinetics for binary and complex mixtures of polycyclic aromatic hydrocarbons. Environ Toxicol Chem 25(7):1746–1756

Leahy JG, Khalid ZM, Quintero EJ, Jones-Meehan JM, Heidelberg JF, Batchelor PJ, Colwell RR (2003) The concentrations of hexadecane and inorganic nutrients modulate the production of extracellular membrane-bound vesicles, soluble protein, and bioemulsifier by *Acinetobacter venetianus* RAG-1 and *Acinetobacter* sp. strain HO1-N. Can J Microbiol 49:569–575

Lindahl M, Faris A, Wadstrom T, Hjerten S (1981) A new test based on 'salting out' to measure relative surface hydrophobicity of bacterial cells. Biochim Biophys Acta 677:471–476

Luning Prak DJ, Pritchard PH (2002) Degradation of polycyclic aromatic hydrocarbons dissolved in tween 80 surfactant solutions by *Sphingomonas paucimobilis* EPA 505. Can J Microbiol 48:151–158

Margesin R, Schinner F (1999) Biodegradation of diesel oil by cold-adapted microorganisms in presence of sodium dodecyl sulphate. Chemosphere 38(15):3463–3472

McLellan SL, Warshawsky D, Shann JR (2002) The effect of polycyclic aromatic hydrocarbons on the degradation of benzo[*a*]pyrene by *Mycobacterium* sp. strain RJGII-135. Environ Toxicol Chem 21:253–259

Mohanty S (2010) Surfactant-aided biodegradation of petroleum hydrocarbons from non-aqueous phase liquids by *Burkholderia* sps. Ph.D. Thesis, IIT Bombay, Mumbai, India

Mohanty G, Mukherji S (2008) Enhancement of NAPL bioavailability by induction of cell-surface hydrophobicity in *Exiguobacterium aurantiacum* and *Burkholderia cepacia*. Ind J Biotechnol 7(3):295–306

Molina MR, Araujo RE, Hodson (1999) Cross-induction of pyrene and phenanthrene in a *Mycobacterium* sp. isolated from PAH contaminated river sediments. Can J Microbiol 45:520–529

Mrozik A, Seget ZP, Labuzek S (2003) Bacterial degradation and bioremediation of polycyclic aromatic hydrocarbons. Pol J Environ Stud 12(1):15–25

Mueller JG, Chapman PJ, Blattmann BO, Pritchard PH (1990) Isolation and characterization of a fluoranthene-utilizing strain of *Pseudomonas paucimobilis*. Appl Environ Microbiol 56: 1079–1086

Mueller JG, Devereux R, Santavy DL, Lantz SE, Willis SG, Pritchard PH (1997) Phylogenetic and physiological comparisons of PAH degrading bacteria from geographically diverse soils. Antonie van Leeuwenhoek 71:329–343

Mukherji S, Weber WJ Jr (1998) Mass transfer effects on microbial uptake of naphthalene from complex NAPLs. Biotechnol Bioeng 60(6):750–760

Mukherji S, Weber WJ Jr (2001) Mass transfer effects on microbial uptake of naphthalene from complex NAPLs. Biotechnol Bioeng 75(1):130

Mukherji S, Swain AK, Venkataraman C (2002) Comparative mutagenicity assessment of aerosols in emissions from biofuel combustion. Atmos Environ 36:5627–5635

Navon-Venezia S, Banin E, Ron EZ, Rosenberg E (1998) The bioemulsifier alasan: role of protein in maintaining structure and activity. Appl Microbiol Biotechnol 49:382–384

Nue T (1996) Significance of bacterial surface-active compounds in interaction of bacteria with interfaces. Microbiol Rev 60(1):151–166

Obayori OS, Ilori MO, Adebusoye SA, Oyetibo GO, Amund OO (2008) Pyrene-degradation potentials of *Pseudomonas* species isolated from polluted tropical soils. World J Microbiol Biotechnol 24:2639–2646

Peters CA, Knightes CD, Brown DG (1999) Long-term composition dynamics of PAH-containing NAPLs and implications for risk assessment. Environ Sci Technol 33(24): 4499–4507

Prabhu Y, Phale PS (2003) Biodegradation of phenanthrene by *Pseudomonas* sp. strain PP2: novel metabolic pathway, role of biosurfactant and cell surface hydrophobicity in hydrocarbon assimilation. Appl Microbiol Biotechnol 61(4):342–351

Rehmann K, Noll HP, Steinberg CE, Kettrup AA (1998) Pyrene degradation by *Mycobacterium* sp. strain KR2. Chemosphere 36:2977–2992

Ron EZ, Rosenberg E (2002) Biosurfactants and oil bioremediation. Curr Opin Biotechnol 13:249–252

Rosenberg M (1981) Bacterial adherence to polystyrene: a replica method of screening for bacterial hydrophobicity. Appl Environ Microbiol 42:375–377

Rosenberg M, Gutnic D, Rosenberg E (1980) Adherence of bacteria to hydrocarbons: a simple method for measuring cell surface hydrophobicity. FEMS Microbiol Lett 9:29–33

Schippers C, Geßner K, Müller T, Scheper T (2000) Microbial degradation of phenanthrene by addition of a sophorolipid mixture. J Biotechnol 83(3):189–198

Schneider J, Grosser R, Jayasimhulu K, Xue W, Warshawsky D (1996) Degradation of pyrene, benz[a]anthracene, and benzo[a]pyrene by *Mycobacterium* sp. strain RJGII-135, isolated from a former coal gasification site. Appl Environ Microbiol 62:13–19

Somtrakoon K, Suanjit S, Pokethitiyook P, Kruatrachue M, Lee H, Upatham S (2008) Phenanthrene stimulates the degradation of Pyrene and fluoranthene by Burkholderia sp. VUN 10013. World J Microbiol Biotechnol 24:523–531

Stringfellow WT, Aitken MD (1995) Competitive metabolism of naphthalene, methylnaphthalenes, and fluorene by phenanthrene-degrading *Pseudomonads*. Appl Environ Microbiol 61:357–362

Thibault SL, Anderson M, Frankenberger WT Jr (1996) Influence of surfactants on pyrene desorption and degradation in soils. Appl Environ Microbiol 62:283–287

Tiehm A, Fritzsche C (1995) Utilization of solubilized and crystalline mixtures of polycyclic aromatic hydrocarbons by a *Mycobacterium* sp. Appl Microbial Biotechnol 42:964–968

Toren A, Navon-Venezia S, Ron EZ, Rosenberg E (2001) Emulsifying activities of purified Alasan proteins from *Acinetobacter radioresistens* KA53. Appl Environ Microbiol 67: 1102–1106

Volkering F, Breure AM, Van Andel JG, Reulkens WH (1995) Influence of non-ionic surfactants on bioavailability and biodegradation of polycyclic aromatic hydrocarbons. Appl Environ Microbiol 61(5):1699–1705

Walter U, Beyer M, Klein J, Rehm HJ (1991) Degradation of pyrene by *Rhodococcus* sp. UW1. Appl Microbiol Biotechnol 34:671–676

Weissenfels WD, Beyer M, Klein J (1990) Degradation of phenanthrene, fluorene and fluoranthene by pure bacterial culture. Appl Microbiol Biotechnol 32:479–484

Weissenfels WD, Beyer M, Klein J (1991) Microbial metabolism of fluoranthene: isolation and identification of ring fission products. Appl Microbiol Biotechnol 34:528–535

Whyte LG, Slagman SJ, Pietrantonio F, Bourbonniere L, Koval SF, Lawrence JR, Inniss WE, Greer CW (1999) Physiological adaptations involved in alkane assimilation at a low temperature by *Rhodococcus* sp. strain Q15. Appl Environ Microbiol 65(7):2961–2968

Wick LY, De Munain AR, Springael D, Harms H (2002) Responses of *Mycobacterium* sp. LB501T to the low bioavailability of solid anthracene. Appl Microbiol Biotechnol 58(3): 378–385

Wick LY, Pasche N, Bernasconi SM, Pelz O, Harms H (2003) Characterization of multiple-substrate utilization by anthracene-degrading *Mycobacterium frederiksbergense* Lb501t. Appl Environ Microbiol 59(10):6133–6142

Willumsen PA, Arvin E (1999) Kinetics of degradation of surfactant solubilized fluoranthene by a *Sphingomonas paucimobilis*. Environ Sci Technol 33:2571–2578

Willumsen PA, Karlson U (1998) Effect of calcium on the surfactant tolerance of a fluoranthene degrading bacterium. Biodegradation 9:369–379

Willumsen PA, Karlson U (2001) Bacterial cell surface hydrophobicity affecting degradation of PAHs in NAPLs. In: Proceedings of 6th international *in situ* and on site bioremediation symposium, San Diego, CA, 4–7 June 2001, Battelle Press, Columbus, Ohio, pp 141–147

Yaws CL (1999) Chemical properties handbook. McGraw Hill, New York
Zhao Z, Selvam A, Wong JWC (2011) Effects of rhamnolipids on cell surface hydrophobicity of PAHs degrading bacteria and the biodegradation of phenanthrene. Bioresour Technol 102(5):3999–4007
Zita A, Hermansson M (1997) Effects of bacterial cell surface structures and hydrophobicity on attachment to activated sludge flocs. Appl Environ Microbiol 63:1168–1170
Zytner RG, Slab AC, Stiver WH (2006) Bioremediation of diesel fuel contaminated soil: comparison of individual compounds to complex mixtures. Soil Seed Cont 15:277–297

Chapter 8
Microbial Degradation of 2,4,6-Trinitrotoluene: Application to Explosives Sensor

Toshinari Maeda and Hiroaki I. Ogawa

8.1 Introduction

2,4,6-Trinitrotoluene (TNT) is a highly energetic compound with the formula $C_6H_2(NO_2)_3CH_3$ and best known as a useful explosive material with convenient handling properties which led to a relatively safe storage due to the low sensitivity to impact shock and heat stimulation compared to other explosives and no metallic corrosion (Boileau et al. 1987). Then, TNT has been used as an explosive for military and industrial purposes and the TNT production reached to its peak during the two World Wars (Harter 1985). It is estimated that TNT is produced close to 1,000,000 kg per year (Harter 1985). Therefore, a high concentration of TNT has been still found in soil and groundwater at former manufacturing sites (Fernando et al. 1990; Hawari et al. 2000; Lewis et al. 2004; Maeda et al. 2006a). Presently soil and groundwater contamination by the explosive is a serious problem in the countries, mainly United States, Germany and Canada (Pennington 1999; Fritsche et al. 2000). Sediments and soils beneath some industrial sites contain large amounts of nitro aromatics with up to 10 g of TNT per kg of soil being reported for some sites (Carpenter et al. 1978; Kaplan and Kaplan 1982; Fernando et al. 1990). The biodegradation studies have indicated that an explosive is highly recalcitrant for microbial biodegradation (Rieger and Knackmuss 1995a, b). Among them, in particular, TNT is more recalcitrant than other nitroaromatic compounds (e.g. mono- and dinitrotoluenes), because three nitro groups are located symmetrically on the aromatic ring which restrict the attack by classic

T. Maeda (✉) · H. I. Ogawa
Department of Biological Functions and Engineering,
Graduate School of Life Science and Systems Engineering,
Kyushu Institute of Technology, 2-4 Hibikino, Wakamatsu-ku,
Kitakyushu 808-0196, Japan
e-mail: toshi.maeda@life.kyutech.ac.jp

S. N. Singh (ed.), *Microbial Degradation of Xenobiotics*,
Environmental Science and Engineering, DOI: 10.1007/978-3-642-23789-8_8,

dioxygenase enzymes involved in the microbial metabolism of aromatic compounds. Hence, TNT has strong cytotoxicity and mutagenicity in various living organisms (Won et al. 1976; Ahlborg et al. 1988; Tan et al. 1992; Berthe-Corti et al. 1998; Letzel et al. 2003; Padda et al. 2003; Saka 2004; Sun et al. 2005) and is listed as class C potential human carcinogen by the US Environmental Protection Agency. In addition, in TNT-exposed humans, notable toxic manifestations have included aplastic anaemia, toxic hepatitis, cataracts, hepatomegaly, and liver cancer (Sabbioni et al. 2007); therefore, it is significant to develop the bioremediation technology for TNT.

To date, many studies have reported on microbial degradation of TNT by aerobic bacteria and anaerobic bacteria. Therein, chemistry of TNT, microbial degradation of TNT (Reductive pathway of nitro groups, polymerization of hydroxylamino-dinitrotoluenes, elimination pathway of nitro group, and oxidative pathway for TNT metabolites), toxicity of TNT and its metabolites, and explosive sensors based on microbial functions were summarized.

8.2 Chemistry of TNT

Unlike toluene which is metabolized through oxidation pathway for its methyl group, 2,4,6-TNT is more resistant to oxidative reaction, because π electrons from the aromatic ring of TNT are removed by the electronegative nitro groups. Therefore, the aromatic ring of TNT has electrophilic property. The detail explanation has been schemed in Fig. 8.1. Basically, methyl group is an electron-releasing substituent, therefore, π electrons from the aromatic ring of toluene increase. The carbon atom of methyl group in toluene is of electron-rich nature and then oxidation reaction is more likely to occur on the carbon atom (Harayama et al. 1989; Harayama and Rekik 1990). Therefore, the toluene is readily converted to benzyl alcohol, benzaldehyde, benzoic acid, and catechol and the catechol is further metabolized through the catalytic reaction by catechol-1,2-dioxygenase or catechol-2,3-dioxygenase (Fig. 8.1a) (Nakazawa et al. 1969a, b; Nakazawa and Yokota 1973). Also, there has been another oxidation pathway by toluene-2,3-dioxygenase (Renganathan 1989; Friemann et al. 2009) which is triggered by the reaction at a carbon skeleton of the aromatic ring having an electron-rich property (Fig. 8.1b). On the other hand, carbon atom of methyl group in TNT is more of electron-poor nature by which the electrons are withdrawn by three nitro groups. Hence, oxidation reaction is energetically unfavorable because this symmetric arrangement of the three nitro groups led to a limited attack by dioxygenase enzymes involved in the microbial metabolism of aromatic compounds (Rieger et al. 1999). Alternatively, reductive reaction or hydride attack is more favorable on the degradation of TNT (Fig. 8.1c). The nitro group consists of two different elements, N and O, which are highly electronegative. However, oxygen is more electronegative than the nitrogen atom and the N atom is charged positively by the electron-attracting capability of oxygen atom. Hence, the nitrogen atom of

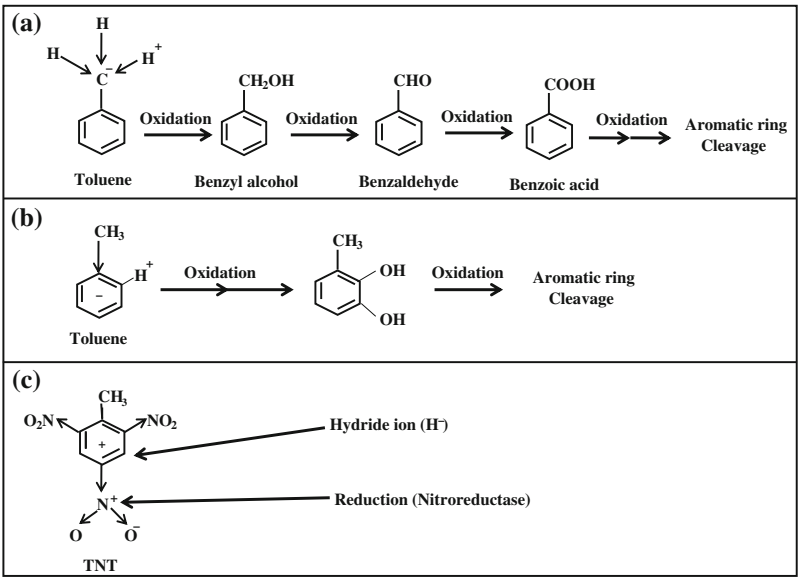


Fig. 8.1 Difference of the electron state within a molecule of toluene (**a** and **b**) and 2,4,6-trinitrotoluene (TNT) (**c**). By the electron-releasing effect of methyl group, the carbon atom at the methyl group of toluene is apparently with electron-rich nature. Therefore, oxidation reaction is more likely to occur on the carbon atom; as a result, the toluene is readily converted to benzyl alcohol, benzaldehyde, and benzoic acid (**a**). In a similar way, the π electrons inside the aromatic ring of toluene are relatively-rich; hence, oxidative reaction by toluene-2,3-dioxygenase can be favorable (**b**). On the other hand, TNT has the quite opposite property with toluene by the electron-withdrawing effect of the three nitro groups; hence, reductive reaction or hydride attack is more favorable on the degradation of TNT (**c**). The nitro group consists of two different elements, N and O, which are both highly electronegative and the oxygen is more electronegative than the nitrogen atom; the N atom is charged positively by the electron-attracting capability of oxygen atom. Hence, the nitrogen atom of the nitro groups in TNT is the target site of reductive reaction and ultimately nitro groups of TNT are easily reducible (**c**)

the nitro groups in TNT is the target site of reductive reaction and ultimately nitro groups of TNT are easily reducible (Fig. 8.1c).

The reductive reaction at the site occurs as a series of two-electron transfers which yield the nitroso, hydroxylamino, and amino derivatives of TNT. The nitroso and hydroxylamino groups are responsible for the toxicity of nitroaromatic compounds, as they react with biological molecules (Carpenter et al. 1978), and hence cause chemical mutagenesis and carcinogenesis (Won et al. 1976; Spanggord et al. 1982; Styles and Cross 1983; Rafii et al. 1994; Honeycutt et al. 1996; Brooks et al. 1997; Vaatanen 1997; Berthe-Corti et al. 1998; Banerjee et al. 1999; Robidoux et al. 1999; Tadros et al. 2000). It has been reported that amino-dinitrotoluenes (ADNTs), in which the nitro group was completely reduced to an amino group, may have lower mutagenic effect than the original compound (Honeycutt et al. 1996; Kennel et al. 2000; Maeda et al. 2006a). However, there is

a possibility to spread the contamination of the compounds into an environment because ADNTs have relatively-higher polarity than TNT (Harter 1985). In the reduction of the nitro groups of TNT, the reduction of the first nitro group is basically much more rapid than that of the rest two nitro groups since the conversion of nitro to amino groups decreases the electron deficiency of the nitroaromatic ring, and consequently a lower redox potential is required to reduce the rest of the nitro group of the molecule. Also in TNT, the *para* position is more easily reduced than *ortho* nitro group, indicating that the production of 4-amino-2,6-dinitrotoluene (4ADNT) is more prefered than that of 2-amino-4, 6-dinitrotoluene (2ADNT), because the electron releasing effect of the methyl group influences the nitro group which is the closest rather than the farthest one to the methyl group. It means that the partially positive charge of the nitrogen atom in the closest nitro group to the methyl group is deactivated by the electron releasing effect compared to that in the farthest one. Hence, the reductive reaction at the farthest nitro group to methyl group will be more incident. The data of single-electron reduction potential of TNT analog showed that 2,4,6-trinitrophenol was more resistant to the reductive reaction than TNT (Maeda et al. 2007a).

8.3 Microbial Degradation of TNT

To date, many review papers have reported on the microbial degradation of TNT (Hawari et al. 2000; Esteve-Núñez et al. 2001; Snellinx et al. 2002; Smets et al. 2007). TNT is degraded by aerobic or anaerobic bacteria and is metabolized through mainly reductive reaction of TNT (Fig. 8.2), polymerization of TNT metabolites (Fig. 8.3), elimination of nitro groups from TNT (Fig. 8.4), and oxidation of TNT metabolites (Fig. 8.5). The details of reductive pathways are given as below:

8.3.1 Reductive Pathway of Nitro Groups

TNT is commonly biotransformed by converting nitro groups into nitroso, hydroxylamino and amino groups (Hawari et al. 2000; Esteve-Núñez et al. 2001; Snellinx et al. 2002), as shown in Fig. 8.2. Thus, because of its high electron deficiency, initial microbial transformations of TNT are characterized by reductive rather than by oxidative reactions (Barrows et al. 1996). In several bacterial species, the major reduction metabolites from TNT are hydroxylamino-dinitrotoluenes [HADNTs; 2-hydroxylamino-4,6-dinitrotoluene (2HADNT) and 4-hydroxylamino-2,6-dinitrotoluene (4HADNT)] and ADNTs that are formed by reducing one of three nitro groups (Vorbeck et al. 1998; Huang et al. 2000; Ahmad and Hughes 2002; Fleischmann et al. 2004; Kroger et al. 2004; Borch et al. 2005; Yin et al. 2005). Also dihydroxylamino-nitrotoluene (DHANT) is accumulated in

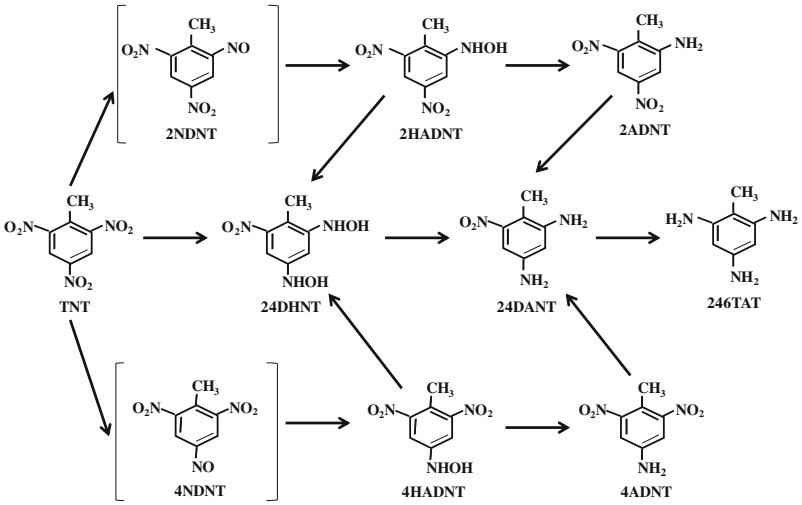


Fig. 8.2 Reductive pathway of TNT by microorganisms. TNT is commonly biotransformed by converting nitro groups into nitroso, hydroxylamino and amino groups by mostly the enzymatic reaction of nitroreductase. The major reduction metabolites from TNT are 2-hydroxylamino-4,6-dinitrotoluene (2HADNT), 4-hydroxylamino-2,6-dinitrotoluene (4HADNT), 2-amino-4,6-dinitrotoluene (2ADNT), 4-amino-2,6-dinitrotoluene (4ADNT), and further 2,4-diamino-6-nitrotoluene (24DANT). Under an anoxic condition, 2,4-dihydroxylamino-nitrotoluene (24DHANT) is produced as well as 2,4,6-triaminotoluene (TAT) which is produced under the strict anaerobic condition. In general, 2-nitroso-4,6-dinitrotoluene (2NDNT) and 4-nitroso-2,6-dinitrotoluene (4 NDNT) are undetectable

some anaerobic bacteria during TNT metabolism (Lewis et al. 1996; Ahmad and Hughes 2002; Fleischmann et al. 2004; Borch et al. 2005; Yin et al. 2005), while aerobically, DHANT is accumulated only by *Pseudomonas pseudoalcalogenes* JS52 (Fiorella and Spain 1997), which is a spontaneous mutant derived from strain JS45 (Nishino and Spain 1993), and *Pseudomonas* sp. TM15 under a partial anoxic condition which is possibly formed during the incubation using a high cell density (Kubota et al. 2008). Then, two ADNTs (2ADNT and 4ADNT) and diamino-nitrotoluene (DANT) are also detected as a metabolite of TNT. Also, under a strict anaerobic condition, TNT is converted to triamino-toluene (TAT) (Funk et al. 1993; Hofstetter et al. 1999). An engineered plant expressing the nitroreductase from *Enterobacter cloacae* holds a great promise as a tool of phytoremediation for a TNT-contaminated soil (Hannink et al. 2001; Hannink et al. 2007).

A key enzyme involved in the reductive reaction of TNT is nitroreductase and this family of nitroreductases can reduce the nitro group of a wide range of nitroaromatic compounds, such as nitrofurazones, nitroarenes, nitrophenols, nitrobenzenes, and explosives (TNT, hexahydro-1,3,5-trinitro-1,3,5-triazine, and glycerol trinitrate). Nitroreductases contain flavin mono-nucleotide (FMN) as the cofactor, use NADPH and/or NADH as the electron donor, and perform sequential two-electron reductions to the nitro group (Bryant and DeLuca 1991;

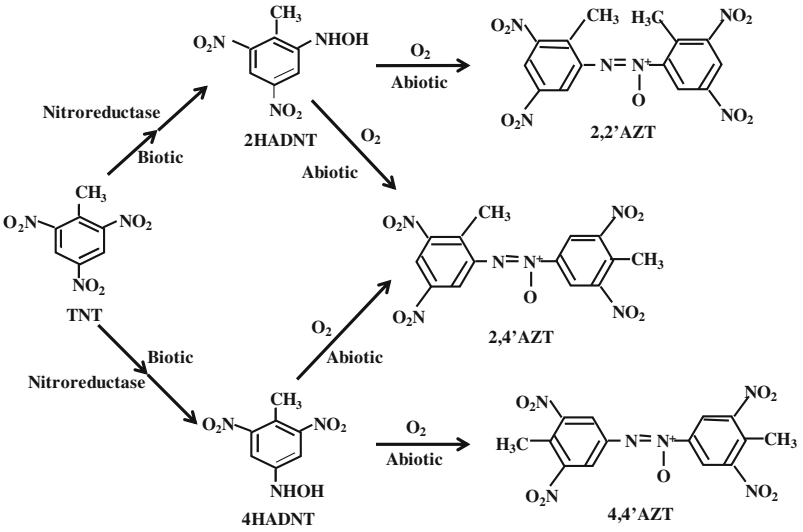


Fig. 8.3 Abiotic degradation of hydroxylamino-dinitrotoluenes (HADNTs). 2HADNT and 4HADNT which are unstable compounds are spontaneously destructed under an oxic condition; thereby, 4,4′,6,6′-Tetranitro-2,2′-azoxytoluene (2,2′AZT), 2,2′,6,6′-tetranitro-4,4′-azoxytoluene, and 4,2′,6,6′-tetranitro-2,4′-azoxytoluene (2,4′AZT) are produced from a mixture of 2HADNT and 4HADNT

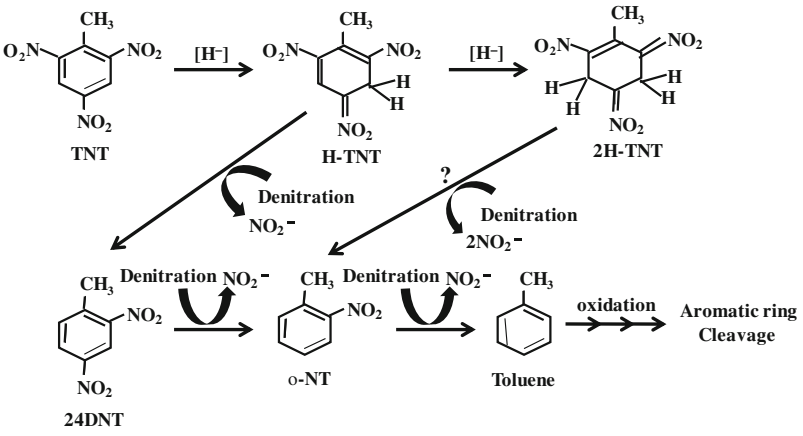


Fig. 8.4 Elimination pathway of nitro group from TNT. Denitration of TNT can occur through hydrogenation of aromatic ring via a Meisenheimer complex. The hydride-Misenheimer complex (H-TNT) is subsequently denitrated to 2,4-dinitrotoluene (24DNT); as a result, nitrite ions are released through the reaction. In a similar way, the dihydride complex of TNT (2H-TNT) is also produced by the attack of two hydride ions. Then, the continuous hydrogenation can trigger the production of *o*-nitrotoluene (NT) and toluene. Ultimately, an engineered strain expressing the oxidative enzymes from TOL plasmid may utilize TNT as the sole carbon and nitrogen source although there was still an unbalance growth

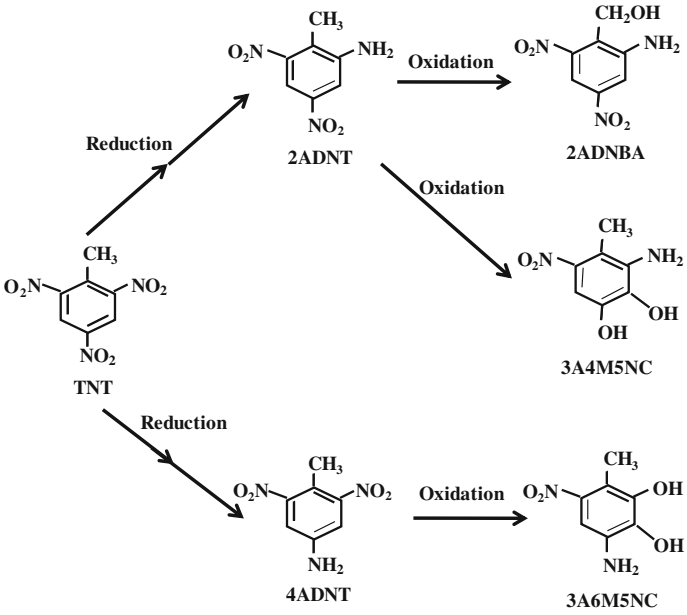


Fig. 8.5 Oxidative pathway for TNT metabolites. Unlike TNT which is relatively-resistant to oxidative reaction, the TNT metabolites such as 2ADNT and 4ADNT are possible to be oxidized by several dioxygenases. The oxidation of 2ADNT by the 24DNT dioxygenase from *Burkholderia* sp. produces 3-amino-4-methyl-5-nitrocatechol (3A4M5NC) as well as 2-amino-4,6-dinitrobenzyl alcohol (2ADNBA). Also, 3-amino-6-methyl-5-nitrocatechol is produced by the oxidative reaction of 4ADNT by a nitrobenzene dioxygenase from *Comamonas* sp. strain. Nitrite ions are produced from the process of these oxidative reactions

Bryant et al. 1991). In addition, nitroreductases have already been identified from many bacteria: NfsA and NfsB in *Escherichia coli* (Zenno et al. 1996a, b; Rau and Stolz 2003), NfsI in *Enterobacter cloacae* (Bryant and DeLuca 1991), Frase I in *Vibrio fisherii* (Zenno et al. 1994), RdxA in *Helicobacter pylori* (Goodwin et al. 1998), SnrA and Cnr in *Salmonella enterica* serovar Typhimurium (Watanabe et al. 1990; Nokhbeh et al. 2002), nitrobenzene nitroreductase in *Pseudomonas pseudoalcaligenes* (Somerville et al. 1995), PnrA and PnrB in *Pseudomonas putida* (Park and Kim 2000; Caballero et al. 2005a), NitA and NitB in *Clostridium acetobutylicum* (Kutty and Bennett 2005), and NADH oxidase in *Thermus thermophilus* (Park et al. 1992). It was demonstrated that the expression of NitA and NitB is inducible in the presence of TNT (Kutty and Bennett 2005) and *pnrA* is constitutively expressed in *P. putida* JLR11 (Caballero et al. 2005a). Whereas TNT degradation by the whole cell system accumulates ADNTs in which one nitro group is reduced to the amino group (Hawari et al. 2000; Esteve-Núñez et al. 2001; Snellinx et al. 2002), in vitro enzyme reaction process by nitroreductases mainly produced HADNTs rather than ADNTs (Riefler and Smets 2000; Watrous et al. 2003; Caballero et al. 2005a; Kutty and Bennett 2005).

Other enzymes that can reduce nitroaromatic compounds include old yellow enzyme (OYE) (Williams et al. 2001), aldehyde oxidase (Wolper et al. 1973), dihydrolipic amide dehydrogenase (Tatsumi et al. 1979), cytochrome b5 reductase (Mason and Holtzman 1975), diaphorases (Kato et al. 1969), hydrogenases (Watrous et al. 2003), and carbon monoxide dehydrogenase (Huang et al. 2000). The OYE family [e.g. XenA and XenB from *Pseudomonas* sp. (Pak et al. 2000; Orville et al. 2004a, b), pentaerythritol tetranitrate (PETN) reductase from *E. cloacae* (Khan et al. 2002), N-ethylmaleimide reductase (NemA) from *E. coli* (González-Pérez et al. 2007), morphinone reductase from *P. putida* M10 (French and Bruce 1995), YqjM from *Bacillus subtilis* (Fitzpatrick et al. 2003), and OYE from *Saccharomyces cerevisiae* (Williams et al. 2004)] has capability to reduce nitro group and the gene expression appears inducible by TNT. While a hydrogenase enzyme derived from *C. acetobutylicum* is basically related to biohydrogen production, a H_2-dependent reduction of TNT produces 2HADNT and 4HADNT, and DHANT by the subsequent reduction of these compounds (Watrous et al. 2003). Purified carbon monoxide dehydrogenase from *C. thermoaceticum* catalyzes the conversion of TNT to 2HADNT and 4HADNT in the presence of CO and cyanide, an inhibitor for the CO/CO_2 oxidation/reduction activity of this enzyme, inhibited the TNT degradation activity by this enzyme (Huang et al. 2000).

8.3.2 Polymerization of Hydroxylamino-Dinitrotoluenes

As shown in Fig. 8.3, the major metabolites produced from TNT, 2HADNT and 4HADNT are unstable compounds under an oxic condition and the spontaneous destruction of 2HADNT and 4HADNT was accelerated by heat treatment and inhibited under argon gas, indicating that dissolved oxygen is responsible for the destruction (Haïdour and Ramos 1996; Maeda et al. 2006b). Further studies demonstrated that tetranitro-azoxytoluenes which are dimer formations of HADNTs are produced from the process of the spontaneous destruction of 2HADNT and 4HADNT (Maeda et al. 2007b). 4,4′,6,6′-Tetranitro-2,2′-azoxytoluene (2,2′AZT) from 2HADNT and 2,2′,6,6′-tetranitro-4,4′-azoxytoluene (4,4′AZT) from 4HADNT were identified as the polymerization products. Also, 4,2′,6,6′-tetranitro-2,4′-azoxytoluene (2,4′AZT), 2,2′AZT, and 4,4′AZT were detected from a mixture of 2HADNT and 4HADNT and the yield of 2,2′AZT was lower than that of 2,4′AZT and 4,4′AZT (Maeda et al. 2007b) probably due to the steric hindrance between a polymerization site and nitro groups. Also through polymerizing reaction of HADNTs, nitrite ions are released and it has been suggested that the nitrite ions can be utilized as a nitrogen source for microbial growth (Wittich et al. 2009). In addition, DHANT, in which two nitro groups of TNT are reduced to two hydroxylamino groups, is also unstable under an oxic condition and the amount of DHANT decreases with time (Kubota et al. 2008). Although the destruction product of DHANT was not detected, perhaps in this case, the same polymerization reaction as HADNTs can occur and it has potential to synthesize

a multimeric complex from DHANT, because this metabolite has two polymerization sites (two hydroxylamino groups) on the aromatic ring.

8.3.3 Elimination Pathway of Nitro Group

Denitration of TNT can occur through hydrogenation of aromatic ring via a Meisenheimer complex (Kaplan and Siedle 1971) (Fig. 8.4). The hydride-Misenheimer complex (H-TNT) is subsequently denitrated to 2,4-dinitrotoluene (24DNT) or 2,6-dinitrotoluene (26DNT). As a result, nitrite ions are also produced in the reaction (Tatsumi et al. 1979; Williams et al. 2004; Ramos et al. 2005). The three nitro groups of TNT trigger the deficiency of electrons in the aromatic nucleus of TNT. Therefore, a non-aromatic structure, such as a Meisenheimer complex, can be formed and the structure can be rearomatized after nitrite release with the formation of 24DNT and 26DNT. An NADPH-dependent F420 reductase derived from *Rhodococcus erythropolis* isolated as a picric acid-degrading strain, has capability to generate H-TNT and dihydride complex of TNT (2H-TNT). However, the subsequent formations of 24DNT or 26DNT were not detected (Ebert et al. 1999). In addition, although XenB, PETN reductase, and NemA have been known to work on the nitroreduction pathway, these enzymes are also related to the denitration of TNT via the formation of H-TNT and 2H-TNT (Meah et al. 2001; Williams et al. 2004). The 2H-TNT is an unstable compound and enzymatically transformed by OYE enzymes with subsequent or concomitant nitrite release (Pak et al. 2000). The enzyme function of these enzymes leaves behind a deep mystery (e.g. nitroreduction versus direct hydride addition). Production of 8 different hydride complexs has been detected as metabolites of TNT by *Yarrowia lipolytica* (Ziganshin et al. 2007). The continual hydrogenation to the aromatic ring with nitro groups or TNT transformation by denitrase may lead to the formation of toluene which is metabolized readily by classic dioxygenases. It has been reported that an engineered strain (*Pseudomonas* sp. clone A harboring TOL plasmid which contributes to the complete degradation of toluene) is able to utilize TNT as the sole carbon and nitrogen source and may mineralize TNT, although it appears there is an unbalanced growth (Duque et al. 1993). Additionally, a recent study showed that a small-molecular-weight catalyst extracted from the culture medium of *P. aeruginosa* ESA-5 works as a denitration catalyst in the presence of NADH (Stenuit et al. 2009). It seems that the nitrite production is through the formation of 2,2′AZT, 2,4′AZT and 4,4′AZT from HADNTs. This small-molecular-weight substance holds a great promise as a green catalyst to initiate TNT denitration. A molecular-based study using random transposon mutagensis for *P. putida* JLR11 which can utilize TNT as the nitrogen source showed that nitrogen from TNT is assimilated via the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway, as evidenced by the inability of GOGAT mutants (*gltB* and *gltD*) to use TNT and other three mutants defective in the master regulator in nitrogen assimilation (*ntrC*), a positive transcriptional regulator of the

nas operon for nitrate and nitrite assimilation (*nasT*), and a LysR-type transcriptional regulator (*cnmA*) failed to grow on nitrite, but grew on TNT, although slower than the wild-type strain (Caballero et al. 2005b). Also, another transposon mutant from *Pseudomonas* sp. TM15 accumulates nitrite ions (Asakawa et al. 2006).

8.3.4 Oxidative Pathway for TNT Metabolites

TNT is relatively resistant to oxidative reaction due to the electron withdrawing effect of three nitro groups and hence TNT is easily reduced to 2ADNT and 4ADNT (Spain et al. 2000). The expression of several dioxygenases in *E. coli* enables the possible oxidative attacks for the TNT metabolites, ADNTs which were disengaged from the critical electron poor status triggered by the three nitro groups. *E. coli* cells expressing the 24DNT dioxygenase from *Burkholderia cepacia* R34 or *Burkholderia* sp. strain DNT are capable of oxidizing 2ADNT at the 3,4 position releasing nitrite to form 3-amino-4-methyl-5-nitrocatechol (3A4M5NC) as well as 2-amino-4,6-dinitrobenzyl alcohol through oxidizing the methyl group of 2ADNT (Johnson et al. 2001). Also, the heterologously-expressed nitrobenzene dioxygenase from *Comamonas* sp. strain JS765 can oxidize 4ADNT at the 2,3 position to generate 3-amino-6-methyl-5-nitrocatechol along with releasing nitrite (Johnson et al. 2001). The oxidative reaction of these recombinant dioxygenases led to the simultaneous oxidation of 2ADNT and 4ADNT, yielding the expected catechols (Keenan and Wood 2006). The activity of these dioxygenases seems to be quite low (less than 0.5 mmol/min/mg-protein), especially for 4ADNT which was more recalcitrant. Hence, it is still unclear if the formed catechols, 3A4M5NC can be enzymatically transformed or remain as dead-end products. On the other hand, in general, the formation of catechols is amenable to the cleavage of the aromatic ring through catechol dioxygenases (Nakazawa et al. 1969a; Harayama and Rekik 1990). Hence, there will be still a possibility to completely cleave the aromatic ring of TNT metabolites.

8.4 Toxicity of TNT and its Metabolites

8.4.1 TNT

TNT is the most toxic substance and is mutagenic in *Salmonella typhimurium* strains TA98 and TA100 with or without exogenous metabolic activity (Berthe-Corti et al. 1998). An unpublished 2-year study was reported in 1984 by the IIT Research Institute, Chicago, IL. Fisher 344 rats were fed diets containing 0.4, 2, 10, or 50 mg-TNT/kg-weight per day. In the urinary bladder, hyperplasia (12 of 47 animals) and carcinoma (11 of 47 animals) were observed at significant levels

in high-dose (50 mg/kg) females and in one or two females, respectively, at 10 mg/kg (Bolt et al. 2006). In addition, it has been reported that TNT inhibits endothelial nitric oxide synthase activity, causing an elevated blood pressure in rats (Sun et al. 2005) and causes methemoglobin formation in human erythrocytes (Maroziene et al. 2001). Hence, TNT is a harmful compound to living organisms including human. Another Ames tests (Won et al. 1976; Tan et al. 1992; Honeycutt et al. 1996) and a *Salmonella* microsuspension bioassay (George et al. 2001) also showed that TNT has high mutagenic effect. However, how these explosives exert strong mutagenicity in cells of living organisms has not been adequately revealed and to our knowledge, very little data about the mutagenic induction of TNT is available in the literature. The results by Spanggord et al. (1982) suggest that the mutagenicity from isomeric TNT can exerted either independently or dependently of nitroreductase, which catalyzes the conversion of nitro group to nitroso, hydroxylamino, and amino group. Also, *O*-acetyltransferase, which catalyzes an *N*-hydroxylamine to *N*-acetoxyarylamine in the presence of acetyl coenzyme A as well as nitroreductase are responsible for inducing the mutagenicity of TNT, however, *O*-acetyltransferase was less effective than nitroreductase (Einisto 1991; Vaatanen 1997). The mechanism of mutagenic activity in nitroaromatic compounds has been discussed as follows. Since TNT is readily reduced to nitroso, hydroxylamino, and amino derivatives as well as the formation of superoxide radical and hydrogen peroxide is enhanced by TNT (Kong et al. 1989), its metabolites or reactive oxygen species might mutagenize the DNA of living organisms through the direct or indirect interaction with DNA or oxidation damage, as shown in the results that the mutagenicity induced by nitroaromatic compounds was correlated either with the reactivity of reduction or with their biodegradability (Maeda et al. 2007a). These insights suggest that the intensity of the mutagenicity in nitroaromatic compounds significantly correlates with the increase of their reductive potentials.

8.4.2 HADNTs and DHANT

Hydroxylamino compounds, which are produced by nitroreductase, may be responsible for oxidizing DNA in living organisms, because 4-hydroxylamino-dinitrotoluene, one of the TNT reduction products, increases the DNA oxidation in the presence of NADH and Cu(II) (Homma-Takeda et al. 2002). However, these chemicals have no cytotoxicity (Maeda et al. 2006a), indicating that such chemicals (high mutagenicity and low cytotoxicity) are critical to living organisms because they may produce organisms with many mutations due to no cytotoxicity. Also, 2,4DHANT has higher mutagenicity than HADNTs (Padda et al. 2003), although whether this compound has no cytotoxicity known.

8.4.3 AZTs

AZTs are polynitroaromatic compounds that can be produced during microbial reduction of TNT. Three major AZTs, 2,2′AZT, 2,4′AZT, and 4,4′AZT were mutagenic in TA100, but not in TA100NR (Spanggord et al. 1995), indicating the need for nitroreductase activity to induce mutagenicity. The mutagenicity of 2,2′AZT was higher than that of the other two AZTs and the parent compound, TNT. Another Ames test also showed that 2,2′AZT is mutagenic in TA100 with or with S9 (Honeycutt et al. 1996). Also, CHO-hprt assay did not show a significant mutagenic activity at a low concentration of these AZTs (Kennel et al. 2000).

8.4.4 ADNTs

Formation of the two monoamino transformation products, 2ADNT and 4ADNT from TNT is favored (Bruns-Nagel et al. 1999; Spain et al. 2000) and they are typically observed in TNT-contaminated sites (soil and ground water). 2ADNT and 4ADNT are more stable in the environment and more mobile, posing a potential threat to human health, and the environment (Harter 1985). Also, in earthworms, the toxicity of 4ADNT was higher than that of TNT and 2ADNT. Since, 2ADNT was bioaccumulated more than other ones (Lachance et al. 2004); hence, it needs consideration when evaluating overall TNT toxicity. In fact, these ADNTs are detected from the urine of workers exposed to TNT (Ahlborg et al. 1988; Sabbioni et al. 2007), therefore, 2ADNT and 4ADNT are the most prevalent metabolites in tissue of animals exposed to TNT. The mutagenicity of 2ADNT and 4ADNT was less than that of TNT, although it remains high (Maeda et al. 2006a; Neuwoehner et al. 2007). Also, the treatment of 2ADNT to MCF-7 human breast cancer cells led to an increased production of p53 tumor suppressor protein (Banerjee et al. 2003).

8.5 Explosive Sensors Based on Microbial Functions

A large number of landmines have been produced through many military activities and it has been estimated that the number of land mines remaining under the soil may be approximately 110 million in 60 countries. To date, there are several ways to explore the land mines: one is a way using an excellent olfactory sense of a dog specially trained and the other is a way using a metal detector. However, there are several problems associated with detection of landmines. In the former case, to sense the smell of explosives by the dog's nose, it incurs a large amount of money for maintaining the dogs. On the other hand, although the metal detector is a useful method, but it takes more time to detect the land mine and shows inability to detect

plastic-type landmines. Therefore, developing a novel technique to efficiently detect a land mine was greatly realized.

So far, several explosive sensors based on biological functions have been developing. Here, 5 methods were developed to detect TNT and the impurity of dinitro-toluene (DNT). Although further research progress is required for the commercial viability, these novel methods will hold a great promise for the promotion of land mine removal.

8.5.1 Algal Biosensor

Algal biosensor is to assay TNT by measuring the difference of chlorophyll fluorescence between the two-type algal strains (one is a strain sensitive to TNT and the other one is a strain resistant to TNT) (Altamirano et al. 2004). In the presence of a toxic compound, such as TNT, photosynthesis of the TNT-sensitive algal strain was inhibited and the resultant fluorescence intensity also decreased according to the concentration of TNT. On the other hand, the TNT-resistant algal strain always exhibited significant higher values of fluorescence in the presence of TNT than the wild-type cells. Thereby, the use of two different genotypes (sensitive and resistant to a given pollutant) is a useful method to detect TNT with algal biosensors.

8.5.2 Nitroreductase Sensor

As described above, nitroreductase is the enzyme to catalyze the reduction of TNT. The nitroreductase sensor is a way to assay TNT or DNT by electrochemically measuring the electrons produced through the reductive reaction of nitro groups by the enzyme (Naal et al. 2002). The TNT biosensor is based on the surface immobilization of a fused protein complex (MBP-NR) between maltose binding protein (MBP) and nitroreductase (NR) onto an electrode modified with an electropolymerized films of N-(3-pyrrol-1-ylpropyl)-4,4′-bipyridine (PPB). Although the wild-type NR enzyme without the MBP domain loses most of its enzyme activity, the MBP domain of MBP-NR exhibits a high and specific affinity towards electropolymerized films of PPB with the immobilized enzyme retaining virtually all of its enzymatic activity.

8.5.3 Surface Plasmon Resonance Immunosensor

A surface plasmon resonance (SPR) immunoassay for the detection of TNT was developed by using an immunoreaction between 2,4,6-trinitrophenol-ovalbumin (TNP-OVA) conjugate and anti-TNP antibody (Shankaran et al. 2005).

The quantification of TNT is based on the principle of indirect competitive immunoassay, in which the immunoreaction between the TNP-OVA conjugate and anti-TNP antibody was inhibited of free TNT in solution. The decrease in the resonance angle shift is proportional to an increase of TNT concentration and the immunoassay exhibited excellent sensitivity for the detection of TNT. The SPR sensor showed a very low sensitivity to some structurally related nitroaromatic derivatives, such as 24DNT, 2ADNT, 4ADNT, and 1,3-dinitrobenzene. A modified TNT sensor has been further fabricated for highly sensitive SPR detection of TNT (Mizuta et al. 2008).

8.5.4 Engineered Olfactory Sensor

The use of *S. cerevisiae* strain containing the primary components of the mammalian olfactory signaling pathway led to development of a unique biomimetic system to detect 24DNT (Radhika et al. 2007). In this engineered yeast strain, WIF-1α, olfactory receptor signaling is coupled to green fluorescent protein (GFP) expression and then using this 'olfactory yeast', and olfactory receptors that can respond the presence of the odorant 24DNT, an explosive residue mimic was screened. With this approach, the novel rat olfactory receptor Olfr226, which is closely related to the mouse olfactory receptors Olfr2 and MOR226-1, was identified as a 2,4-dinitrotoluene–responsive receptor. Hence, this engineered yeast strain can produce the GFP fluorescence in the presence of 24DNT.

8.5.5 Engineered Regulator-Based Sensor

The TNT-sensing way is based on transcriptional activity of the engineered toluene-responsive *P. putida* XylR regulator that is reassembled to respond to DNT and TNT (Garmendia et al. 2008). By using mutaion-prone PCR and DNA sequence shuffling, variants of the effector recognition domain of the XylR were evolved and screened to find a novel variant able to be activated in the presence of nitroaromatic compounds, since the original XylR showed inability of the transcriptional activation by TNT (TNT did not bind to the effector recognition site of theXylR). When strains bearing transcriptional fusions to reporters with an optical output (*luxAB* or GFP) were spread on soil spotted with nitrotoluenes, the signal triggered by promoter activation allowed localization of the target compounds on the soil surface. Hence, this work was to report that non-natural transcriptional factors evolved to respond to nitroaromatics can be engineered in soil bacteria and inoculated on a target site to pinpoint the presence of explosive. This approach is a usable way to tackle this gigantic humanitarian problem.

8.6 Conclusion

TNT has been biotransformed easily to HADNTs, ADNTs, DHANT, and DANT, which are the metabolites produced by the reductive reaction of TNT by nitroreductases or other potential reductases. Then, HADNTs are converted spontaneously to AZTs under an oxic condition, and nitrite ions are released through Meisenheimer-complexes formation. Also, it has been shown that the reductive metabolites from TNT can be further metabolized through the oxidative reaction by classic dioxygenases. However, an evidence for TNT mineralization by microorganisms is still scarce.

On the other hand, protein engineering of XylR protein had great potential to evolve a well-known protein to be adaptive to DNT and TNT. In addition, an engineered strain was successful to oxidize TNT metabolites. Thus, there will be still a possibility in the future to develop a bacterium able to completely mineralize TNT through a natural selection process or an induction of spontaneous mutations under a heavy TNT contamination, since TNT and its metabolites both are toxic and mutagenic to living organisms. In other words, such a microorganism will be able to evolve from TNT-polluted sites around the world to mineralize it completely.

Several brilliant methods to detect explosives are developed based on microbial functions which have been worked out through the extensive research over the years. However, further research is still needed to develop novel methods which will be useful to tackle the gigantic problem of explosives disposal.

References

Ahlborg G Jr, Einisto P, Sorsa M (1988) Mutagenic activity and metabolites in the urine of workers exposed to trinitrotoluene (TNT). Br J Ind Med 45:353–358

Ahmad F, Hughes JB (2002) Reactivity of partially reduced arylhydroxylamine and nitrosoarene metabolites of 2,4,6-trinitrotoluene (TNT) toward biomass and humic acids. Environ Sci Technol 36:4370–4381

Altamirano M, Garcia-Villada L, Agrelo M, Sanchez-Martin L, Martin-Otero L, Flores-Moya A (2004) A novel approach to improve specificity of algal biosensors using wild-type and resistant mutants: an application to detect TNT. Biosens Bioelectron 19:1319–1323

Asakawa H, Maeda T, Ogawa HI, Haruyama T (2006) A cellular bioassay for TNT detection using engineered *Pseudomonas* sp. strain TM101 for systematic bioremediation. J Biol Phys Chem 6

Banerjee HN, Verma M, Hou LH, Ashraf M, Dutta SK (1999) Cytotoxicity of TNT and its metabolites. Yale J Biol Med 72:1–4

Banerjee H, Hawkins Z, Dutta S, Smoot D (2003) Effects of 2-amino-4,6-dinitrotoluene on p53 tumor suppressor gene expression. Mol Cell Biochem 252:387–389

Barrows SE, Cramer CJ, Truhlar DG, Elovitz MS, Weber EJ (1996) Factors controlling regioselectivity in the reduction of polynitroaromatics in aqueous solution. Environ Sci Technol 30:3028–3038

Berthe-Corti L, Jacobi H, Kleihauer S, Witte I (1998) Cytotoxicity and mutagenicity of a 2,4, 6-trinitrotoluene (TNT) and hexogen contaminated soil in *S. typhimurium* and mammalian cells. Chemosphere 37:209–218

Boileau J, Fauquigonon C, Napoly C (1987) Explosives. In: Gerhartz W, Yamamoto YS, Kaudy L, Rounsaville JF, Schulz G (eds) Ullmann's encyclopedia of industrial chemistry. VCH, Weinheim, pp 145–172

Bolt HM, Degen GH, Dorn SB, Plottner S, Harth V (2006) Genotoxicity and potential carcinogenicity of 2,4,6-TNT trinitrotoluene: structural and toxicological considerations. Rev Environ Health 21:217–228

Borch T, Inskeep WP, Harwood JA, Gerlach R (2005) Impact of ferrihydrite and anthraquinone-2,6-disulfonate on the reductive transformation of 2,4,6-trinitrotoluene by a gram-positive fermenting bacterium. Environ Sci Technol 39:7126–7133

Brooks LR, Jacobson RW, Warren SH, Kohan MJ, Donnelly KC, George SE (1997) Mutagenicity of HPLC-fractionated urinary metabolites from 2,4,6-trinitrotoluene-treated Fischer 344 rats. Environ Mole Mutagen 30:298–302

Bruns-Nagel D, Schmidt TC, Drzyzga O, von Low E, Steinbach K (1999) Identification of oxidized TNT metabolites in soil samples of a former ammunition plant. Environ Sci Pollut Res 6:7–10

Bryant C, De Luca M (1991) Purification and characterization of an oxygen-insensitive NAD(P)H nitroreductase from *Enterobacter cloacae*. J Biol Chem 266:4119–4125

Bryant C, Hubbard L, McElroy WD (1991) Cloning, nucleotide sequence, and expression of the nitroreductase gene from *Enterobacter cloacae*. J Biol Chem 266:4126–4130

Caballero A, Esteve-Nunez A, Zylstra GJ, Ramos JL (2005a) Assimilation of nitrogen from nitrite and trinitrotoluene in *P. putida* JLR11. J Bacteriol 187:396–399

Caballero A, Lazaro JJ, Ramos JL, Esteve-Nunez A (2005b) PnrA, a new nitroreductase-family enzyme in the TNT-degrading strain *P. putida* JLR11. Environ Microb 7:1211–1219

Carpenter DF, McCormick NG, Cornell JH, Kaplan AM (1978) Microbial transformation of ^{14}C-labeled 2,4,6-trinitrotoluene in an activated-sludge system. Appl Environ Microbiol 35:949–954

Duque E, Haïdour A, Godoy F, Ramos JL (1993) Construction of a *Pseudomonas* hybrid strain that mineralizes 2,4,6-trinitrotoluene. J Bacteriol 175:2278–2283

Ebert S, Rieger PG, Knackmuss HJ (1999) Function of coenzyme F420 in aerobic catabolism of 2,4,6-trinitrophenol and 2,4-dinitrophenol by Nocardioides simplex FJ2–1A. J Bacteriol 181:2669–2674

Einisto P (1991) Role of bacterial nitroreductase and O-acetyltransferase in urine mutagenicity assay of rats exposed to 2,4,6-trinitrotoluene (TNT). Mutat Res 262:167–169

Esteve-Núñez A, Caballero A, Ramos JL (2001) Biological degradation of 2,4,6-trinitrotoluene. Microbiol Mol Biol Rev 65:335–352

Fernando T, Bumpus JA, Aust SD (1990) Biodegradation of TNT (2,4,6-trinitrotoluene) by *Phanerochaete chrysosporium*. Appl Environ Microbiol 56:1666–1671

Fiorella PD, Spain JC (1997) Transformation of 2,4,6-trinitrotoluene by *Pseudomonas pseudoalcaligenes* JS52. Appl Environ Microbiol 63:2007–2015

Fitzpatrick TB, Amrhein N, Macheroux P (2003) Characterization of YqjM, an Old Yellow Enzyme homolog from *Bacillus subtilis* involved in the oxidative stress response. J Biol Chem 278:19891–19897

Fleischmann TJ, Walker KC, Spain JC, Hughes JB, Morrie Craig A (2004) Anaerobic transformation of 2,4,6-TNT by bovine ruminal microbes. Biochem Biophys Res Commun 314:957–963

French CE, Bruce NC (1995) Bacterial morphinone reductase is related to Old Yellow Enzyme. Biochem J 312(Pt 3):671–678

Friemann R, Lee K, Brown EN, Gibson DT, Eklund H, Ramaswamy S (2009) Structures of the multicomponent Rieske non-heme iron toluene 2,3-dioxygenase enzyme system. Acta Crystallogr D Biol Crystallogr 65:24–33

Fritsche W, Scheibner K, Herre A, Hofrichter M (2000) Fungal degradation of explosives: TNT and related nitroaromatic compounds. In: Spain JC, Hughes JB, Knackmuss H-J (eds) Biodegradation of nitroaromatic compounds and explosives. CRC Press, Boca Raton, pp 213–238

Funk SB, Roberts DJ, Crawford DL, Crawford RL (1993) Initial-phase optimization for bioremediation of munition compound-contaminated soils. Appl Environ Microbiol 59:2171–2177

Garmendia J, de las Heras A, Galvão TC, de Lorenzo V (2008) Tracing explosives in soil with transcriptional regulators of *P. putida* evolved for responding to nitrotoluenes. Microbiol Biotechnol 1:236–246

George SE, Huggins-Clark G, Brooks LR (2001) Use of a *Salmonella* microsuspension bioassay to detect the mutagenicity of munitions compounds at low concentrations. Mutat Res Genet Toxicol Environ Mutagen 490:45–56

González-Pérez MM, van Dillewijn P, Wittich RM, Ramos JL (2007) *Escherichia coli* has multiple enzymes that attack TNT and release nitrogen for growth. Environ Microbiol 9:1535–1540

Goodwin A, Kersulyte D, Sisson G, van Zanten SJOV, Berg DE, Hoffman PS (1998) Metronidazole resistance in *Helicobacter pylori* is due to null mutations in a gene (*rdxA*) that encodes an oxygen-insensitive NADPH nitroreductase. Mol Microbiol 28:383–393

Haïdour A, Ramos JL (1996) Identification of products resulting from the biological reduction of 2,4,6-trinitrotoluene, 2,4-dinitrotoluene, and 2,6-dinitrotoluene by *Pseudomonas* sp. Environ Sci Technol 30:2365–2370

Hannink N, Rosser SJ, French CE, Basran A, Murray JA, Nicklin S, Bruce NC (2001) Phytodetoxification of TNT by transgenic plants expressing a bacterial nitroreductase. Nat Biotechnol 19:1168–1172

Hannink NK, Subramanian M, Rosser SJ, Basran A, Murray JA, Shanks JV, Bruce NC (2007) Enhanced transformation of TNT by tobacco plants expressing a bacterial nitroreductase. Intl J Phytoremediation 9:385–401

Harayama S, Rekik M (1990) The meta cleavage operon of TOL degradative plasmid pWW0 comprises 13 genes. Mol Gen Genet 221:113–120

Harayama S, Rekik M, Wubbolts M, Rose K, Leppik RA, Timmis KN (1989) Characterization of five genes in the upper-pathway operon of TOL plasmid pWW0 from *P. putida* and identification of the gene products. J Bacteriol 171:5048–5055

Harter DR (1985) The use and importance of nitroaromatic compounds in the chemical industry. Heisphere Publishing, Washington, DC

Hawari J, Beaudet S, Halasz A, Thiboutot S, Ampleman G (2000) Microbial degradation of explosives: biotransformation versus mineralization. Appl Microbiol Biotechnol 54:605–618

Hofstetter TB, Heijman CG, Haderlein SB, Holliger C, Schwarzenbach RP (1999) Complete reduction of TNT and other (poly)nitroaromatic compounds under iron reducing subsurface conditions. Environ Sci Technol 33:1479–1487

Homma-Takeda S, Ohkuma Y, Hiraku Y, Oikawa S, Murata M, Ogawa K (2002) 2,4, 6-trinitrotoluene-induced reproductive toxicity via oxidative DNA damage by its metabolite. Free Radic Res 36:555–566

Honeycutt ME, Jarvis AS, McFarland VA (1996) Cytotoxicity and mutagenicity of 2,4, 6-trinitrotoluene and its metabolites. Ecotoxicol Environ Saf 35:282–287

Huang S, Lindahl PA, Wang C, Bennett GN, Rudolph FB, Hughes JB (2000) 2,4,6-trinitrotoluene reduction by carbon monoxide dehydrogenase from *Clostridium thermoaceticum*. Appl Environ Microbiol 66:1474–1478

Johnson GR, Smets BF, Spain JC (2001) Oxidative transformation of aminodinitrotoluene isomers by multicomponent dioxygenases. Appl Environ Microbiol 67:5460–5466

Kaplan DL, Kaplan AM (1982) Thermophilic biotransformations of 2,4,6-trinitrotoluene under simulated composting conditions. Appl Environ Microbiol 44:757–760

Kaplan LA, Siedle AR (1971) Studies on boron hydrides IV. Stable hydride Meisenheimer adducts. J Org Chem 36:937–939

Kato R, Oshima T, Takanaka A (1969) Studies on the mechanism of nitro reduction by rat liver. Mol Pharmacol 5:487–498

Keenan BG, Wood TK (2006) Orthric Rieske dioxygenases for degrading mixtures of 2,4-dinitrotoluene/naphthalene and 2-amino-4,6-dinitrotoluene/4-amino-2,6-dinitrotoluene. Appl Microbiol Biotechnol 73:827–838

Kennel SJ, Foote LJ, Morris M, Vass AA, Griest WH (2000) Mutation analyses of a series of TNT-related compounds using the CHO-hprt assay. J Appl Toxicol 20:441–448

Khan H, Harris RJ, Barna T, Craig DH, Bruce NC, Munro AW (2002) Kinetic and structural basis of reactivity of pentaerythritol tetranitrate reductase with NADPH, 2-cyclohexenone, nitroesters, and nitroaromatic explosives. J Biol Chem 277:21906–21912

Kong LY, Jiang QG, Qu QS (1989) Formation of superoxide radical and hydrogen peroxide enhanced by trinitrotoluene in rat liver, brain, kidney, and testicle in vitro and monkey liver in vivo. Biomed Environ Sci 2:72–77

Kroger M, Schumacher ME, Risse H, Fels G (2004) Biological reduction of TNT as part of a combined biological-chemical procedure for mineralization. Biodegradation 15:241–248

Kubota A, Maeda T, Nagafuchi N, Kadokami K, Ogawa HI (2008) TNT biodegradation and production of dihydroxylamino-nitrotoluene by aerobic TNT degrader *Pseudomonas* sp. strain TM15 in an anoxic environment. Biodegradation 19:795–805

Kutty R, Bennett GN (2005) Biochemical characterization of trinitrotoluene transforming oxygen-insensitive nitroreductases from *Clostridium acetobutylicum* ATCC 824. Arch Microbiol 184:158–167

Lachance B, Renoux AY, Sarrazin M, Hawari J, Sunahara GI (2004) Toxicity and bioaccumulation of reduced TNT metabolites in the earthworm *Eisenia andrei* exposed to amended forest soil. Chemosphere 55:1339–1348

Letzel S, Goen T, Bader M, Angerer J, Kraus T (2003) Exposure to nitroaromatic explosives and health effects during disposal of military waste. Occup Environ Med 60:483–488

Lewis TA, Goszczynski S, Crawford RL, Korus RA, Admassu W (1996) Products of anaerobic 2,4,6-trinitrotoluene (TNT) transformation by *Clostridium bifermentans*. Appl Environ Microbiol 62:4669–4674

Lewis TA, Newcombe DA, Crawford RL (2004) Bioremediation of soils contaminated with explosives. J Environ Manage 70:291–307

Maeda T, Kadokami K, Ogawa HI (2006a) Characterization of 2,4,6-trinitrotoluene (TNT)-metabolizing bacteria isolated from TNT-polluted soils in the Yamada Green Zone. J Environ Biotechnol 6:33–39

Maeda T, Nagafuchi N, Kubota A, Kadokami K, Ogawa HI (2006b) One-step isolation and identification of hydroxylamino-dinitrotoluenes, unstable products from 2,4,6-trinitrotoluene metabolites, with thin-layer chromatography and laser time-of-flight mass spectrometry. J Chromatogr Sci 44:96–100

Maeda T, Nagafuchi N, Kubota A, Kadokami K, Ogawa HI (2007a) Identification of spontaneous conversion products of unstable 2,4,6-trinitrotoluene metabolites, hydroxylamino-dinitrotoluenes, by combination of thin-layer chromatography and laser time-of-flight mass spectrometry. J Chromatogr Sci 45:345–349

Maeda T, Nakamura R, Kadokami K, Ogawa HI (2007b) Relationship between mutagenicity and reactivity or biodegradability for nitroaromatic compounds. Environ Toxicol Chem 26:237–241

Maroziene A, Kliukiene R, Sarlauskas J, Cenas N (2001) Methemoglobin formation in human erythrocytes by nitroaromatic explosives. Z Naturforsch C 56:1157–1163

Mason RP, Holtzman JL (1975) The role of catalytic superoxide formation in the O_2 inhibition of nitroreductase. Biochem Biophys Res Commun 67:1267–1274

Meah Y, Brown BJ, Chakraborty S, Massey V (2001) Old yellow enzyme: reduction of nitrate esters, glycerin trinitrate, and propylene 1,2-dinitrate. Proc Natl Acad Sci USA 98:8560–8565

Mizuta Y, Onodera T, Singh P, Matsumoto K, Miura N, Toko K (2008) Development of an oligo(ethylene glycol)-based SPR immunosensor for TNT detection. Biosens Bioelectron 24:191–197

Naal Z, Park JH, Bernhard S, Shapleigh JP, Batt CA, Abruna HD (2002) Amperometric TNT biosensor based on the oriented immobilization of a nitroreductase maltose binding protein fusion. Anal Chem 74:140–148

Nakazawa T, Yokota T (1973) Benzoate metabolism in *P. putida*(arvilla) mt-2: demonstration of two benzoate pathways. J Bacteriol 115:262–267

Nakazawa A, Nakazawa T, Kotani S, Nozaki M, Hayaishi O (1969a) Studies on pyrocatechase. 3. Circular dichroism and optical rotatory dispersion. J Biol Chem 244:1527–1532

Nakazawa T, Nozaki M, Hayaishi O, Yamano T (1969b) Sttudies on pyrocatechase II. Electron spin resonance and other properties of iron in the active center. J Biol Chem 244:119–125

Neuwoehner J, Schofer A, Erlenkaemper B, Steinbach K, Hund-Rinke TK, Eisentraeger A (2007) Toxicological characterization of 2,4,6-trinitrotoluene, its transformation products, and two nitramine explosives. Environ Toxicol Chem 26:1090–1099

Nishino SF, Spain JC (1993) Degradation of nitrobenzene by a *Pseudomonas pseudoalcaligenes*. Appl Environ Microbiol 59:2520–2525

Nokhbeh MR, Boroumandi S, Pokorny N, Koziarz P, Paterson ES, Lambert IB (2002) Identification and characterization of SnrA, an inducible oxygen-insensitive nitroreductase in *Salmonella enterica* serovar *Typhimurium* TA1535. Mutat Res 508:59–70

Orville AM, Manning L, Blehert DS, Fox BG, Chambliss GH (2004a) Crystallization and preliminary analysis of xenobiotic reductase B from *Pseudomonas fluorescens* I-C. Acta Crystallogr D Biol Crystallogr 60:1289–1291

Orville AM, Manning L, Blehert DS, Studts JM, Fox BG, Chambliss GH (2004b) Crystallization and preliminary analysis of xenobiotic reductase A and ligand complexes from *P. putida* II-B. Acta Crystallogr D Biol Crystallogr 60:957–961

Padda RS, Wang C, Hughes JB, Kutty R, Bennett GN (2003) Mutagenicity of nitroaromatic degradation compounds. Environ Toxicol Chem 22:2293–2297

Pak JW, Knoke KL, Noguera DR, Fox BG, Chambliss GH (2000) Transformation of 2,4, 6-trinitrotoluene by purified xenobiotic reductase B from *Pseudomonas fluorescens* I-C. Appl Environ Microbiol 66:4742–4750

Park HS, Kim HS (2000) Identification and characterization of the nitrobenzene catabolic plasmids pNB1 and pNB2 in *P. putida* HS12. J Bacteriol 182:573–580

Park HJ, Reiser CO, Kondruweit S, Erdmann H, Schmid RD, Sprinzl M (1992) Purification and characterization of a NADH oxidase from the thermophile *Thermus thermophilus* HB8. Eur J Biochem 205:881–885

Pennington JC (1999) Explosive. In: Anderson WC, Loehr RC, Smith BP (eds) Environmental availability in soils chlorinated organics explosives, metals. American Academy of Environmental Engineers Publication, New York, pp 85–109

Radhika V, Proikas-Cezanne T, Jayaraman M, Onesime D, Ha JH, Dhanasekaran DN (2007) Chemical sensing of DNT by engineered olfactory yeast strain. Nat Chem Biol 3:325–330

Rafii F, Selby AL, Newton RK, Cerniglia CE (1994) Reduction and mutagenic activation of nitroaromatic compounds by a *Mycobacterium* sp. Appl Environ Microbiol 60:4263–4267

Ramos JL, González-Pérez MM, Caballero A, van Dillewijn P (2005) Bioremediation of polynitrated aromatic compounds: plants and microbes put up a fight. Curr Opin Biotechnol 16:275–281

Rau J, Stolz A (2003) Oxygen-insensitive nitroreductases NfsA and NfsB of *Escherichia coli* function under anaerobic conditions as lawsone-dependent azo reductases. Appl Environ Microbiol 69:3448–3455

Renganathan V (1989) Possible involvement of toluene-2,3-dioxygenase in defluorination of 3-fluoro-substituted benzenes by toluene-degrading *Pseudomonas* sp. strain T-12. Appl Environ Microbiol 55:330–334

Riefler RG, Smets BF (2000) Enzymatic reduction of 2,4,6-trinitrotoluene and related nitroarenes: Kinetics linked to one-electron redox potentials. Environ Sci Technol 34:3900–3906

Rieger P-G, Knackmuss H-J (1995a) Basic knowledge and perspectives on biodegradation of 2,4,6-trinitrotoluene and related nitroaromatic compounds in contaminated soil. Plenum Press, New York

Rieger P-G, Knackmuss H-J (1995b) Basic knowledge and perspectives on biodegradation of 2,4,6-trinitrotoluene and related nitroaromatic compounds in comtaminated soil. In: Spain JC (ed) Biodegradation of nitroaromatic compounds. Plenum Press, New York, pp 1–8

Rieger PG, Sinnwell V, Preuss A, Francke W, Knackmuss HJ (1999) Hydride-Meisenheimer complex formation and protonation as key reactions of 2,4,6-trinitrophenol biodegradation by *Rhodococcus erythropolis*. J Bacteriol 181:1189–1195

Robidoux PY, Hawari J, Thiboutot S, Ampleman G, Sunahara GI (1999) Acute toxicity of 2,4,6-trinitrotoluene in earthworm (*Eisenia andrei*). Ecotoxicol Environ Saf 44:311–321

Sabbioni G, Sepai O, Norppa H, Yan H, Hirvonen A, Zheng Y (2007) Comparison of biomarkers in workers exposed to 2,4,6-trinitrotoluene. Biomarkers 12:21–37

Saka M (2004) Developmental toxicity of p,p′-dichlorodiphenyltrichloroethane, 2,4,6-trinitrotoluene, their metabolites, and benzo[a]pyrene in *Xenopus laevis* embryos. Environ Toxicol Chem 23:1065–1073

Shankaran DR, Gobi KV, Sakai T, Matsumoto K, Toko K, Miura N (2005) Surface plasmon resonance immunosensor for highly sensitive detection of 2,4,6-trinitrotoluene. Biosens Bioelectron 20:1750–1756

Smets BF, Yin H, Esteve-Nunez A (2007) TNT biotransformation: when chemistry confronts mineralization. Appl Microbiol Biotechnol 76:267–277

Snellinx Z, Nepovim A, Taghavi S, Vangronsveld J, Vanek T, van der Lelie D (2002) Biological remediation of explosives and related nitroaromatic compounds. Environ Sci Pollut Res Intl 9:48–61

Somerville CC, Nishino SF, Spain JC (1995) Purification and characterization of nitrobenzene nitroreductase from *Pseudomonas pseudoalcaligenes* JS45. J Bacteriol 177:3837–3842

Spain JC, Hughes JB, Knackmuss HJ (2000) Biodegradation of nitroaromatic compounds and explosives. Lewis, Boca Raton

Spanggord RJ, Mortelmans KE, Griffin AF, Simmon VF (1982) Mutagenicity in *Salmonella typhimurium* and structure-activity relationships of wastewater components emanating from the manufacture of trinitrotoluene. Environ Mutagen 4:163–179

Spanggord RJ, Stewart KR, Riccio ES (1995) Mutagenicity of tetranitroazoxytoluenes: a preliminary screening in *Salmonella typhimurium* strains TA100 and TA100NR. Mutat Res 335:207–211

Stenuit B, Eyers L, Rozenberg R, Habib-Jiwan JL, Matthijs S, Cornelis P, Agathos SN (2009) Denitration of 2,4,6-trinitrotoluene in aqueous solutions using small-molecular-weight catalyst(s) secreted by *Pseudomonas aeruginosa* ESA-5. Environ Sci Technol 43:2011–2017

Styles JA, Cross MF (1983) Activity of 2,4,6-trinitrotoluene in an in vitro mammalian gene mutation assay. Cancer Lett 20:103–108

Sun Y, Iemitsu M, Shimojo N, Miyauchi T, Amamiya M, Sumi D (2005) 2,4,6-Trinitrotoluene inhibits endothelial nitric oxide synthase activity and elevates blood pressure in rats. Arch Toxicol 79:705–710

Tadros MG, Crawford A, Mateo-Sullivan A, Zhang C, Hughes JB (2000) Toxic effects of hydroxylamino intermediates from microbial transformation of trinitrotoluene and dinitrotoluene on algae *Selenastrum capricornutum*. Bull Environ Contam Toxicol 64:579–585

Tan EL, Ho CH, Griest WH, Tyndall RL (1992) Mutagenicity of trinitrotoluene and its metabolites formed during composting. J Toxicol Environ Health 36:165–175

Tatsumi K, Koga N, Kitamura S, Yoshimura H, Wardman P, Kato Y (1979) Enzymic *cis/trans* isomerization of nitrofuran derivatives: isomerizing activity of xanthine oxidase, lipoyl dehydrogenase, DT-diaphorase and liver microsomes. Biochim Biophys Acta 567:75–87

Vaatanen AR (1997) Spectrum of spontaneous and 2,4,6-trinitrotoluene (TNT)-induced mutations in *Salmonealla typhymurium* strains with different nitroreductase and o-acetyltransferase activities. Mutat Res 379:185–190

Vorbeck C, Lenke H, Fischer P, Spain JC, Knackmuss HJ (1998) Initial reductive reactions in aerobic microbial metabolism of 2,4,6-trinitrotoluene. Appl Environ Microbiol 64:246–252

Watanabe M, Ishidate M Jr, Nohmi T (1990) Nucleotide sequence of *Salmonella typhimurium* nitroreductase gene. Nucleic Acids Res 18:1059

Watrous MM, Clark S, Kutty R, Huang S, Rudolph FB, Hughes JB, Bennett GN (2003) 2,4,6-trinitrotoluene reduction by an Fe-only hydrogenase in *Clostridium acetobutylicum*. Appl Environ Microbiol 69:1542–1547

Williams RE, Rathbone DA, Moody PC, Scrutton NS, Bruce NC (2001) Degradation of explosives by nitrate ester reductases. Biochem Soc Symp 68:143–153

Williams RE, Rathbone DA, Scrutton NS, Bruce NC (2004) Biotransformation of explosives by the old yellow enzyme family of flavoproteins. Appl Environ Microbiol 70:3566–3574

Wittich RM, Ramos JL, van Dillewijn P (2009) Microorganisms and explosives: mechanisms of nitrogen release from TNT for use as an N-source for growth. Environ Sci Technol 43:2773–2776

Wolper MK, Althans JR, Johns DG (1973) Nitroreductase activity of mammalian liver aldehyde oxidase. J Pharmacol Exp Ther 185:202–213

Won WD, DiSalvo LH, Ng J (1976) Toxicity and mutagenicity of 2,4,-6-trinitrotoluene and its microbial metabolites. Appl Environ Microbiol 31:576–580

Yin H, Wood TK, Smets BF (2005) Reductive transformation of TNT by *Escherichia coli*: pathway description. Appl Microbiol Biotechnol 67:397–404

Zenno S, Saigo K, Kanoh H, Inouye S (1994) Identification of the gene encoding the major NAD (P)H-flavin oxidoreductase of the bioluminescent bacterium *Vibrio fischeri* ATCC 7744. J Bacteriol 176:3536–3543

Zenno S, Koike H, Kumar AN, Jayaraman R, Tanokura M, Saigo K (1996a) Biochemical characterization of NfsA, the *Escherichia coli* major nitroreductase exhibiting a high amino acid sequence homology to Frp, a *Vibrio harveyi* flavin oxidoreductase. J Bacteriol 178:4508–4514

Zenno S, Koike H, Tanokura M, Saigo K (1996b) Conversion of NfsB, a minor *Escherichia coli* nitroreductase, to a flavin reductase similar in biochemical properties to FRase I, the major flavin reductase in *Vibrio fischeri*, by a single amino acid substitution. J Bacteriol 178:4731–4733

Ziganshin AM, Gerlach R, Borch T, Naumov AV, Naumova RP (2007) Production of eight different hydride complexes and nitrite release from 2,4,6-trinitrotoluene by *Yarrowia lipolytica*. Appl Environ Microbiol 73:7898–7905

Chapter 9
Biodegradation of Military Explosives RDX and HMX

Rita Singh and Antaryami Singh

9.1 Introduction

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) are important military explosives. They are toxic anthropogenic compounds and persist as environmental pollutants as a result of weapon manufacture, deployment, and decommissioning. Production, testing and use of these compounds has resulted in numerous acres of contaminated soils and groundwater near many munitions facilities (Pennington et al. 2005; Wingfors et al. 2006). Therefore, economical and efficient methods for treatment of wastewater and clean-up of soils or groundwater containing RDX and HMX are needed. The physico–chemical remedial strategies to clean up sites contaminated with these xenobiotic compounds are neither cost effective nor very efficient. Therefore, research is increasingly being focused on the biological methods for the degradation and elimination of these pollutants. Although RDX and HMX are xenobiotic compounds, microorganisms have been found capable of biotransforming them. Biodegradation is regarded today as a potential method for the treatment of explosives like RDX and HMX. A diverse range of microorganisms can utilize these xenobiotics as substrates, often mineralizing them or converting them into harmless products, and thus cleaning the environment. This chapter provides an overview of RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) and HMX (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine) biodegradation and

R. Singh (✉) · A. Singh
Defence Laboratory, Defence Research and Development Organization, Jodhpur, 342 011, INDIA
e-mail: singhritadr@yahoo.com

A. Singh
e-mail: antaryamisingh@yahoo.com

S. N. Singh (ed.), *Microbial Degradation of Xenobiotics*, Environmental Science and Engineering, DOI: 10.1007/978-3-642-23789-8_9,

biotransformation pathways. Biodegradation studies on these energetic chemicals under both aerobic and anaerobic conditions have been discussed.

9.1.1 Structure and Properties of RDX and HMX

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) are two widely used cyclic nitramine explosives. The nitramines are the most recently introduced class of organic nitrate explosives. RDX (Research Department Explosive) and HMX (High Melting Explosive) are the most prominent members of this class. RDX and HMX are considered to be oligomers, $[(CH_2NNO_2)_n$; n = 3 or 4, respectively] of the same repeating structural moiety, CH_2NNO_2. HMX is a big ring molecule, same as RDX but with an extra CH_2NNO_2 unit (Fig. 9.1).

Research Department Explosive, also known less commonly as cyclonite, hexogen and T4, and chemically as cyclotrimethylenetrinitramine, is an explosive nitramine widely used in military and industrial applications. RDX was first time synthesized by Hans Hemming in 1899. In 1920, it was patented as an explosive, and its further development at the War Department in Woolwich, UK led to its renaming as Royal Demolition Explosive. RDX is currently the most widely used military explosive (Rosenblatt et al. 1991). It is stable in storage and is considered as one of the most powerful and brisant of the military high explosives. The chemical formula for RDX is $C_3H_6N_6O_6$ and the molecular weight is 222.117. It has density of 1.806 g/cc and detonation velocity of 8.70 km/s. Its melting point is 205°C. RDX has very low solubility in water and has an extremely low volatility. RDX does not get sorbed to soil particles very strongly and hence can move into the groundwater from the soil. RDX is obtained by reacting concentrated nitric acid with hexamine (Luo et al. 2002). RDX is second in strength to nitroglycerin among common explosive substances. RDX has both military and civilian applications. As a military explosive, RDX can be used alone as a base charge for detonators or mixed with another explosive such as TNT to form cyclotols, which produce a bursting charge for aerial bombs, mines, and torpedoes. Common military uses of RDX have been as an ingredient in plastic bonded explosives, or plastic explosives which have been used as explosive fill in almost all types of munition compounds. Civilian applications of RDX include use in fireworks, in demolition blocks, as a heating fuel for food rations, and as an occasional rodenticide.

HMX, also called octogen, is the most powerful military explosive in current use. Like RDX, the name has been variously listed as High Melting explosive, Her Majesty's explosive, High-velocity Military explosive, or High-Molecular-weight RDX (Cooper 1996). Also known as cyclotetramethylene-tetranitramine, tetrahexamine tetranitramine, or octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine, HMX was first synthesized in 1930. Its molecule is an eight-membered ring of alternating carbon and nitrogen atoms, with a nitro group attached to each nitrogen atom. Because of its high molecular weight, it is one of the most powerful

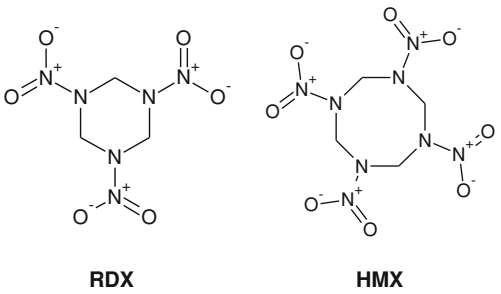


Fig. 9.1 Structure of RDX and HMX

chemical explosives manufactured. However, the high performance of HMX compared to RDX is offset by its high cost of production. The chemical formula is $C_4H_8N_8O_8$ and molecular weight is 296.20. It has density of 1.902 g/cc and detonation velocity of 9.11 km/s. It is a colorless solid with a melting point of 276–286°C. HMX is a man made chemical and does not occur naturally in the environment. HMX is made by the nitration of hexamine with ammonium nitrate and nitric acid in an acetic acid/acetic anhydride solvent. A small amount of HMX is also formed in making cyclotrimethylene-trinitramine (RDX). High Melting Explosive (HMX) explodes violently at high temperatures (534°F and above). Consequently, HMX is used exclusively for military purposes to implode fissionable material in nuclear devices, as a component of plastic-bonded explosives, as a component of rocket propellant, and as a high explosive burster charge.

9.1.2 RDX and HMX as Environmental Pollutants

Heterocyclic nitramines, hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), are powerful highly energetic chemicals. They commonly make up the bulk of modern explosive formulations because of a high stability and detonation power. Activities associated with manufacturing, training, waste disposal, and closures of bases have resulted in severe soil and groundwater contamination with these two explosives (Haas et al. 1990; Myler and Sysk 1991; Spain et al. 2000; Wingfors et al. 2006). RDX and HMX are extensively used for various military applications, particularly high explosive compositions for warheads to produce mass destructive effect and as an additive to produce high energy in the most advanced solid propellants for guns and rockets. RDX and HMX are used by the military in high-yield munitions, often in combination. Manufacture and testing of explosives have resulted in soil contamination with mixtures of these nitramine explosives. Large amounts of RDX and HMX contaminated waste water are produced in the activities of manufacturing, testing, training and demilitarizing. Careless disposal of munitions waste has created serious soil and groundwater contamination problems at numerous military sites (Testud et al. 1996). In the past, water used to wash production equipment was discarded into unlined trenches or lagoons where contaminants accumulated at very high levels (Garg et al. 1991). These explosives are very

persistent in the natural environment and can migrate from contaminated soil to surface water and groundwater (Monteil-Rivera et al. 2003; Clausen et al. 2004). Because of their solubility in water and weak binding affinity with soil, both chemicals migrate through sub-surface soil (Sheremata et al. 1999) to cause groundwater contamination. RDX and HMX are also widely used by many naval defence departments worldwide. Various naval military activities including testing, training, and demilitarization have resulted in the deposition of munitions and unexploded ordnance (UXO) in seas and waterways. Undersea deposition of unexploded ordnance (UXO) constitutes a potential source for contamination of marine environments by RDX and HMX (Bhatt et al. 2005). Munitions compounds leached from ruptured UXO are a major source of contamination because they can accumulate in sediment and aquatic organisms (Darrach et al. 1998). RDX and HMX, therefore, pose the largest potential threat to the environment, as they are produced and used in defence in high quantities. Widespread contamination of land and groundwater resulting from the use, manufacture, and storage of these military explosives, needs their safe removal from the environment.

9.1.3 Toxicity of RDX and HMX

RDX poses a significant risk to the human health. The effects of RDX on mammals are generally characterized by convulsions. There have been several reported cases of RDX toxicity to humans. Workers in RDX factories in Germany, Italy and U.S.A. have been seen suffering with symptoms including convulsions, unconsciousness, vertigo and vomiting after exposure, usually through the inhalation of RDX powder (Kaplan et al. 1965). Ingestion of RDX can adversely affect the central nervous system, gastro-intestinal tract, and kidneys (Rosenblatt 1980). RDX exposure to both dogs and rats results in irritability and convulsions as symptoms of chronic toxicity, and death in the rats was associated with congestion in the gastro-intestinal tract and lungs (Burdette et al. 1988). A study on a child who ingested plasticized RDX and developed seizures found that RDX can transport easily into the central nervous system (Woody et al. 1986). RDX toxicity can also cause weight loss associated with a reduction of food intake in rats (Levine et al. 1981). RDX is also used as a rodenticide. It has also been demonstrated to be toxic to other mammals, algae, invertebrates and fish (Testud et al. 1996). Based on its ecotoxicity, the U.S. Environmental Protection Agency (EPA) has classified RDX as a possible human carcinogen and priority pollutant for remediation.

Information on the adverse health effects of HMX is limited. HMX affects central nervous system (CNS) similar to those of RDX, but at considerably higher doses (Rosenblatt et al. 1991). HMX exposure has been investigated in several studies on animals. Studies in rats, mice, and rabbits indicate that HMX may be harmful to the liver and central nervous system, if it is swallowed or contacts the skin. However, mechanism by which HMX causes adverse effects on the liver and nervous system is not understood. HMX is poorly absorbed by ingestion. When

applied to the dermis, it induces mild skin irritation, but not delayed contact sensitization. Various acute and sub-chronic neurobehavioral effects have been reported in rabbits and rodents, including ataxia, sedation, hyperkinesia, and convulsions. The chronic effects of HMX that have been documented through animal studies include decreased hemoglobin, increased serum alkaline phosphatase, and decreased albumin. Pathological changes were also observed in animal livers and kidneys. The reproductive and developmental effects of HMX have not been well studied in humans or animals. At present, information needed to determine if HMX causes cancer is still insufficient (Rosenblatt et al. 1991). Due to the lack of information, EPA has not yet determined whether HMX causes human carcinogenicity or not.

The toxicity of RDX and HMX has been documented for most classes of organisms, including bacteria (Drzyzga et al. 1995), algae (Burton et al. 1994), plants (Robidoux et al. 2003), earthworms (Robidoux et al. 2000), aquatic invertebrates (Peters et al. 1991), and animals including mammals and humans (Goldberg et al. 1992; Testud et al. 1996; Lachance et al. 1999). Health advisory levels have been established by the US Environmental Protection Agency (USEPA) for several explosives, including HMX and RDX (USEPA 2004). The Drinking Water Equivalent Levels (DWELs) is 100 μg/L for RDX, while DWEL for HMX is significantly higher at 2 mg/L. EPA has also recommended long-term health advisories for HMX exposure for a 10 kg child and a 70 kg adult at 5 mg/L and 20 mg/L, respectively (McLellan et al. 1992). The Office of the Surgeon General has recommended a 24 h average maximum allowable concentration of 0.3 mg/L for RDX (Rosenblatt et al. 1991). The US Environmental Protection Agency has listed the cyclic nitramine RDX as a priority pollutant and HMX as a contaminant of concern (USEPA 2004). RDX and HMX are toxic hazardous compounds, which need to be removed from affected environments.

9.1.4 Treatment Methods for RDX and HMX Contamination

Explosive compounds, including hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), pose the largest potential threat to the environment as they are produced and used in defence in very large quantities. RDX and HMX are widespread contaminants at many current and former military facilities. Explosives contamination is of major concern due to the large number of affected sites (Levsen et al. 1993; Griest et al. 1995) and also because of their toxicity and mutagenicity (Lachance et al. 1999; Talmage et al. 1999). Many methods for the remediation of RDX and HMX have been reported. Current practices to treat RDX and HMX contaminated soils include incineration, composting, alkaline hydrolysis/oxidation, and aqueous thermal decomposition (Garg et al. 1991). These ex situ approaches, however, are expensive and have technical hurdles. Open burning and open detonation is not encouraged by regulatory agencies for disposal of explosives. Hence, there is need for a safe technology to degrade these contaminants.

Ex situ and in situ processes have been employed for the remediation of RDX and HMX contaminated groundwater. Ex situ processes include the treatment of pumped groundwater in granular activated carbon units, anaerobic bioreactors, electrochemical cells, and UV-oxidation reactors. However, all these practices have the disadvantage of high pumping and re-injection costs (Vanderloop et al. 1997; Hansen et al. 2001). Although granular activated carbon (GAC) effectively eliminates RDX and HMX from the groundwater (Carter et al. 1992; Morley et al. 2005), this treatment method requires long term pumping of contaminated groundwater, resulting in excessive operation, maintenance, and disposal costs. Because of the limitations of pump and treat groundwater remediation, methods for in situ groundwater remediation are being explored to reduce the time and costs for remediation. However, in situ reduction processes using zero-valent iron (Oh et al. 2001; Naja et al. 2008) have the disadvantages of high materials expense and repeated injections for soluble electron donor technologies, such as molasses, lactate, and hydrogen-release compound (Heaston et al. 2001). This necessitates the development of a relatively cheap and passive groundwater remediation alternative for treating RDX and HMX.

Traditional treatments of toxic ammunition wastes (e.g., open burning and open detonation, adsorption onto activated carbon, photooxidation, etc.) are costly and damaging for the environment, and in many cases, practically infeasible. There is, therefore, a considerable interest in developing cost-effective biological alternatives based on microorganisms. Bioremediation has been proposed as a safe and also cost-effective method of cleaning explosive contaminated sites (Kaplan 1990; Myler and Sysk 1991). Energetic compounds may undergo transformation through biotic or abiotic degradation. Numerous microorganisms have been isolated with the ability to degrade/transform energetic compounds as a sole carbon source, sole nitrogen source, or through co-metabolic processes under aerobic or anaerobic conditions. The microbial process can be used by itself, or it can augment or support other technologies for the treatment of energetic materials.

9.2 Biodegradation of RDX and HMX

The non-aromatic cyclic nitramine explosives hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) have weak C–N bonds. Initial enzymatic attack able to change N–NO_2 or C–H bonds of the cyclic nitramine can readily destabilize the cyclic structure and cause further molecular fragmentation. Several chemical studies (Zhao et al. 1988; Brill 1990; Sewell and Thompson 1991; Wight and Botcher 1992) have shown that once the non-aromatic cyclic nitramine RDX or HMX undergoes a change in its molecular structure, the ring collapses to produce small nitrogen-containing (N_2O, NO_2, NH_3) and small carbon-containing (HCHO, HCOOH and CO_2) products. The behaviour of RDX and HMX is distinguishable from that of the aromatic compound, TNT, which is biotransformed under several aerobic and anaerobic conditions to produce stable intermediates (amines, acetyl derivatives, azo and azoxy

compounds) while maintaining its stable aromatic ring structure (Hawari et al. 1998, 1999).

Biodegradation of RDX and HMX has been demonstrated under both aerobic and anaerobic conditions (McCormick et al. 1981; Kitts et al. 1994; Young et al. 1997; Hawari 2000; Price et al. 2001; Fuller et al. 2009; Halasz et al. 2010). RDX and HMX are cyclic nitrogen-containing compounds that are moderately resistant to aerobic degradation and undergo ring cleavage and extensive mineralization (Hawari 2000). Anaerobic degradation of RDX and HMX involves direct microbial reduction of the nitro functional groups on the cyclic structure. This process has been used to develop remedial strategies employing microbial degradation to address RDX and HMX contamination (Doppalapudi et al. 2002; Morley et al. 2002; Kwon and Finneran 2006; Young et al. 2006). Possible microbial metabolic functions of RDX and HMX include (a) electron donor for metabolic and respiratory redox reactions (b) carbon substrate for growth and metabolism, or (c) nitrogen substrate for growth and metabolism (Hawari 2000). The potential for anaerobic microbial degradation of RDX has been demonstrated under nitrate-reducing (Freedman and Sutherland 1998), sulfate-reducing (Boopathy et al. 1998a, b), and methanogenic conditions (Boopathy et al. 1998b). Biodegradation uses composting systems (Isbister et al. 1984; Griest et al. 1995), soil slurries (Lenke et al. 1998; Shen et al. 1998) or with pure cultures of bacteria, both aerobic (Binks et al. 1995) and anaerobic (Kitts et al. 1994). However, RDX and HMX with nitrogen-containing heterocyclic molecular structure, is moderately recalcitrant to microbial attack under oxygen-rich conditions (Kwon and Finneran 2006). Partial mineralization by microorganisms leads to formation of end products like CO_2, N_2O, NO_2^- and NH_3.

In general, RDX is more amenable to biodegradation than HMX (McCormick et al. 1981; Kitts et al. 1994). RDX and HMX are structurally similar and are constructed of $CH_2 = N–NO_2$ monomeric units. However, HMX is less water soluble (5 mg/L) than RDX (40 mg/L) (Yinon 1990) and is chemically more stable (Akhavan 1998). RDX, which exists preferentially in a chair conformation, and HMX, which exists in a crown type conformation, resemble their corresponding cyclohexyl and cyclooctyl compounds in reactivity. The transition state of HMX experiences more steric effects than RDX due to the crowding of atoms in the $CH_2–N–NO_2$ reacting group of the nitramine. Therefore, the reaction involving loss of NO_2 in HMX demands more energy than the corresponding reaction of RDX (Croce and Okamoto 1979). Consequently, differences are also observed in the biodegradability of RDX and HMX (Jones et al. 1995b).

9.2.1 Biodegradation of RDX and HMX under Anaerobic Conditions

Biodegradation of RDX and HMX under anaerobic condition has been extensively studied (McCormick et al. 1981; Hawari et al. 2000a; Adrian and Arnett 2004; Bhatt et al. 2005). Anaerobic reduction of RDX and HMX can be achieved in

several different ways. RDX biodegradation under anaerobic conditions was first reported by McCormick et al. (1981), who proposed RDX biodegradation by reduction of the RDX nitro (–NO_2) groups to nitroso (–NO) groups to produce hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX); hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX); and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX). The nitroso compounds undergo further transformation to unstable hydroxylamine derivatives, HONH-RDX, that subsequently undergo ring cleavage to eventually yield methanol, 1,1-dimethylhydrazine, 1,2-dimethylhydrazine, and formaldehyde. McCormick et al. (1981) also included methylenedinitramine (III) [$CH_2(NHNO_2)_2$] and nitramine (NH_2NO_2) in their degradation pathway. However, nitrous oxide (N_2O) and carbon dioxide were not observed despite previous chemical evidence that both methylenedinitramine (III) and nitramine decompose in water to produce N_2O (Lamberton et al. 1949a, b). Major end products formed during RDX biodegradation in a municipal anaerobic sludge were N_2O and CO_2 (Hawari et al. 2000b) similar to those formed during the thermal, chemical, photolytic and hydrolytic cleavage of RDX (Croce and Okamoto 1979; Zhao et al. 1988; Agrawal and Tratnyek 1996; Singh et al. 1998). McCormick et al. (1981) reported that ring cleavage occurred only via the primary hydroxylamino derivatives of RDX, HOHN–RDX, rather than through RDX itself. Other mechanisms including the enzymatic cleavage of the inner -N–C–N- or outer N–NO_2 bonds in RDX are strong possibilities. In several recent studies on the examination of hypothetical metabolites proposed in McCormick's pathway, only a few were confirmed, several intermediates were excluded, and many other new metabolites were identified (Hawari et al. 2000b; Adrian and Chow 2001; Zhang and Hughes 2003). The full product analysis of RDX biodegradation is particularly challenging because it involves gas-phase mineralization products, unstable nitroso- and hydroxyamino intermediates, as well as small molecules such as formaldehyde and methanol. Hawari et al. (2000b) proposed that RDX biodegradation is due to a combination of biological and chemical processes. Enzymatic attack on the inner C–N bond of RDX produces two unstable metabolites, methylenedinitramine and bis-(hydroxymethyl) nitramine. These two metabolites hydrolyze rapidly to produce nitramine, formic acid, and formaldehyde, which are further degraded biotically and abiotically to carbon dioxide, nitrous oxide, methane, and water.

Biodegradation of RDX and HMX in soil and water under anaerobic conditions has been extensively reported (Funk et al. 1993; Gorontzy et al. 1994; Boopathy et al. 1997; Guiot et al. 1999; Hawari et al. 2000b; Adrian and Arnett 2004; Bradley and Dinicola 2005). RDX and HMX can be degraded by either anaerobic sludge (McCormick et al. 1981), electron-accepting agents under nitrate reducing, sulfidogenic, acetogenic and methanogenic conditions (Boopathy et al. 1998a, b; Freedman and Sutherland 1998; Adrian et al. 2003; Bradley and Dinicola 2005; Sherburne et al. 2005) or mixed microbial cultures (Funk et al. 1993; Young et al. 1997; Harkins 1998; Huang 1998). Kitts et al. (1994) used three soil isolates (*Providencia rettgeri, Citrobacter freundii, and Morganella morganii)* of the Enterobacteriaceae family to degrade both RDX and HMX under O_2-depleting conditions. They identified only the nitroso derivatives (MNX, DNX and TNX) as

metabolites. They did not identify the carbon containing (HCHO and HCOOH) or nitrogen containing [N_2O, NH_2NH_2 $(CH_3)_2NNH_2$] ring cleavage intermediates. Degradation initiated by nitroreductases has been suggested as a potential degradation route for cyclic nitramines under anaerobic conditions (Kitts et al. 2000). The authors reported the degradation of RDX by the enteric bacteria *Morganella morganii* via the oxygen-insensitive type I nitroreductase (a two-electron transfer process). Boopathy et al. (1998a) studied the metabolism of RDX and HMX using the sulphate reducing bacterium *Desulfovibrio* sp. and observed the formation of ammonia which was subsequently used as a nitrogen source by the degrading microorganism. Young et al. (1997) studied the biological breakdown of RDX in mixed soil slurry reactors under anaerobic conditions using either indigenous microorganisms in horse manure or the single isolate *M. morganii*. A complicated three-phase kinetic model was derived to postulate the degradation pathway of RDX based on the appearance and disappearance of the three nitroso metabolites (MNX, DNX and TNX) of RDX. Shen et al. (2000) compared native soil microbial populations and unadapted municipal anaerobic sludges for nitramine explosive degradation in microcosm assays under various conditions. Microbial populations from an explosive-contaminated soil were only able to mineralize 12% RDX (at a concentration of 800 mg/kg slurry) or 4% HMX (at a concentration of 267 mg/kg slurry). In contrast, municipal anaerobic sludges were able to mineralize them to carbon dioxide, with efficiencies of up to 65%. Reduction of RDX and HMX into their corresponding nitroso-derivatives was notably faster than their mineralization. The biodegradation of HMX was delayed by the presence of RDX in the microcosm, confirming RDX is used as an electron acceptor preferentially to HMX. RDX is preferentially degraded over HMX which is in line with their chemical stabilities (Akhavan 1998).

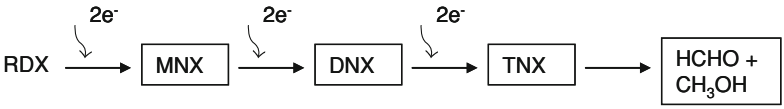
Zhao et al. (2002) investigated degradation of RDX by a facultative anaerobic bacterium which was isolated from the anaerobic sludge and identified as *Klebsiella pneumoniae* strain SCZ-1. Strain SCZ-1 degraded RDX to formaldehyde (HCHO), methanol (CH_3OH), carbon dioxide (CO_2), and nitrous oxide (N_2O) through intermediary formation of methylenedinitramine ($O_2NNHCH_2NHNO_2$). Likewise, hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) was degraded to HCHO, CH_3OH, and N_2O. These findings suggested the possible involvement of a common initial reaction, possibly denitration, followed by ring cleavage and decomposition in water. The trace amounts of MNX detected during RDX degradation and the trace amounts of hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine detected during MNX degradation suggested that another minor degradation pathway was also present that reduced ONO_2 groups to the corresponding ONO groups. Zhao et al. (2004a) evaluated in situ degradation of the two nitramine explosives in cold marine sediment under anaerobic and oligotrophic conditions. The RDX concentration (14.7 mg/L) in the aqueous phase was reduced by half in 4 days, while reduction of HMX concentration (1.2 mg/L) by half required 50 days. Supplementation with the carbon sources like glucose, acetate, or citrate did not affect the removal rate of RDX, but improved removal of HMX. Optimal mineralization of RDX and HMX was obtained in the presence of glucose.

The disappearance of RDX was accompanied by the formation of the mononitroso derivative hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) and formaldehyde (HCHO) that subsequently disappeared. The disappearance of HMX was also accompanied by the formation of the mononitroso derivative. Based on the distribution of products, the authors suggested that the sediment microorganisms degrade RDX and HMX via an initial reduction to the corresponding mononitroso derivative, followed by denitration and ring cleavage.

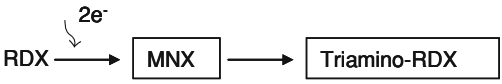
An et al. (2010) investigated the performance of anaerobic mesophilic granular sludge for the degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). Anaerobic granular sludge exhibited good performance in treating RDX as the sole substrate. Biodegradation was the main mechanism responsible for RDX removal. Ammonium had no significant promoting effect on the degradation process. The presence of glucose was found to enhance the degradation of RDX by anaerobic granular sludge, while the addition of sulfate and nitrate had adverse effects on the reductive transformation of RDX. RDX is difficult to degrade because its heterocyclic ring is firm and hard to cleave. Under anaerobic conditions, the microorganisms in granular sludge are capable of degrading RDX and using it as the sole source of carbon in the absence of a co-substrate. Various anaerobic bacteria in the granular sludge, including hydrolytic, fermentative, acetogenic, and methanogenic groups, may use a wide range of metabolic products. Beside N-nitrosoderivatives, it has been observed that RDX could be transformed to many other metabolites, such as formaldehyde, formic acid and methanol, either as the product of direct ring cleavage or further breakdown of nitroso intermediates. Such reducing equivalents, which are known as growth substrates, could also serve as the source of energy for RDX transformation by anaerobic bacteria in granular sludge. Authors concluded that RDX is capable of acting as electron acceptor as well as electron donor in the absence of suitable exogenous donors. For anaerobic degradation of RDX, detections of the nitroso intermediates, such as MNX, DNX, TNX, in culture, indicate the existence of similar intermediates between granular sludge microorganisms and those found in anaerobic sewage sludge from municipal wastewater treatment plant (McCormick et al. 1981). Thus, the granular sludge microorganisms are able to degrade RDX via sequential reduction of nitro groups to nitroso groups and ring cleavage. Biodegradation of RDX by anaerobic granular sludge is a complex process, involving a number of bacteria and steps in a sequential manner. Various groups of microorganisms in anaerobic granular sludge could utilize RDX with high efficiency.

9.2.1.1 Biotransformation Pathways of RDX

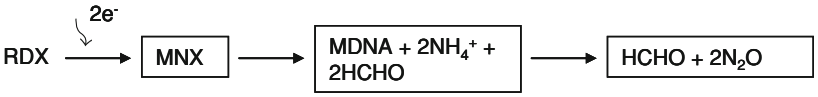
Metabolic pathways involved in the biodegradation of the cyclic nitramines RDX and HMX have been reviewed (Hawari et al. 2000a; Crocker et al. 2006). The potential degradation routes known for RDX are presented in Fig. 9.2. Three mechanisms for the transformation of RDX have been proposed: two-electron reduction, single-electron reduction/denitration, and direct enzymatic cleavage.



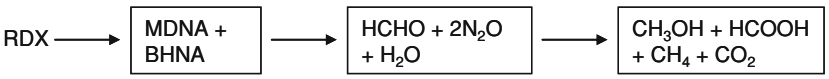
Path A: Reduction of RDX to nitroso derivatives before ring cleavage (McCormick et al, 1981)



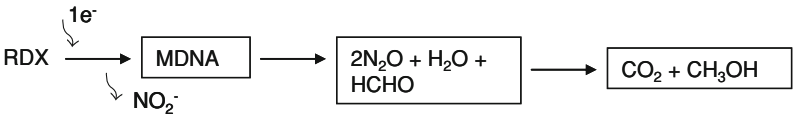
Path B: Reduction of RDX to 1,3,5-triamino-1,3,5-triazine (Zhang and Hughes, 2003)



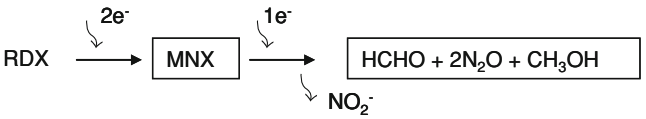
Path C: Reduction of RDX via nitrate oxidoreductase enzyme (Bhushan et al, 2002)



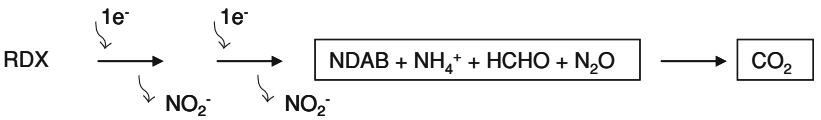
Path D: Direct enzymatic cleavage of RDX (Hawari et al, 2000b)



Path E: Anaerobic denitration of RDX (Zhao et al, 2002)



Path F: Denitration of RDX via the reductive intermediate MNX (Zhao et al, 2003)



Path G: Aerobic denitration of RDX (Bhushan et al, 2003a; Fournier et al, 2002)

Fig. 9.2 Proposed biodegradation pathways for RDX. *MNX* hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine, *DNX* hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine, *TNX* hexahydro-1,3,5-trinitroso-1,3,5-triazine, *MDNA* methylenedinitramine, *BHNA* bis-(hydroxymethyl)nitramine, *NDAB* 4-nitro-2,4-diazabutanal

The two-electron reductive pathway (Fig. 9.2, Path A), as originally proposed by McCormick et al. (1981), follows the reduction of RDX in a stepwise process to hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX). Nitroso metabolites are postulated to undergo further reduction prior to ring cleavage and transformed into hydroxylamino-RDX intermediate, which is followed by ring cleavage yielding hydrazines, methanol and formaldehyde. This two-electron reduction pathway serves as a minor RDX degradation route in some bacteria like *Klebsiella pneumoniae* strain SCZ-1, *Clostridium bifermentans* strain HAW-1, *Shewanella halifaxensis* strain HAW-EB4, and *Shewanella* sp. HAW-EB5 (Zhao et al. 2002, 2003, 2004b, 2006). However, the pathway is reported to be the major route of degradation in *Methylobacterium* sp., enterobacteria, *Shewanella* sp. HAW-EB2 (Kitts et al. 1994; Van Aken et al. 2004; Zhao et al. 2004b; Fournier et al. 2005).

Two-electron reduction pathway that diverges from MNX in the McCormick pathway has also been proposed as an alternate mechanism (Fig. 9.2, Path B). RDX is transformed through MNX, hydroxylamino, and amino intermediates to 1,3,5-triamino-1,3,5-triazine in the presence of H_2 by the cell-free extracts of *Clostridium acetobutylicum* (Zhang and Hughes 2003). Ring cleavage is not observed, and the triamino compound accumulated account for 91% of the carbon mass balance. Similarly, a nitrate oxidoreductase from *Aspergillus niger* has been shown to reduce RDX to ammonium, nitrous oxide and formaldehyde in a cell-free system (Bhushan et al. 2002). The reduced intermediate, MNX, and a ring cleavage product, methylenedinitramine (MDNA), were formed as transient intermediates, but DNX and TNX were not observed (Fig. 9.2, Path C). MNX was shown to be the precursor of MDNA and it was presumed to be reduced by additional reductive steps.

A pathway based on the direct enzymatic cleavage of inner C–N, N–NO_2, or methylene C–H bonds has been proposed (Hawari et al. 2000b; Halasz et al. 2002) resulting in the formation of MDNA and bis-(hydroxymethyl)nitramine as the major intermediates in the anaerobic biotransformation of RDX by sewage sludge (Fig. 9.2, Path D). MDNA is proposed to be produced due to the cleavage of a C–N bond by a hydrolase or the enzymatic α-hydroxylation of a methylene bond. Bis-(hydroxymethyl)nitramine and MDNA did not accumulate in the sludge microcosms, but disappeared to form nitrous oxide, traces of nitrogen gas, formaldehyde, formic acid, methanol, carbon dioxide, and methane (Hawari et al. 2000b). The MDNA was shown to spontaneously decompose in water to nitrous oxide and formaldehyde (Halasz et al. 2002), and bis-(hydroxymethyl)nitramine was also proposed to spontaneously decompose in water to nitrous oxide and formaldehyde (Hawari et al. 2000b). Denitration, has also been proposed as the major route of RDX biotransformation (Fig. 9.2, Paths E and F). Anaerobic denitration involves a single-electron transfer that may occur by an oxygen-sensitive, type II nitroreductase generating a free-radical anion (RDX^-) and subsequently releasing a nitro group. The loss of the nitro group destabilizes the molecule leading to ring cleavage and the formation of one molecule of MDNA

and other unknown intermediates. The MDNA spontaneously decomposes in water to nitrous oxide and formaldehyde (Halasz et al. 2002). The formaldehyde, in turn, can be biotransformed to methanol, formate, or carbon dioxide by various bacteria. *Klebsiella pneumoniae* strain SCZ-1 and *Clostridium bifermentans* strain HAW-1 are reported to use denitration as the main pathway for biodegradation of RDX and MNX. In *Klebsiella pneumoniae* strain SCZ-1, the denitration of RDX is the major pathway (Fig. 9.2, Path E), while reduction to MNX followed by denitration of MNX (Fig. 9.2, Path F) is a minor route (Zhao et al. 2002). In contrast, in *Clostridium bifermentans* strain HAW-1, the reduction of RDX to MNX occurs before denitration (Fig. 9.2, Path F), and methanol, formaldehyde, carbon dioxide and nitrous oxide are formed (Zhao et al. 2003).

9.2.1.2 Biotransformation Pathways of HMX

The biodegradation mechanism for HMX has been found to be similar to that of RDX. Under anaerobic conditions, nitroso derivatives are formed as intermediates from the two-electron reduction of the nitro groups on the HMX ring (Fig. 9.3, Path A). Usually, octahydro-1-nitroso-3,5,7-trinitro-1,3,5,7-tetrazocine (1NO–HMX) (Zhao et al. 2004a, b) is formed, but formation of octahydro-1, 3-dinitroso-5,7-dinitro-1,3,5,7-tetrazocine or octahydro-1,5-dinitroso-3,7-dinitro-1,3,5,7-tetrazocine (2NO–HMX) (Hawari et al. 2001; Zhao et al. 2004b), and occasionally octahydro-1,3,5-trinitroso-7-nitro-1,3,5,7-tetrazocine (3NO–HMX) (Boopathy 2001; Zhao et al. 2004b) and octahydro-1,3,5,7-tetranitroso-1,3,5,7-tetrazocine (4NO–HMX) (Monteil-Rivera et al. 2003), are also reported. The fate of these nitroso derivatives is unknown, as they are produced transiently but in trace amounts.

Hydroxylamino-HMX derivatives and other proposed intermediates from the McCormick pathway (McCormick et al. 1981) have not been observed (Hawari et al. 2001; Bhushan et al. 2003a; Monteil-Rivera et al. 2003). HMX is biotransformed by the sewage sludge to the ring cleavage intermediates MDNA and bis(hydroxymethyl) nitramine (Fig. 9.3, Path B), which was later transformed to formaldehyde, formic acid, and nitrous oxide (Hawari et al. 2001). The sludge is expected to contain acetogenic, methanogenic, and denitrifying bacteria which may be responsible for the production of carbon dioxide and methane from formic acid, and nitrogen gas from nitrous oxide. The enzymatic study with xanthine oxidase from buttermilk provided an evidence for the single-electron reduction/denitration of HMX (Bhushan et al. 2003a). The in vitro biotransformation of HMX was accompanied by the transient accumulation of the ring cleavage products MDNA and NDAB, and the end products nitrite, nitrous oxide, ammonium, formaldehyde, and formic acid (Fig. 9.3, Path C). The absence of nitroso intermediates and the presence of nitrite and NDAB were taken as an evidence for single-reductive denitration mechanism, analogous to RDX denitration.

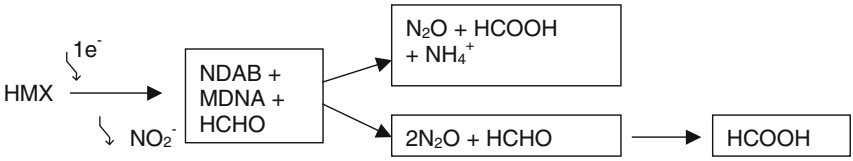
9.2.2 Biodegradation of RDX and HMX under Aerobic Conditions

Several groups have studied aerobic metabolism of RDX and HMX and have demonstrated the potential for these energetic chemicals to be mineralized (Binks et al. 1995; Jones et al. 1995a, b; Greer et al. 1997; Coleman et al. 1998; Tekoah and Abeliovich 1999; Sheremata and Hawari 2000). Binks et al. (1995) showed that *Stenotrophomonas maltophilia* can degrade RDX, but not HMX when the cyclic nitramine is used as a nitrogen source. Harkins et al. (1999) reported the formation of five nitroso derivatives (two di-isomers, and mono-, tri- and tetra-nitroso derivatives) when HMX was treated in water under aerobic conditions using livestock manure and indigenous microorganisms from a contaminated site. Several aerobic bacteria have been isolated based on their ability to use RDX as a sole nitrogen source incorporating three of the six RDX nitrogen atoms into biomass. Aerobic RDX degraders, such as *Stenotrophomonas* strain PB1 (Binks et al. 1995), *Rhodococcus* sp. strain A (Jones et al. 1995b), *Rhodococcus* sp. strain DN22 (Coleman et al. 1998), and *Rhodococcus rhodochrous* strain 11Y (Seth-Smith et al. 2002) grow on RDX using as sole nitrogen source. Van Aken et al. (2004) have also reported that aerobic *Methylobacterium* sp. strain BJ001 was able to metabolize RDX and HMX under aerobic conditions.

Products from biodegradation of cyclic nitramine explosives under aerobic conditions are poorly understood, particularly ring cleavage products (Binks et al. 1995; Hawari 2000). Jones et al. (1995b) isolated a *Rhodococcus* sp. strain A from explosives-contaminated soil and demonstrated its potential for the degradation of RDX, but did not report any products. Binks et al. (1995) reported the formation of two products from the degradation of RDX with *Stenotrophomonas maltophilia*. One product was identified as methylene-N-(hydroxymethyl)-hydroxylamine-N-(hydroxymethyl) nitramine and the second as the chloride salt of methylene-N-nitroamino-N-acetoxyammonium chloride. Coleman et al. (1998) reported the isolation and characterization of another *Rhodococcus* sp. strain DN22, which efficiently degrades RDX with the production of NO_2^-. No other products have been identified during RDX biodegradation with DN22. Several authors (Binks et al. 1995; Jones et al. 1995b; Coleman et al. 1998; Coleman and Duxbury 1999; Tekoah and Abeliovich 1999) did not observe any of the nitroso derivatives under aerobic conditions. Coleman and Duxbury (1999) suggested the involvement of cytochrome P-450 in the degradation of RDX by *Rhodococcus* sp. Strain DN22. No products were identified other than nitrite. Tekoah and Abeliovich (1999) also reported the involvement of cytochrome P-450 in the degradation of RDX by *Rhodococcus* sp. strains YH11. However, the study did not identify any metabolites other than nitrite. Harkins (1998) employed mixed cultures from horse manure to degrade RDX and HMX separately using dextrose and alfalfa as supplementary carbon sources. Gram-negative bacteria (*Alcaligenes* sp., *Hydrogenophaga flava* and *Xanthomonas oryzae*) and several facultative bacteria (*Escherichia coli*, *Kingella kingae* and *Capnocytophaga canimorus*) were tested. The nitramines RDX and HMX disappeared in 9 days and produced the

Path A: Reduction of HMX to nitroso derivatives before ring cleavage (McCormick et al, 1981)

Path B: Direct enzymatic cleavage of HMX (Hawari et al, 2001)



Path C: Denitration of HMX via xanthine oxidase enzyme (Bhushan et al, 2003a)

Fig. 9.3 Proposed biodegradation pathways for HMX. *1NO-HMX* octahydro-1-nitroso-3,5,7-trinitro-1,3,5,7-tetrazocine, *2NO-HMX* octahydro-1,3-dinitroso-5,7-dinitro-1,3,5,7-tetrazocine or octahydro-1,5-dinitroso-3,7-dinitro-1,3,5,7-tetrazocine, *3NO-HMX* octahydro-1,3,5-trinitroso-7-nitro-1,3,5,7-tetrazocine, *4NO-HMX* octahydro-1,3,5,7-tetranitroso-1,3,5,7-tetrazocine, *MDNA* methylenedinitramine, *BHNA* bis-(hydroxymethyl)nitramine, *NDAB* 4-nitro-2,4-diazabutanal

corresponding nitroso derivatives. Harkins (1998) concluded that the nitramine explosives were neither used as a carbon source nor as a nitrogen source. Fournier et al. (2002) detected nitrite, nitrous oxide, ammonia, formaldehyde and CO_2 in addition to a dead-end metabolite ($C_2H_5N_3O_3$), possibly a structural isomer of formyl-methylamino-nitroamine. The initial denitration of the RDX molecule by Rhodococci requires the involvement of a cytochrome P-450 (Coleman et al. 2002; Seth-Smith et al. 2002; Bhushan et al. 2003b). Denitration is followed by ring cleavage and the production of nitrous oxide (N_2O), ammonia (NH_3), formaldehyde (HCHO), and a dead-end product identified as 4-nitro-2,4-diazabutanal (NDAB) (Fournier et al. 2004a). Hawari et al. (2000b) assumed that following any initial enzymatic attack on RDX leading to the cleavage of any of the bonds in RDX (inner NOC or external $NONO_2$ and COH bond), the resulting intermediate becomes very unstable. Once an external bond in RDX is cleaved, some of the inner CON bonds would spontaneously decompose to produce N_2, N_2O, HCHO, and HCOOH. The fate of the resulting intermediates will be determined by competitive microbial and chemical processes. Most of the products are thermally unstable and undergo fast hydrolytic cleavage in water.

Some potential pathways have been postulated for the biodegradation of cyclic nitramines under aerobic conditions based on the information of the product distribution and the enzymes involved. Reduction of the nitro groups in RDX by nitroreductases can take place either via two electron transfer process to produce nitroso derivatives or via a one electron transfer process to produce the free anion radical of RDX (or HMX). It has also been reported that RDX nitro anion radical reverts back to the nitro compound in the presence of oxygen (McCalla et al. 1970; Peterson et al. 1979). The denitration of the anion radical to give the corresponding RDX (or HMX) radical is also proposed. This later transient intermediate would then undergo rapid ring cleavage to produce the end product. The absence of nitroso RDX derivatives as degradation products in several studies (Binks et al. 1995; Coleman et al. 1998) supports the occurrence of a denitration mechanism. Furthermore, the superoxide anion radical, O_2^-, generated from the reaction of oxygen with RDX anion radical is extremely reactive and its presence may contribute to the degradation of the cyclic nitramine. Several studies identified nitrite as the metabolite during biodegradation of RDX under aerobic conditions (Binks et al. 1995; Coleman et al. 1998). However, as the nitrite did not accumulate, it was suspected that the microorganisms used this ion as their nitrogen source. Jones et al. (1995a) showed that the disappearance of RDX initially produced nitrite that subsequently disappeared with the detection of traces of ammonium ions. Coleman et al. (1998) isolated and identified *Rhodococcus* sp. Strain D22 from an RDX-contaminated site that used nitrite liberated from RDX as its sole nitrogen source. The liberated nitrite was transformed to ammonium enzymatically prior to its use by the microorganism. In contrast, the presence of ammonium (NH_4^+) has been shown to inhibit RDX mineralization (Yang et al. 1983; Binks et al. 1995; Coleman et al. 1998). Ammonium competes with RDX as a nitrogen source, and thus RDX degradation is repressed. Fournier et al. (2002) have proposed a pathway for aerobic RDX degradation by analogy to the abiotic alkaline hydrolysis of RDX, in which initial denitration yields unstable cyclohexenyl derivatives (Hoffsommer et al. 1977).

Denitration of RDX by aerobic bacteria involves two one-electron transfer steps and releases two nitro groups before ring cleavage (Fig. 9.2, Path G). This results in the formation of one molecule of 4-nitro-2,4-diazabutanal (NDAB) along with nitrous oxide, ammonium, formaldehyde, and carbon dioxide (Fournier et al. 2002; Bhushan et al. 2003b). RDX appears to be mineralized according to this mechanism and the nitrite generated is utilized as a nitrogen source for growth by the aerobic bacteria *Rhodococcus rhodochrous* strain 11Y (Seth-Smith et al. 2002), *Rhodococcus* sp. strain DN22 (Coleman et al. 1998; Fournier et al. 2002), *Williamsia* sp. strain KTR4 (Thompson et al. 2005), and *Gordonia* sp. strain KTR9 (Thompson et al. 2005). The NDAB produced is apparently not further metabolized by strains 11Y, DN22, KTR4, or KTR9 and hence gets accumulated (Fournier et al. 2002, 2004a; Thompson et al. 2005). However, it has recently been shown that NDAB may not be an environmentally recalcitrant end-product, as it can be biodegraded by a methylotrophic bacterium producing nitrous oxide and carbon dioxide (Fournier et al. 2004a, 2005).

9.2.3 Biodegradation of RDX and HMX by Fungi

Fungi have potential application in the degradation of cyclic nitramines owing to their widespread distribution in the environment, particularly in soil, and their capability to produce several extracellular enzymes. Fungi can degrade chemicals using several enzymes, such as peroxidases that are known to catalyze a number of free radical reactions (Stahl and Aust 1995). The electronegative $–NO_2$ group in RDX readily accepts a free electron to form an anion radical. The elimination of nitrite anion $–NO_2^-$ would leave an unstable free radical intermediate behind to continue the decomposition process. Fungal degradation of RDX and HMX has not been studied as extensively as the bacterial degradation. Several studies using white rot fungi *Phanerochaete chrysosporium* have been carried out to elucidate the mechanisms for RDX and HMX biodegradation (Sheremata and Hawari 2000; Fournier et al. 2004a, b). Fernando and Aust (1991) studied biodegradation of RDX by the white rot fungus *Phanerochaete chrysosporium*. In RDX contaminated liquid cultures (1.25 nmoles/10 ml cultures), 66.6 ± 4.1% of the [^{14}C] RDX was converted to $^{14}CO_2$ over a 30 day period. Similarly, 76.0 ± 3.9% of the [^{14}C] RDX added (1.25 nmoles/10 g soil), was degraded to $^{14}CO_2$ in 30 days. On the basis of high levels of mineralization, the authors concluded that degradation of [^{14}C]-RDX occurred by an oxidative mechanism, presumably by the lignin peroxidases. Sheremata and Hawari (2000) investigated biodegradation of RDX in liquid cultures (initially at 62 mg/L) using the white rot fungus *Phanerochaete chrysosporium*. With RDX as the main source of nitrogen, complete disappearance of RDX occurred after 60 days. Although traces of MNX were detected, the major products of RDX mineralization were CO_2 and N_2O. An average of 52.9% [UL-^{14}C]-RDX was mineralized to CO_2, 10.7% was taken up in the biomass, and an average of 62% nitrogen in RDX was transformed to N_2O. The authors reported mineralization of RDX without the production of NDAB. Fournier et al. (2004a) showed that *Phanerochaete chrysosporium* also biodegraded NDAB to nitrous oxide, carbon dioxide and traces of nitramide. The degradation of NDAB by *Phanerochaete chrysosporium* was attributed to a manganese-dependent peroxidase, as the enzyme was able to transform NDAB in vitro.

Bhatt et al. (2006) have reported fungi isolated from marine sediments capable of aerobically biodegrading RDX to HCHO, CO_2 and N_2O through both direct ring cleavage and reduction to MNX prior to ring-cleavage. Four aerobic RDX-degrading fungi were identified as members of *Rhodotorula, Bullera, Acremonium* and *Penicillium*. The four fungal isolates mineralized 15–34% of RDX in 58 days as determined by liberated $^{14}CO_2$. *Acremonium* was selected to determine biotransformation pathway of RDX. Methylenedinitramine (MEDINA), N_2O and HCHO were detected on incubation of RDX with resting cells of *Acremonium.* Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) together with trace amounts of hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX) and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) was also detected. Under the same conditions, MNX produced N_2O and HCHO together with trace amounts of DNX and

TNX. TNX did not degrade with *Acremonium*. Based on the experimental findings, it is, therefore, proposed that RDX was degraded via at least two major initial routes. One route involved direct ring cleavage to MEDINA and another involved reduction to MNX prior to ring cleavage. Nitrite was only detected in trace amounts suggesting that degradation via initial denitration did take place, but not significantly.

9.3 Microorganisms Transforming RDX and HMX

A number of microorganisms have been identified in a wide variety of contaminated environments capable of transforming RDX and HMX. Several research groups have employed mixed anaerobic microbial cultures that included methanogens (Adrian and Lowder 1999; Adrian and Chow 2001), acetogens (Beller 2002), nitrate reducers (Freedman and Sutherland 1998), and individual isolates to biotransform RDX and HMX. A few terrestrial mesophilic microorganisms, such as *Rhodococcus* sp. (Coleman et al. 1998; Fournier et al. 2002; Seth-Smith et al. 2002; Halasz et al. 2010), *Klebsiella pneumoniae* strain SCZ-1 (Zhao et al. 2002) and the fungus *Phanerochaete chrysosporium* (Fernando and Aust 1991; Bayman et al. 1995; Sheremata and Hawari 2000; Stahl et al. 2001) degrade RDX significantly. Under anaerobic conditions, bacteria, such as *Clostridium* (Regan and Crawford 1994; Zhao et al. 2003; Zhang and Hughes 2003), *Desulfovibrio* (Boopathy et al. 1998a, b) and some members of the Enterobacteriaceae family (Kitts et al. 1994), removed RDX with little mineralization. Microorganisms capable of degrading RDX and HMX, such as those of the *Clostridium* genus and other organisms displaying nitroreductase activity, are generally considered to be ubiquitous in terrestrial environments (Regan and Crawford 1994; Ederer et al. 1997; Ahmad and Hughes 2002).

Kitts et al. (1994) isolated three species of the family Enterobacteriaceae from nitramine explosive-contaminated soil that biochemically reduced RDX and HMX. Two isolates, identified as *Morganella morganii* and *Providencia rettgeri*, completely transformed both RDX and the nitroso-RDX reduction intermediates. The third isolate, identified as *Citrobacter freundii*, partially transformed RDX and generated high concentrations of nitroso-RDX intermediates. All three isolates produced $^{14}CO_2$ from labeled RDX under O_2-depleted culture conditions. Young et al. (1997) studied the biotransformation of RDX by a consortium of bacteria found in horse manure. Five types of bacteria were found to predominate in the consortium. The most effective of these isolates at transforming RDX was *Serratia marcescens*. The biotransformation of RDX by all of these bacteria was found to occur only in the anoxic stationary phase. Degradation of RDX by *Serratia marcescens* was found to proceed through stepwise reduction of the three nitro groups to nitroso groups. An aerobic, gram-positive bacterium was isolated by Coleman et al. (1998) from explosives-contaminated soil by enrichment culture, using the nitramine explosive, RDX, as the sole added N source. The organism was

identified by 16S rDNA analysis as a *Rhodococcus* sp. strain DN22. Resting cells grown on RDX showed the highest degradative activity, compared to cells grown on alternative N sources, indicating that the RDX degradation system is inducible. *Clostridium bifermentans* has also been reported to degrade RDX (Regan and Crawford 1994). The bioconversion of RDX occurs under anaerobic conditions both in the consortium and in pure culture without the need of an added reductant. Bacterium, *Stenotrophomonas maltophilia* PB1, isolated from the culture used RDX as a sole source of nitrogen for growth (Binks et al. 1995). Three moles of nitrogen was used per mole of RDX, yielding a metabolite identified by mass spectroscopy and 1H nuclear magnetic resonance analysis as methylene-N-(hydroxymethyl)-hydroxylamine-N-(hydroxymethyl) nitroamine. The bacterium also used s-triazine as a sole source of nitrogen but not the structurally similar compounds octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine. Boopathy et al. (1998a) studied the metabolism of RDX and HMX by a sulfate-reducing bacterial consortium, *Desulfovibrio* spp. The results indicated that the *Desulfovibrio* spp. used all of the explosive compounds studied as their sole source of nitrogen for growth. The concentrations of RDX and HMX in the culture media dropped to below the detection limit (<0.5 ppm) within 18 days of incubation. Singh et al. (2009) have reported degradation of RDX and HMX by denitrifying bacteria isolated from high explosive production effluent. The denitrifying bacteria were identified as *Pseudomonas* and *Bacillus.*

The capacity to metabolize nonaromatic explosives appears to be reasonably widespread across bacterial genera (Roh et al. 2009). Huang (1998) found that the thermophilic bacterium *Caldicellulosiruptor owensensis* can degrade HMX (30 mg/L) at 75°C under anaerobic conditions. They concluded that 94% of the initial radioactivity from [$U^{14}C$]-HMX remained in the aqueous phase with no mineralization. Lee and Brodman (2005) have reported biodegradation of RDX by two new species of bacteria *Rhizobium rhizogenes* BL and *Burkholderia* sp. It was found that these organisms along with an added carbon source could degrade RDX to simple gaseous products. An aerobic RDX-degrading bacterium, identified as *Gordonia* sp., was isolated from the soil. It degraded RDX aerobically and produced 4-nitro-2,4-diazabutanal (Ronen et al. 2008). Bhushan et al. (2004) reported chemotaxis-mediated biodegradation of RDX and HMX by an obligate anaerobic bacterium *Clostridium* sp. strain EDB2 isolated from marine sediment. The explosives were biotransformed by strain EDB2 via N-denitration with concomitant release of NO_2^-. Van Aken et al. (2004) reported biodegradation of RDX and HMX by a *Methylobacterium* sp. associated with poplar tissues. Seth-Smith et al. (2008) have isolated nineteen strains of *Rhodococcus* spp. capable of utilizing RDX as the sole nitrogen source. Zhao et al. (2008) studied the effect of terminal electron acceptors (TEA) on the growth of *Shewanella halifaxensis* HAW-EB4 and on the enzymic processes involved in RDX metabolism. The results demonstrated that aerobic conditions were optimal for bacterial growth, but that anaerobic conditions in the presence of trimethylamine *N*-oxide (TMAO) or in the absence of TEA favoured RDX metabolism. Bhatt et al. (2005) screened 22 marine sediment bacterial isolates for RDX and HMX biodegradation activity under

anaerobic conditions. Five marine sediment bacteria capable of RDX degradation were identified as *Halomonas* (HAW-OC4), *Marinobacter* (HAW-OC1), *Pseudoalteromonas* (HAW-OC2 and HAW-OC5) and *Bacillus* (HAW-OC6).

9.4 Conclusion

Contamination of soil and water by military explosives, RDX and HMX is widespread. Biodegradation of cyclic nitramines RDX and HMX under both aerobic and anaerobic conditions is feasible. The two cyclic nitramines RDX and HMX undergo ring cleavage following initial transformation of the molecules. Once the ring in cyclic nitramine cleaves, the resulting degradation products are expected to be thermally unstable and to hydrolyze readily in water. RDX and HMX can be biodegraded under anaerobic or anoxic conditions by facultative or anaerobic microorganisms. Under aerobic conditions, RDX and HMX can be used as a sole source of nitrogen by aerobic bacteria or by fungi. In many laboratory studies, it has been established that anaerobic metabolism occurs more readily than aerobic metabolism. Due to different conformations, HMX is chemically more stable and therefore, less amenable to biodegradation than RDX. Several biodegradation pathways have been proposed based mainly on end-product characterization. Biodegradation mechanisms for cyclic nitramines include formation of a nitramine free radical and loss of nitro functional groups; reduction of nitro functional groups; direct enzymatic cleavage; α-hydroxylation; or hydride ion transfer. Biodegradation of explosives is an approach that offers a potential solution for many RDX and HMX contaminated sites. Research has shown that RDX and HMX are biodegraded by a wide range of microorganisms in different environments. Advances in the identification of metabolic intermediates and suggested pathways demonstrate the potential of the biodegradation technology for bioremediation of explosive-contaminated sites.

Acknowledgments Authors express sincere gratitude to Dr. Narendra Kumar, Director, Defence Laboratory, Jodhpur for the support and encouragement.

References

Adrian NR, Arnett CM (2004) Anaerobic biodegradation of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) by *Acetobacterium malicum* strain HAAP-1 isolated from a methanogenic mixed culture. Curr Microbiol 48:332–340

Adrian NR, Chow T (2001) Identification of hydroxylamino-dinitroso-1, 3, 5-triazine as a transient intermediate formed during the anaerobic biodegradation of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine. Environ Toxicol Chem 20:1874–1877

Adrian NR, Lowder A (1999) Biodegradation of RDX and HMX by a methanogenic enrichment culture. In: Alleman BC, Leeson A (eds) Bioremediation of nitroaromatic and haloaromatic compounds, vol 7. Battelle Press, Columbus, pp 1–6

Adrian NR, Arnett CM, Hickey RF (2003) Stimulating the anaerobic biodegradation of explosives by the addition of hydrogen or electron donors that produce hydrogen. Water Res 37:3499–3507

Agrawal A, Tratnyek P (1996) Reduction of nitroaromatic compounds by zero-valent iron metal. Environ Sci Technol 30:153–160

Ahmad F, Hughes JB (2002) Reactivity of partially reduced arylhydroxylamine and nitrosoarene metabolites of 2, 4, 6 trinitrotoluene (TNT) towards biomass and humic acids. Environ Sci Technol 36:4370–4381

Akhavan J (1998) The chemistry of explosives. Royal Society of Chemistry, Cambridge

An CJ, He YL, Huang GH, Yang SC (2010) Degradation of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) by anaerobic mesophilic granular sludge from a UASB reactor. J Chem Technol Biotechnol 85:831–838

Bayman P, Ritchey SD, Bennett JW (1995) Fungal interactions with the explosive RDX (hexahydro-1, 3, 5-trinitro- 1, 3, 5-triazine). J Ind Microbiol 15:418–423

Beller HR (2002) Anaerobic biotransformation of RDX (hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine) by aquifer bacteria using hydrogen as the sole electron donor. Water Res 36:2533–2540

Bhatt M, Zhao JS, Monteil-Rivera F, Hawari J (2005) Biodegradation of cyclic nitramines by tropical marine sediment bacteria. J Ind Microbiol Biotechnol 32:261–267

Bhatt M, Zhao JS, Halasz A, Hawari J (2006) Biodegradation of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine by novel fungi isolated from unexploded ordnance contaminated marine sediment. J Ind Microbiol Biotechnol 33:850–858

Bhushan B, Halasz A, Spain JC, Thiboutot S, Ampleman G, Hawari J (2002) Biotransformation of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine catalyzed by a NAD(P)H: nitrate oxidoreductase from *Aspergillus niger*. Environ Sci Technol 36:3104–3108

Bhushan B, Paquet L, Halasz A, Spain JC, Hawari J (2003a) Mechanism of xanthine oxidase catalyzed biotransformation of HMX under anaerobic conditions. Biochem Biophys Res Commun 306:509–515

Bhushan B, Trott S, Spain JC, Halasz A, Paquet L, Hawari J (2003b) Biotransformation of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) by a rabbit liver cytochrome P450: insight into the mechanism of RDX biodegradation by *Rhodococcus* sp. strain DN22. Appl Environ Microbiol 69:1347–1351

Bhushan B, Halasz A, Thiboutot S, Ampleman G, Hawari J (2004) Chemotaxis-mediated biodegradation of cyclic nitramine explosives RDX, HMX, and CL-20 by *Clostridium* sp. EDB2. Biochem Biophys Res Commun 316:816–821

Binks PR, Nicklin S, Bruce NC (1995) Degradation of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) by *Stenotrophomonas maltophilia* PB1. Appl Environ Microbiol 61:1318–1322

Boopathy R (2001) Enhanced biodegradation of cyclotetramethylenetetranitramine (HMX) under mixed electron-acceptor condition. Biores Technol 76:241–244

Boopathy R, Manning J, Kulpa CF (1997) Anaerobic bioremediation of explosive contaminated soil: a laboratory study. In: Wise DL (ed) Global environmental biotechnology, vol 39. Kluwer Academic Publishers, Dordrecht, pp 463–474

Boopathy R, Kulpa CF, Manning J (1998a) Anaerobic biodegradation of explosives and related compounds by sulfate-reducing and methanogenic bacteria: a review. Biores Technol 63:81–89

Boopathy RB, Gurgas M, Ullian J, Manning JF (1998b) Metabolism of explosive compounds by sulfate-reducing bacteria. Curr Microbiol 37:127–131

Bradley PM, Dinicola RS (2005) RDX (hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine) biodegradation in aquifer sediments under manganese-reducing conditions. Biorem J 9:1–8

Brill BT (1990) Structure-thermolysis relationships for energetic materials. In: Bulusu SN (ed) Chemistry and physics of energetic materials. Kluwer Academic Publishers, Dordrecht, pp 277–326

Burdette LJ, Cook LL, Dyer RS (1988) Convulsant properties of cyclotrimethylenetrinitramine (RDX): spontaneous, audiogenic, and amygdaloid kindled seizure activity. Toxicol Appl Pharmacol 92:436–444

Burton DT, Turley SD, Peters GT (1994) The toxicity of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) to the freshwater green alga *Selenastrum capricornutum*. Water Air Soil Pollut 76:449–457

Carter MC, Weber WJ Jr, Olmstead KP (1992) Effects of background dissolved organic matter on TCE adsorption by GAC. J Am Water Works Assoc 84:1–91

Clausen J, Robb J, Curry D, Korte N (2004) A case study of contaminants on military ranges: Camp Edwards, Massachusetts, USA. Environ Pollut 129:13–21

Coleman NV, Duxbury T (1999) Biodegradation of RDX by *Rhodococcus* sp. strain DN22. 2nd international symposium biodegradation of nitroaromatic compounds and explosives, Leesburg, VA, 8–9 September, Abstract, p 13

Coleman NV, Nelson DR, Duxbury T (1998) Aerobic biodegradation of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) as a nitrogen source by a *Rhodococcus* sp. strain DN22. Soil Biol Biochem 30:1159–1167

Coleman NV, Spain JC, Duxbury T (2002) Evidence that RDX biodegradation by *Rhodococcus* strain DN22 is plasmid-borne and involves a cytochrome P-450. J Appl Microbiol 93:463–472

Cooper PW (1996) Explosives engineering. Wiley, New York

Croce M, Okamoto Y (1979) Cationic micellar catalysis of the aqueous alkaline hydrolyses of 1, 3, 5-triaza-1, 3, 5-trinitrocyclohexane and 1, 3, 5, 7-tetraaza-1, 3, 5, 7- tetranitrocyclooctane. J Organic Chem 44:2100–2103

Crocker FH, Indest KJ, Fredrickson HL (2006) Biodegradation of the cyclic nitramine explosives RDX, HMX and CL-20. Appl Microbiol Biotechnol 73:274–290

Darrach MR, Chutjian A, Plett GA (1998) Trace explosives signatures from World War II unexploded undersea ordnance. Environ Sci Technol 32:1354–1358

Doppalapudi RB, Sorial GA, Maloney SW (2002) Electrochemical reduction of simulated munitions wastewater in a bench-scale batch reactor. Environ Eng Sci 19:115–130

Drzyzga O, Gorontzy T, Schmidt A, Blotevogel KH (1995) Toxicity of explosives and related compounds to the luminescent bacterium *Vibrio fischeri* NRRL-B-11177. Arch Environ Contam Toxicol 28:229–235

Ederer MM, Lewis TA, Crawford RL (1997) 2, 4, 6-trinitrotoluene (TNT) transformation by clostridia isolated from a munition-fed bioreactor: comparison with non-adapted bacteria. J Ind Microbiol Biotechnol 18:82–88

Fernando T, Aust SD (1991) Biodegradation of munition waste, TNT (2, 4, 6-trinitrotoluene) and RDX (hexahydro-1, 3, 5- trinitro-1, 3, 5-triazine) by *Phanerochaete chrysosporium*. In: Tedder DW, Pohland FG (eds) Emerging technologies in hazardous waste management. American Chemical Society, Washington, pp 214–232

Fournier D, Halasz A, Spain J, Fiurasek P, Hawari J (2002) Determination of key metabolites during biodegradation of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine with *Rhodococcus* sp. Strain DN22. Appl Environ Microbiol 68:166–172

Fournier D, Halasz A, Spain J, Spanggord RJ, Bottaro JC, Hawari J (2004a) Biodegradation of the hexahydro-1, 3, 5-trinitro-1, 3, 5- triazine ring cleavage product 4-nitro-2, 4-diazabutanal by *Phanerochaete chrysosporium*. Appl Environ Microbiol 70:1123–1128

Fournier D, Halasz A, Thiboutot S, Ampleman G, Manno D, Hawari J (2004b) Biodegradation of octahydro-1, 3, 5, 7-tetranitro-1, 3, 5, 7 tetrazocine (HMX) by *Phanerochaete chrysosporium*: new insight into the degradation pathway. Environ Sci Technol 38:4130–4133

Fournier D, Trott S, Hawari J, Spain J (2005) Metabolism of the aliphatic nitramine 4-nitro-2, 4-diazabutanal by *Methylobacterium* sp. strain JS178. Appl Environ Microbiol 71:4199–4202

Freedman DL, Sutherland KW (1998) Biodegradation of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) under nitrate-reducing conditions. Water Sci Technol 38:33–40

Fuller ME, McClay K, Hawari J, Paquet L, Malone TE, Fox BG, Steffan RJ (2009) Transformation of RDX and other energetic compounds by xenobiotic reductases XenA and XenB. Appl Microbiol Biotechnol 84:535–544

Funk SB, Roberts DJ, Crawford DL, Crawford RL (1993) Initial-phase optimization for bioremediation of munition compound-contaminated soils. Appl Environ Microbiol 59:2171–2177

Garg R, Grasso D, Hoag G (1991) Treatment of explosives contaminated lagoon sludge. Hazard Waste Hazard Mater 8:319–340

Goldberg DJ, Green ST, Nathwani D, McMenamin J, Hamlet N, Kennedy DH (1992) RDX intoxication causing seizures and a widespread petechial rash mimicking meningococcemia. J Res Soc Med 85:181

Gorontzy T, Drzyzga O, Kahl MW, Bruns-Nagel D, Breitung J, von Leow E, Blotevogel KH (1994) Microbial degradation of explosives and related compounds. Crit Rev Microbiol 20:265–284

Greer CW, Godbout J, Zilber B, Labelle S, Sunahara G, Hawari J, Ampleman G, Thiboutot S, Dubois C (1997) Bioremediation of RDX/HMX-contaminated soil: from flask to field. In: Hinchee R, Hoeppel RE, Anderson DB (eds) In situ and ex situ bioremediation. Battelle Press, Columbus, pp 393–398

Griest WH, Tyndall RL, Stewart AJ, Caton JE, Vass AA, Ho CH, Caldwell WM (1995) Chemical characterization and toxicological testing of windrow composts from explosives-contaminated sediments. Environ Toxicol Chem 14:51–59

Guiot SR, Shen CF, Paquet L, Breton J, Hawari J (1999) Pilot-scale anaerobic bioslurry remediation of RDX and HMX contaminated soils. In: Alleman B, Leeson A (eds) Bioremediation of nitroaromatic and haloaromatic compounds. Battelle Press, Columbus, p 15

Haas R, Schreiber I, von Low E, Stork G (1990) Conception for the investigation of contaminated munitions plants. 2. Investigation of former RDX-plants and filling stations. Fresenius J Anal Chem 338:41–45

Halasz A, Spain J, Paquet L, Beaulieu C, Hawari J (2002) Insights into the formation and degradation mechanisms of methylenedinitramine during the incubation of RDX with anaerobic sludge. Environ Sci Technol 36:633–638

Halasz A, Manno D, Strand SE, Bruce NC, Hawari J (2010) Biodegradation of RDX and MNX with *Rhodococcus* sp. strain DN22: New insights into the degradation pathway. Environ Sci Technol 44:9330–9336

Hansen LD, Davis JL, Escalon L (2001) Reductive transformation of RDX in a bench-scale simulated aquifer. In: Magar VS, Johnson G, Ong SK, Leeson A (eds) The 6th International in situ and on site bioremediation conference, Battelle Press, San Diego, California, pp 51–58

Harkins VAR (1998) Aerobic biodegradation of HMX (Octahydro-1,3,5,7-tetranitro- 1,3,5,7-tetrazocine) with supplemental study of RDX (Hexahydro-1,3,5-trinitro-1,3,5- triazine). Dissertation, Texas Technical University, Lubbock

Harkins VR, Mollhagen T, Heintz C, Rainwater K (1999) Aerobic biodegradation of high explosives, phase 1-HMX. Bioremed J 3:285–290

Hawari J (2000) Biodegradation of RDX and HMX: from basic research to field application. In: Spain JC, Hughes JB, Knackmuss HJ (eds) Biodegradation of nitroaromatic compounds and explosives. Lewis Publishers, Boca Raton, pp 277–310

Hawari J, Halasz A, Paquet L, Zhou E, Spencer B, Ampleman G, Thiboutot S (1998) Characterization of metabolites in the biotransformation of 2, 4, 6-trinitrotoluene with anaerobic sludge: role of triaminotoluene. Appl Environ Microbiol 64:2200–2206

Hawari J, Halasz A, Beaudet S, Paquet L, Ampleman G, Thiboutot S (1999) Biotransformation of 2, 4, 6-trinitrotoluene (TNT) with *Phanerochaete chrysosporium* in agitated cultures at pH 4.5. Appl Environ Microbiol 65:2977–2986

Hawari J, Beaudet S, Halasz A, Thiboutot S, Ampleman G (2000a) Microbial degradation of explosives: biotransformation versus mineralization. Appl Microbiol Biotechnol 54:605–618

Hawari J, Halasz A, Sheremata T, Beaudet S, Groom C, Paquet L, Rhofir C, Ampleman G, Thiboutot S (2000b) Characterization of metabolites during biodegradation of hexahydro-1, 3,

5-trinitro-1, 3, 5-triazine (RDX) with municipal anaerobic sludge. Appl Environ Microbiol 66:2652–2657

Hawari J, Halasz A, Beaudet S, Paquet L, Ampleman G, Thiboutot S (2001) Biotransformation routes of octahydro-1, 3, 5, 7-tetranitro-1, 3, 5, 7-tetrazocine by municipal anaerobic sludge. Environ Sci Technol 35:70–75

Heaston MS, Barnes PW, Alvestad KR (2001) Reductive biotransformation of nitrate and explosives compounds in groundwater. In: Magar VS, Johnson G, Ong SK, Leeson A (eds) The 6th International in situ and on site bioremediation conference, Battelle Press, San Diego, California, pp 17–24

Hoffsommer JC, Kubose DA, Glover DJ (1977) Kinetic isotope effects and intermediate formation for the aqueous alkaline hydrolysis of 1, 3, 5-triaza-1, 3, 5-trinitrocyclohexane (RDX). J Phys Chem 81:380–385

Huang CY (1998) The anaerobic biodegradation of the high explosive octahydro- 1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) by an extremely thermophilic anaerobe *Caldicellulosiruptor owensensis* sp., Dissertation, University of California, Los Angeles

Isbister JD, Anspach GL, Kitchens JF, Doyle RC (1984) Composting for decontamination of soils containing explosives. Microbiolgica 7:47–73

Jones AM, Greer CW, Ampleman G, Thiboutot S, Lavigne J, Hawari J (1995a) Biodegradability of selected highly energetic pollutants under aerobic conditions. In: Hinchee RE, Anderson DB, Hoeppel RE (eds) Bioremediation of recalcitrant organics. 3rd International in situ bioremediation symposium. Battelle Press, Columbus, Ohio, pp 251–257

Jones AM, Labelle S, Paquet L, Hawari J, Rho D, Samson R, Greer CW, Lavigne J, Thiboutot S, Ampleman G, Lavertu R (1995b) Assessment of the aerobic biodegradation potential of RDX, TNT, GAP and NC. In: Moo-Young M, Anderson WA, Chakrabarty AM (eds) Environmental biotechnology: principles and applications. Kluwer Academic Publisher, Dordrecht, pp 368–381

Kaplan DL (1990) Biotransformation pathways of hazardous energetic organo-nitro compounds. In: Kamely D, Chakrabarty A, Omenn GS (eds) Biotechnology and biodegradation. Advances in applied biotechnology series, vol 4. Portfolio Publishing, Woodlands, pp 155–181

Kaplan AS, Berghout CF, Peczenik A (1965) Human intoxication from RDX. Arch Environ Health 10:877–883

Kitts CL, Cunningham DP, Unkefer PJ (1994) Isolation of three hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine degrading species of the family enterobacteriaceae from nitramine explosive-contaminated soil. Appl Environ Microbiol 60:4608–4611

Kitts CL, Green CE, Otley RA, Alvarez MA, Unkefer PJ (2000) Type I nitroreductases in soil enterobacteria reduce TNT (2, 4, 6,-trinitrotoluene) and RDX (hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine). Can J Microbiol 46:278–282

Kwon MJ, Finneran KT (2006) Microbially mediated biodegradation of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine by extracellular electron shuttling compounds. Appl Environ Microbiol 72:5933–5941

Lachance B, Robidoux PY, Hawari J, Ampleman G, Thiboutot S, Sunahara GI (1999) Cytotoxic and genotoxic effects of energetic compounds on bacterial and mammalian cells in vitro. Mutat Res 444:25–39

Lamberton AH, Lindley C, Owston PG, Speakman JC (1949a) Studies of nitroamines. Part V. Some properties of hydroxymethyl- and aminomethyl-nitroamines. J Chem Soc 355:1641–1646

Lamberton AH, Lindley C, Speakman JC (1949b) Studies of nitroamines. Part VII. The decomposition of methylenedinitroamine in aqueous solutions. J Chem Soc 357:1650–1656

Lee SY, Brodman BW (2005) Biodegradation of 1, 3, 5-Trinitro-1, 3, 5-triazine (RDX). J Environ Sci Health 39:61–75

Lenke H, Warrelmann J, Daun G, Hund K, Sieglen U, Walter U, Knackmuss HJ (1998) Biological treatment of TNT contaminated soil. 2. Biologically induced immobilization of the contaminants and full-scale application. Environ Sci Technol 32:1964–1971

Levine BS, Furedi EM, Gordon DE, Burns JM, Lish PM (1981) Thirteen week toxicity study of hexahydro-1, 3, 5-trinitro-1, 3, 5- triazine (RDX) in Fischer 344 rats. Toxicol Lett 8:241–245

Levsen K, Mussmann P, Berger-Priess E, Priess A, Volmer D, Wunsch G (1993) Analysis of nitroaromatics and nitramines in ammunition waste water and in aqueous samples from former ammunition plants and other military sites. Acta Hydrochimica Hydrobiologica 21:153–166

Luo KM, Lin SH, Chang JG, Huang TH (2002) Evaluations of kinetic parameters and critical runaway conditions in the reaction system of hexamine-nitric acid to produce RDX in a non-isothermal batch reactor. J Loss Prev Process Indust 15(2):119–127

McCalla DR, Reuvers A, Kaiser C (1970) Mode of action of nitrofurazone. J Bacteriol 104:1126–1134

McCormick NJ, Cornell JH, Kaplan AM (1981) Biodegradation of hexahydro-l, 3, 5-trinitro-1, 3, 5-triazine. Appl Environ Microbiol 42:817–823

McLellan WL, Hartley WR, Brower ME (1992) Octahydro-l, 3, 5, 7-tetranitro-l, 3, 5, 7-tetrazocine (HMX). In: Hartley WR, Roberts WC (eds) Drinking water health advisory: munitions. CRC Press, Lewis Publishers, Boca Raton, pp 247–273

Monteil-Rivera F, Groom C, Hawari J (2003) Sorption and degradation of octahydro-1, 3, 5, 7-tetranitro-1, 3, 5, 7-tetrazocine in soil. Environ Sci Technol 37:3878–3884

Morley MC, Shammas SN, Speitel GE Jr (2002) Biodegradation of RDX and HMX mixtures: batch screening experiments and sequencing batch reactors. Environ Eng Sci 19:237–250

Morley MC, Henke JL, Speitel GE Jr (2005) Adsorption of RDX and HMX in rapid small-scale column tests: Implications for full-scale adsorber applications. J Environ Eng 131:29–37

Myler CA, Sysk W (1991) Bioremediation of explosives contaminated soils. In: Sayler GS (ed) Environmental biotechnology for waste treatment. Plenum Press, New York, pp 137–196

Naja G, Halasz A, Thiboutot S, Ampleman G, Hawari J (2008) Degradation of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) using zerovalent iron nanoparticles. Environ Sci Technol 42:4364–4370

Oh BT, Just L, Alvarez PJJ (2001) Hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine mineralization by zero valent iron and mixed anaerobic cultures. Environ Sci Technol 35:4341–4346

Pennington JC, Jenkins TF, Thiboutot S, Ampleman G, Clausen J, Hewitt AD, Lewis J, Walsh MR, Walsh ME, Ranney TA, Silverblatt B, Marois A, Gagnon A, Brousseau P, Zufelt JE, Poe K, Bouchard M, Martel R, Walker DD, Ramsey CA, Hayes CA, Yost SL, Bjella KL, Trepanier L, Berry TE Jr, Lambert DJ, Dubé P, Perron NM (2005) Distribution and fate of energetics on DoD test and training ranges: interim report 5. U.S. Army Engineer Research and Development Center, Vicksburg, MS, Technical Report ERDC TR-05-2

Peters GT, Burton DT, Paulson RL, Turley SD (1991) The acute and chronic toxicity of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) to three freshwater invertebrates. Environ Toxicol Chem 10:1073–1081

Peterson FG, Mason RP, Hovesepian J, Holtzman JL (1979) Oxygen-sensitive and-insensitive nitroreduction by *Escherichia coli* and rat hepatic microcosms. J Biol Chem 254:4009–4014

Price CB, Brannon JM, Yost SL, Hayes CA (2001) Relationship between redox potential and pH on RDX transformation in soil water slurries. J Environ Eng 127:26–31

Regan KM, Crawford RL (1994) Characterization of *Clostridium biofermentans* and its biotransformation of 2, 4, 6-trinitrotoluene (TNT) and 1, 3, 5-triaza-1, 3, 5-trinitrocyclohexane (RDX). Biotechnol Lett 16:1081–1086

Robidoux PY, Svendsen C, Caumartin J, Hawari J, Ampleman G, Thiboutot S, Weeks JM, Sunahara GI (2000) Chronic toxicity of energetic compounds in soil determined using the earthworm (*Eisenia andrei*) reproduction test. Environ Toxicol Chem 19:1764–1773

Robidoux PY, Bardai G, Paquet L, Ampleman G, Thiboutot S, Hawari J, Sunahara GI (2003) Phytotoxicity of 2, 4, 6-trinitrotoluene (TNT) and octahydro-1, 3, 5, 7-tetranitro-1, 3, 5, 7-tetrazocine (HMX) in spiked artificial and natural forest soils. Arch Environ Contam Toxicol 44:198–209

Roh H, Yu CP, Fuller ME, Chu KH (2009) Identification of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine degrading microorganisms via ^{15}N-stable isotope probing. Environ Sci Technol 43:2505–2511

Ronen Z, Yanovich Y, Goldin R, Adar E (2008) Metabolism of the explosive hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) in a contaminated vadose zone. Chemosphere 73:1492–1498

Rosenblatt DH (1980) Toxicology of explosives and propellants. In: Kaye SM (ed) Encyclopedia of explosives and related items. US Army Armament Research Development Committee, Dover, pp 332–345

Rosenblatt DH, Burrows EP, Mitchell WR, Parmer DL (1991) Organic explosives and related compounds. In: Hutzinger O (ed) The handbook of environmental chemistry Vol. 3 Part G. Springer, Berlin, pp 196–225

Seth-Smith HMB, Rosser SJ, Basran A, Travis ER, Dabbs ER, Nicklin S, Bruce NC (2002) Cloning, sequencing, and characterization of the hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine degradation gene cluster from *Rhodococcus rhodochrous*. Appl Environ Microbiol 68:4764–4771

Seth-Smith HMB, Edwards J, Rosser SJ, Rathbone DA, Bruce NC (2008) The explosive degrading cytochrome P450 systems is highly conserved among strains of *Rhodococcus* spp. Appl Environ Microbiol 74:4550–4552

Sewell TD, Thompson DL (1991) Classical dynamics study of unimolecular dissociation of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine. J Phys Chem 95:6228–6242

Shen CF, Guiot SR, Thiboutot S, Ampleman G, Hawari J (1998) Complete degradation of RDX and HMX in anoxic soil slurry bioreactors: laboratory and pilot-scale experiments, Proceedings of 6th International FZK/TNO conference on contaminated soil, Edinburgh, UK, pp 513–522

Shen CF, Hawari J, Ampleman G, Thiboutot S, Guiot SR (2000) Enhanced biodegradation and fate of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) and octahydro-1, 3, 5, 7-tetranitro-1, 3, 5, 7-tetrazocine (HMX) in anaerobic soil slurry bioprocess. Biorem J 4:27–39

Sherburne LA, Shrout JD, Alvarez PJJ (2005) Hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) degradation by *Acetobacterium paludosum*. Biodegradation 16:539–547

Sheremata TW, Hawari J (2000) Mineralization of RDX by the white rot fungus *Phanerochaete chrysosporium* to carbon dioxide and nitrous oxide. Environ Sci Technol 34:3384–3388

Sheremata TW, Halasz A, Ampleman G, Thiboutot S, Hawari J (1999) Fate of 2, 4, 6-trinitrotoluene and its metabolites in natural and model soil systems. Environ Sci Technol 33:4002–4008

Singh J, Comfort SD, Shea PJ (1998) Remediating RDX-contaminated water and soil using zero-valent iron. J Environ Qual 27:1240–1245

Singh R, Soni P, Kumar P, Purohit S, Singh A (2009) Biodegradation of high explosive production effluent containing RDX and HMX by denitrifying bacteria. World J Microbiol Biotechnol 25:269–275

Spain JC, Hughes JB, Knachmuss HJ (2000) Biodegradation of nitroaromatic compounds and explosives. Lewis Publishers, New York

Stahl JD, Aust SD (1995) Biodegradation of 2, 4, 6-trinitrotoluene by the white rot fungus *Phanerochaete chrysosporium*. In: Spain JC (ed) Biodegradation of nitroaromatic compounds. Plenum Press, New York, pp 117–134

Stahl JD, Van Aken B, Cameron MD, Aust SD (2001) Hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) biodegradation in liquid and solid-state matrices by *Phanerochaete chrysosporium*. Biorem J 5:13–25

Talmage SS, Opresko DM, Maxwell CJ, Welsh CJ, Cretella FM, Reno PH, Daniel FB (1999) Nitroaromatic munition compounds: environmental effects and screening values. Rev Environ Contam Toxicol 161:1–156

Tekoah Y, Abeliovich NA (1999) Participation of cytochrome P450 in the biodegradation of RDX by a *Rhodococcus* strain. 2nd international symposium on biodegradation of nitroaromatic compounds and explosives, Leesburg, VA, 8–9 September, Abstract, p 7

Testud F, Glanclaude JM, Descotes J (1996) Acute hexogen poisoning after occupational exposure. J Toxicol Clin Toxicol 34:109–111

Thompson KT, Crocker FH, Fredrickson HL (2005) Mineralization of the cyclic nitramine explosive hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine by *Gordonia* and *Williamsia* spp. Appl Environ Microbiol 71:8265–8272

USEPA (2004) US environmental protection agency, drinking water standards and health advisories. Publication EPA 822-R-04–005, Office of Water, USEPA, Washington

Van Aken B, Yoon JM, Schnoor JL (2004) Biodegradation of nitro-substituted explosives 2, 4, 6-trinitrotoluene, hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine, and octahydro-1, 3, 5, 7-tetranitro-1, 3, 5-tetrazocine by a phytosymbiotic *Methylobacterium* sp. associated with poplar tissues (*Populus deltoides* x nigra DN34). Appl Environ Microbiol 70:508–517

Vanderloop SL, Suidan MT, Berchtold SR, Moteleb MA, Maloney SW (1997) Effects of molecular oxygen on GAC adsorption of energetics. Water Sci Technol 35:197–204

Wight CA, Botcher TR (1992) Thermal decomposition of solid RDX begins with N–N bond scission. J Am Chem Soc 114:8303–8304

Wingfors H, Edlund C, Hagglund L, Waleij A, Sjöstrom J, Karlsson RM, Leffler P, Qvarfort U, Ahlberg M, Thiboutot S, Ampleman G, Martel R, Duvalois W, Creemers A, van Ham N (2006) Evaluation of the contamination by explosives and metals in soils at the Alvdalen shooting range. Part II: Results and discussion. FOI-Swedish Defence Research Agency, NBC Defence, SE-901 82 Umeå, Report FO1-R-1877-SE

Woody RC, Kearns GL, Brewster MA, Turley CP, Sharp GB, Lake RS (1986) The neurotoxicity of cyclotrimethylenetrinitramine (RDX) in a child: a clinical and pharmacokinetic evaluation. J Toxicol Clin Toxicol 24:305–319

Yang YX, Wang P, Yin P, Li W, Zhou P (1983) Studies on three strains of *Corynebacterium* degrading cyclotrimethylenetrinitro. Acta Microbiol Sin 23:251–256

Yinon J (1990) Toxicity and metabolism of explosives. CRC Press, Boca Raton

Young DM, Unkefer PJ, Ogden KL (1997) Biotransformation of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) by a prospective consortium and its most effective isolate *Serratia marcescens*. Biotechnol Bioeng 53(5):515–522

Young TSM, Morley MC, Snow DD (2006) Anaerobic biodegradation of RDX and TCE: single- and dual-contaminant batch tests. Pract Periodical Hazard, Toxic, Radioactive Waste Manage 10(2):94–101

Zhang C, Hughes JB (2003) Biodegradation pathways of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) by *Clostridium acetobutylicum* cell-free extract. Chemosphere 50:665–671

Zhao X, Hintsa EJ, Lee YT (1988) Infrared multiphoton dissociation of RDX in a molecular beam. J Chem Phys 88:801–810

Zhao JS, Halasz A, Paquet L, Beaulieu C, Hawari J (2002) Biodegradation of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine and its mononitroso derivative hexahydro-1-nitroso-3, 5-dinitro-1, 3, 5-triazine by *Klebsiella pneumoniae* strain SCZ-1 isolated from an anaerobic sludge. Appl Environ Microbiol 68:5336–5341

Zhao JS, Paquet L, Halasz A, Hawari J (2003) Metabolism of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine through initial reduction to hexahydro-1-nitroso-3, 5-dinitro-1, 3, 5-triazine followed by denitration in *Clostridium bifermentans* HAW-1. Appl Microbiol Biotechnol 63:187–193

Zhao JS, Greer CW, Thiboutot S, Ampleman G, Hawari J (2004a) Biodegradation of the nitramine explosives hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine and octahydro-1, 3, 5, 7-tetranitro-1, 3, 5, 7-tetrazocine in cold marine sediment under anaerobic and oligotrophic conditions. Can J Microbiol 50:91–96

Zhao JS, Spain J, Thiboutot S, Ampleman G, Greer C, Hawari J (2004b) Phylogeny of cyclic nitramine-degrading psychrophilic bacteria in marine sediment and their potential role in the natural attenuation of explosives. FEMS Microbiol Ecol 49:349–357

Zhao JS, Manno D, Leggiadro C, O'Neil D, Hawari J (2006) *Shewanella halifaxensis* sp. nov., a novel obligately respiratory and denitrifying psychrophile. Intl J Syst Evol Microbiol 56:205–212

Zhao JS, Manno D, Hawari J (2008) Regulation of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) metabolism in *Shewanella halifaxensis* HAW-EB4 by terminal electron acceptor and involvement of c-type cytochrome. Microbiology 154:1026–1037

Chapter 10
Microbial Degradation of PAHs: Organisms and Environmental Compartments

Elisa Rojo-Nieto and José A. Perales-Vargas-Machuca

10.1 Introduction

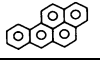
Polycyclic aromatic hydrocarbons (PAHs) represent a major class of organic compounds (Xue and Warshawsky 2005), that consist of over 100 individual moieties (Rehmann et al. 2008). Because of their toxicity and wide spread occurrence, PAH represent one of the most important groups of environmental pollutants (Eggen and Majcherczyk 1998). They consist of two or more fused benzene rings in linear, angular or cluster arrangements. The persistence of these chemicals in the environment is mainly due to their low solubility in water and stable polycondensed aromatic structure. Hydrophobicity and recalcitrance of PAHs to microbial degradation generally increase as the molecular weight increases. Besides being toxic to animals, some PAHs with four or more benzene rings, such as benzo[*a*]anthracene, chrysene and benzo[*a*]pyrene, have been shown to be carcinogenic (Bezalel et al. 1996).

Historically, human exposures to a variety of these complex mixtures have been associated with an increased incidence of cancer cases. Many PAHs and their heterocyclic analogs have been found to be tumorigenic in humans or experimental animals (Xue and Warshawsky 2005). Early, in 1761, the physician John Hill documented the high incidence of nasal cancer as consequence of excessive use of tobacco snuff (Cerniglia 1984). Pott (1775) reported high rate of scrotal skin cancer in chimney sweeps (compounds contained in the soot). Yamagiwa and Ichikawa (1915) induced tumors on the ears of rabbits by repeated application of coal tar. Due to their toxic, mutagenic and/or carcinogenic properties, sixteen PAHs (Table 10.1) have

E. Rojo-Nieto (✉) · J. A. Perales-Vargas-Machuca
Department of Environmental Technologies,
Andalusian Centre of Marine Science and Technology (CACYTMAR),
University of Cadiz, Puerto Real, 11510 Cadiz, Spain
e-mail: elisa.rojo@uca.es

S. N. Singh (ed.), *Microbial Degradation of Xenobiotics*,
Environmental Science and Engineering, DOI: 10.1007/978-3-642-23789-8_10,

Table 10.1 General characteristics and structures of the PAHs (16 PAHs listed as priority pollutants by USEPA)

PAH (USEPA)	CAS no.	Molecular formula	Structure	Molecular weight (g/mol)	Meelting point (°C)[a]	Vapor pressure (P^s/Pa)[a]	Solubility in water (g/m^3)[a]	log K_{ow} [a]	IARC group [b]
Naphthalene	91-20-3	$C_{10}H_8$		128.171	80.26	10.4	31	3.37	2B
Acenaphtylene	208-96-8	$C_{12}H_8$		152.192	91.8	0.9	16.1	4.00	
Acenaphthene	83-32-9	$C_{12}H_{10}$		154.207	93.4	0.3	3.80	3.92	3
Fluorene	86-73-7	$C_{13}H_{10}$		166.218	114.77	0.09	1.90	4.18	3
Phenanthrene	85-01-8	$C_{14}H_{10}$		178.229	99.24	0.02	1.10	4.57	3
Anthracene	120-12-7	$C_{14}H_{10}$		178.229	215.76	0.001	0.045	4.54	3
Pyrene	129-00-0	$C_{16}H_{10}$		202.250	150.62	0.0006	0.132	5.18	3
Fluoranthene	206-44-0	$C_{16}H_{10}$		202.250	110.19	0.00123	0.26	5.22	3
Chrysene	218-01-9	$C_{18}H_{12}$		228.288	255.5	5.70×10^{-7}	0.002	5.60	2B
Benzo(a)Anthracene	56-55-3	$C_{18}H_{12}$		228.288	160.5	2.80×10^{-5}	0.011	5.91	2B
Benzo(b)Fluoranthene	205-99-2	$C_{20}H_{12}$		252.309	168		0.0015	5.80	2B
Benzo(k)Fluoranthene	207-08-9	$C_{20}H_{12}$		252.309	217	5.20×10^{-8}	0.0008	6.00	2B
Benzo(a)Pyrene	50-32-8	$C_{20}H_{12}$		252.309	181.1	7.00×10^{-7}	0.0038	6.04	1

(continued)

Table 10.1 (continued)

PAH (USEPA)	CAS no.	Molecular formula	Structure	Molecular weight (g/mol)	Meelting point (°C)[a]	Vapor pressure (P^s/Pa)[a]	Solubility in water (g/m^3)[a]	log K_{ow} [a]	IARC group [b]
Benzo(g,h,i)Perylene	191-24-2	$C_{22}H_{12}$		276.330	272.5		0.00026	6.40	3
Indeno(1,2,3-cd)Pyrene	193-39-5	$C_{22}H_{12}$		276.330	162				2B
Dibenzo(a,h)Anthracene	50-70-53	$C_{22}H_{14}$		278.346	269.5	3.70×10^{-10}	0.0006	6.75	2A

Source: [a] Mackay et al. (2006), [b] IARC (2010)

been listed as priority pollutants by US Environmental Protection Agency (EPA), which should be regularly monitored in terrestrial and aquatic ecosystems (Chen et al. 2008), their concentration does not exceed the threshold in soil and water.

The main sources of PAHs in the environment include: incomplete combustion at higher temperatures of recent and fossil organic matter (pyrolytic source), slow maturation of organic matter under geochemical gradient conditions (petrogenic source) and short-term diagenetic degradation of biogenic precursors (diagenesis) (Neff 1979; McElroy et al. 1989). As a result of anthropogenic activities as accidental oil leaks and spills, contamination of air, soil, freshwater (surface water and groundwater), and marine environments has been often reported (Bumpus 1989). They are unique contaminants in the environment because they are generated continuously by the inadvertently incomplete combustion of organic matter, for instance in forest fires, home heating, traffic, and waste incineration (Johnsen et al. 2005).

The majority of PAHs released into the environment are subjected to processes like volatilization, chemical oxidation, bioaccumulation and adsorption on soil particles. The main pathway for their removal is currently considered to be microbial transformation and degradation (Ambrosoli et al. 2005). PAHs were in the past considered to be recalcitrant to biodegradation in the absence of oxygen as electron acceptor, but they have recently been proven to be biodegradable under a variety of anaerobic conditions. There are many studies on anaerobic microbial degradation of aliphatic and monoaromatic hydrocarbons. However, only a few studies are available on the anaerobic biodegradation of polyaromatic hydrocarbons (Fuchedzhieva et al. 2008).

The extent and rate of biodegradation depend on many factors including pH, temperature oxygen, microbial population, previous microbial acclimation, accessibility of nutrients, chemical structure, cellular transport properties, and chemical partitioning in growth the medium (Haritash and Kaushik 2009).

In the last decades, biological degradation of PAHs has aroused significant interest since these contaminants have been included among priority pollutants in the United States (Code of Federal Regulation 1982). It has been suggested that the persistence of PAHs in the environment might be due to their low susceptibility to nucleophilic attack owing to the presence of dense clouds of pi-electrons on both sides of the ring structures. In addition, some physical properties of PAHs, such as low aqueous solubility and high solid/water distribution ratios, hamper microbial degradation of these compounds, thereby resulting in their accumulation in soils and sediments (Covino et al. 2010a). PAHs also are usually associated with contaminants, such us BTEX, aliphatic hydrocarbons and heavy metals, which make their biodegradation more difficult (Reineke 2001).

As biodegradation is one of the main routes of PAHs removal from the environment, in this chapter a global point of view of the microbial degradation of PAHs in different environmental compartments has been provided.

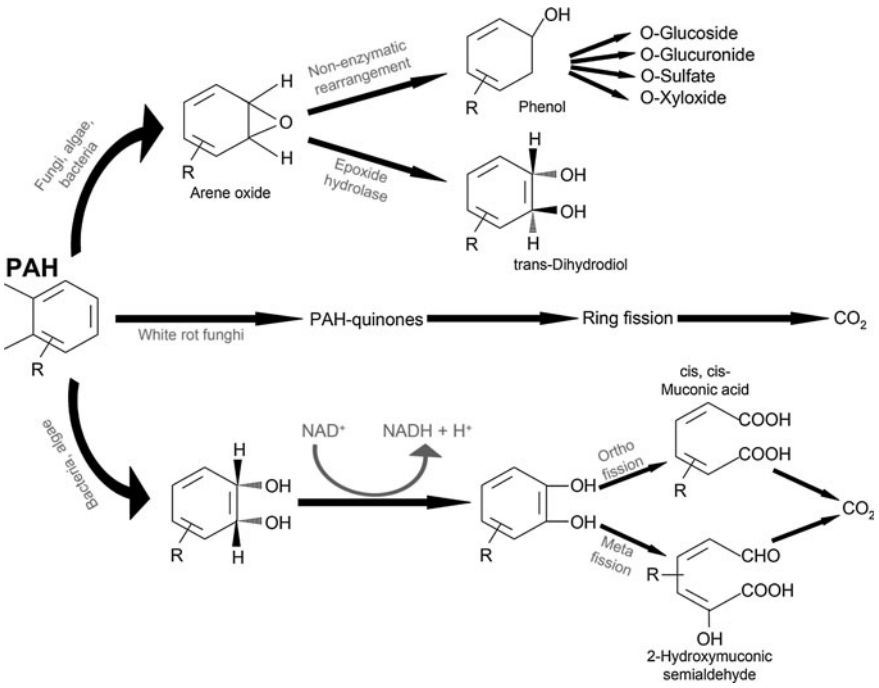


Fig. 10.1 General pathways for PAHs degradation (Redraw from Bamforth and Singleton 2005)

10.2 PAHs Degrading Organisms

The PAH-degrading microorganisms could be algae, bacteria and fungi. It involves breakdown of organic compounds through biotransformation into less complex metabolites, and through mineralization into inorganic minerals, H_2O, CO_2 (aerobic) or CH_4 (anaerobic) (Haritash and Kaushik 2009)

Schematic metabolic pathways to degrade PAHs of these microorganisms are shown in the Fig. 10.1. In aerobic pathways, the main strategy of microorganisms to degrade aromatic pollutants is to use a range of peripheral enzymes, which convert the substances to a key intermediate. Anaerobic degradation of aromatic compounds is carried out by phototrophic bacteria, by fermenting, manganese-reducing, iron-reducing, nitrate-reducing, and sulphate-reducing bacteria, and by methanogenic consortia (Bosma et al. 2001). Metabolism of organic compounds by respiration leads to a much more efficient use of potential chemical energy than anaerobic conversion (Reineke 2001). Although both aerobic and anaerobic biodegradation contribute significantly to remove aromatic pollutants from the environment, the aerobic mechanism is preferred because the process is faster and

Table 10.2 A list of PAH utilizing bacteria, fungi, cyanobacteria and microalgae

Some PAHs utilizing bacteria	Some PAHs utilizing fungi		Some PAHs utilizing cyanobacteria and microalgae
Acinetobacter calcoaceticus	*Absidia glauca*	*Mortierella ramanniana*	*Agmenellum quadruplicatum*
Acinetobacter sp.	*Acheremonium* sp.	*Mortierella verrucosa*	*Agmenellum* sp.
Aeromonas sp.	*Aspergillus niger*	*Mucor hiernalis*	*Anabaena* sp. (strain CA)
Agrobacterium sp.	*Aspergillus ochraceus*	*Mucor racemosus*	*Anabaena* sp. (strain 1F)
Alcaligenes denitrificans	*Aspergillus* sp.	*Neurospora crassa*	*Amphora* sp.
Alcaligenes faecalis	*Basidiobolus ranarum*	*Penicillum chrysogenum*	*Aphanocapsa* sp.
Arthrobacter polychromogenes	*Candida maltosa*	*Penicillum* sp.	*Chlorella autothrophica*
Arthrobacter sp.	*Candida tropicalis*	*Pestalotia* sp.	*Chlorella sorokiniana*
Bacillus cereus	*Candida utilis*	*Phanaerochaete chrysosporium*	*Chorella vulgaris*
Bacillus sp.	*Choanephora campinter*	*Phanaerochaete sordid*	*Chlamydomonas angulosa*
Beijerinckia sp.	*Chrysosphorum pannorum*	*Phlebia radiate*	*Coccochloris elabrens*
Burkhoderia sp.	*Circinella* sp.	*Phlyctochytrium reinboldtae*	*Cylindrotheca* sp.
Corynebacterium renale	*Claviceps paspali*	*Phytophthora cinnamomi*	*Dunaliella tertiolecta*
Flavobacterium sp.	*Cokeromices poitrassi*	*Rhizoctonia solani*	*Microcoleus chthonoplastes*
Haemophilus sp.	*Conidiobolus gonimodes*	*Rhizophlyctis harderi*	*Navicula* sp.
Klebuiella sp.	*Cunninghamella bainieri*	*Rhizopus oryzae*	*Nitzschia* sp.
Micrococcus sp.	*Cunninghamella blakesleeana*	*Rhizopus stolonifer*	*Nostoc* sp.
Moraxella sp.	*Cunninghamella echinulata*	*Saccharomices cerevisiae*	*Oscillatoria sp.* (strain JMC)
Mycobacterium flavescens	*Cunninghamella elegans*	*Saprolegnia parasitica*	*Oscillatoria sp.* (strain MEV)
Mycobacterium sp.	*Cunninghamella japonica*	*Smittium culicis*	*Phrototheca zopfii*
Nocardia sp.	*Emencellopsis* sp.	*Smittium culisetae*	*Porphyridium cruentum*
Paenobacillus sp.	*Epicoccum nigrum*	*Smittium simuli*	*Scenedesmus platydiscus*
Pseudomonas cepacia	*Fusarium oxyporum*	*Sordana fimicola*	*Selenastrum capricornutum*
Pseudomonas fluorescens	*Gilbertella persicaria*	*Syncephalastrum racemosum*	*Skeletonema costatum*
Pseudomonas paucimobilis	*Gliocadium* sp.	*Thamnidium anomalum*	*Synedra* sp.

(continued)

Table 10.2 (continued)

Some PAHs utilizing bacteria	Some PAHs utilizing fungi		Some PAHs utilizing cyanobacteria and microalgae
Pseudomonas putrid	*Helicostylum piriforme*	*Thichocladium canadense*	
Pseudomonas testeroni	*Hyphochytrium catenoides*	*Trichoderma viride*	
Pseudomonas vesicularis	*Irpex lateus*	*Verticillum* sp.	
Pseudomonas sp.	*Linderina pennispora*	*Zygorhynchus moelleri*	
Rhodococcus sp.			
Rhodotorula glutinis			
Staphylococcus auriculans			
Sphingomonas paucimobilis			
Sphingomonas sp.			
Streptomyces griseus			
Streptomyces sp.			
Vibrio sp.			

Source Modified after Cerniglia (1993)

substantive and the initial introduction of oxygen into the aromatic hydrocarbons via hydration in the anaerobic processes is thermodynamically highly unfavorable. As a result, aerobic catabolism of aromatic pollutants is more prevalent in the biosphere (Cao et al. 2009).

10.2.1 Aerobic

10.2.1.1 Bacteria

Bacteria are able to utilize PAHs as the sole source of carbon and energy (Reineke 2001). They exist as gram-positive and gram-negative bacteria with the ability to metabolize some PAHs (Cerniglia 1993).

According to Johnsen et al. (2005), bacteria growing in suspended, shaken cultures with crystalline PAHs in concentrations exceeding the aqueous solubility as the sole source of energy and carbon exhibit characteristic growth curves. These curves can be divided into three phases: exponential phase (maximum rate limited by the physiology of the bacteria, bioavailability number >1), pseudo-linear growing (maximum rate limited by the concentration of PAHs, bioavailability

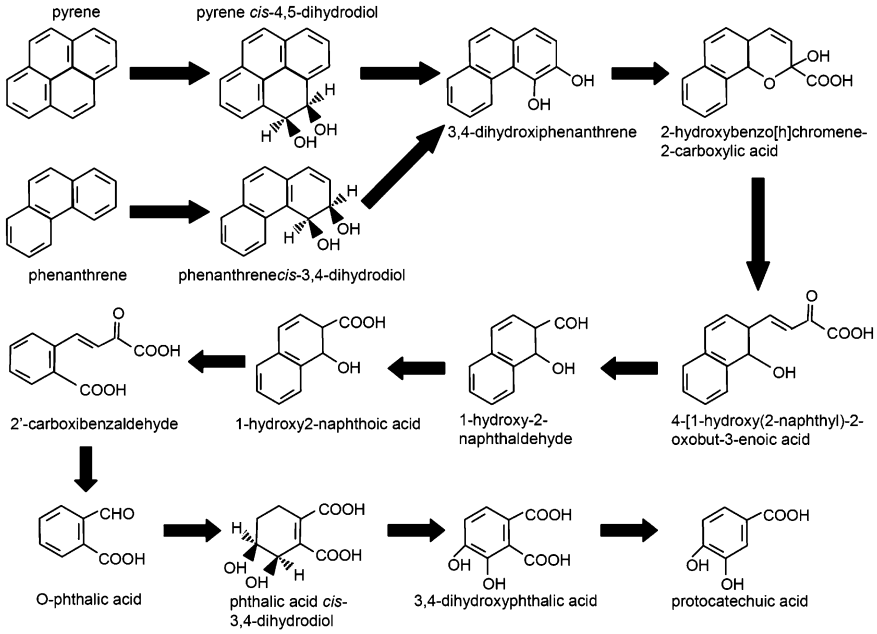


Fig. 10.2 Phenanthrene biodegradation pathway in *Mycobacterium* species (Redrawn from Pagnout et al. 2007)

number <1) and pseudo-stationary. However, real heterogeneous media do not have the same characteristics as these idealized conditions have.

Most of PAH-degrading bacteria oxidize PAHs using dioxygenases. A few bacteria, such as *Mycobacterium* sp. are also capable of oxidizing the PAHs aromatic rings via cytochrome P_{450} monooxygenase enzyme to form *trans*-dihydrodiols rather than *cis*-dihydrodiols (Cao et al. 2009) (Fig. 10.1). Principal well-know PAHs utilizing bacteria are shown in Table 10.2. In the Fig. 10.2, is shown a bacterial biodegradation pathway (Pagnout et al. 2007).

Some authors found that the co-metabolism is an important feature of the degradation of PAH. In some cases, there exists an inhibition on the biodegradation when two or more PAHs are presents while in other cases, an enhancement in the biodegradation is observed. Bouchez et al. (1995) conducted a study where six bacterial strains were isolated. Each of these strains was capable of using PAHs as sole carbon and energy source, at least one of them: naphthalene, phenanthrene, anthracene, fluorene, fluoranthene and pyrene. When more than one PAH were present in the test, an inhibition of the degradation occurs. They related this to PAH specific inhibitory capacity effect and solubility. Dean-Ross et al. (2002) found that *Mycobacterium flavescens* biodegraded fluoranthene in the presence of pyrene, although metabolization of pyrene was slower in the presence of

fluoranthene than in its absence. On the other hand, *Rhodococcus* sp. metabolized fluoranthene in the presence of anthracene, although the presence of fluoranthene slowed the rate of anthracene biodegradation. Walter et al. (1991) found that *Rhodococcus* sp. UW1 could use phenanthrene, anthracene, fluoranthene and chrysene as sole sources of carbon and energy, whereas naphthalene and fluorene were only co-metabolized.

In the field of interaction between photosynthetic organisms and bacteria, Anokhina et al. (2004) studied the consumption of phenanthrene in soil by model plant–microbial associations including natural and transconjugant plasmid-bearing rhizospheric strains of *Pseudomonas fluorescens* and *P. aureofaciens* degrading polycyclic aromatic hydrocarbons. They found that the inoculation of barley seeds with both natural and transconjugant plasmid-bearing *Pseudomonas* strains were able to degrade PAH protecting plants from the phytotoxic action of phenanthrene and favored its degradation in soil. Yutthammo et al. (2010) investigated PAHs biodegradation by Phyllosphere bacteria of ornamental plants (mainly consisting of bacteria, such as *Acinetobacter*, *Pseudomonas*, *Pseudoxanthomonas*, *Mycobacterium,* and uncultured bacteria). They studied ten evergreen ornamental plants: *Ixora* sp. (ixora), *M. paniculata* (orange jasmine), *W. religiosa* (water jasmine), *Bougainvillea* sp. (bougainvillea), *Jasminum sambac* (L.) Ait. (jasmine), *Codiaeum variegatum* (Croton), *Ficus* sp. (ficus), *Streblus asper* Lour. (toothbrush tree), *Pseuderanthemum graciliflorum* (Nees) Ridl. (blue crossandra), and *Hibiscus rosa-sinensis* L. (hibiscus). The results indicated that phyllosphere bacteria on unsterilized leaves, were able to enhance the activity of leaves for phenanthrene removal. Daane et al. (2001) carried out a study that indicated that the rhizosphere of salt marsh plants contained a diverse population of PAH-degrading bacteria, and the use of plant-associated microorganisms has the potential for bioremediation of contaminated sediments. One year later, Daane et al. (2002) described *Paenibacillus naphthalenovorans* sp. nov., a naphthalene-degrading bacterium from the rhizosphere of salt marsh plants. Other synergistic studies (Borde et al. 2003) have also demonstrated a relationship in algal-bacterial microcosms for the treatment of phenanthrene.

There are some studies focusing on the effect of concentration. (Mahanty et al. 2010) observed that *Mycobacterium frederiksbergense* degraded anthracene, naphthalene and pyrene. Experiments were conducted according to a 2^3 factorial design at the low (1 mg l^{-1}) and high (50 mg l^{-1}) levels of the PAHs in combination, in an artificial media (Bushnell Hass (BH)). The results showed that PAH removals varied 54–81% when each PAH was at low concentrations in the mixture and 67–89% at their higher concentration combinations.

Taking into consideration bioremediation studies, Das and Mukherjee (2007) found that *B.subtilis* DM-04 and *P. aeruginosa* M and NM strains could be useful in bioremediation of sites highly contaminated with crude petroleum-oil hydrocarbons. The thermophilic nature of these bacteria could add further advantage for their use in bioremediation of petroleum contaminated soils in tropical countries. Lin et al. (2010) found that a novel *Bacillus fusiformis* (BFN) isolated from the sludge in petroleum-contaminated wastewater can be used to effectively

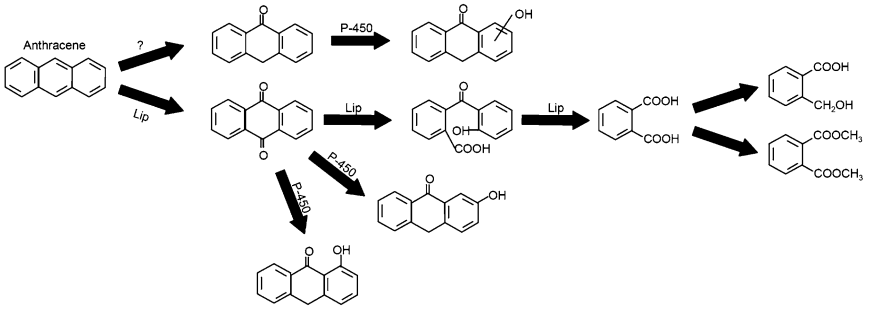


Fig. 10.3 Anthracene degradation by *I. lacteus* (Redrawn from Catjthaml et al. 2002)

biodegrade naphthalene. It emerged that the degradation of naphthalene rose to 99.1% within 4 days under optimum conditions (temperature 30°C, pH 7.0, inoculum concentration of 0.2% and C/N ratio of 1 at initial naphthalene concentration of 50 mg/L). According to Navarro et al. (2008), *Sphingomonas* sp. is able to degrade anthracene, phenanthrene, pyrene and benzo[a]pyrene, in aqueous deoxyribonucleic acid solution. In their study, aqueous DNA solution was applied for the remediation of a PAH-contaminated soil by sequential soil washing and lixiviates biodegradation.

The toxicity of PAHs metabolites during bacterial degradation has been little studied. It has been reported that the metabolites of some PAHs are potentially more bioavailable and could be more toxic than the precursors. Oxy-PAHs accumulated were more toxic and more persistent than the parent compounds, highlighting the importance of complete mineralization of PAHs without accumulation of the catabolic intermediates (Cao et al. 2009).

10.2.1.2 Fungi

A diverse group of lignolytic and non-lignolytic fungi are able to oxidize PAH (Table 10.2). Fungi do not utilize PAHs as a sole source of carbon and energy, but transform PAHs co-metabolically (Cerniglia 1993).

The microbial degradation by lignolytic fungi has been intensively studied during past few years due to their ability to degrade lignin (an amorphous and complex biopolymer with an aromatic structure similar to the aromatic molecular structure of some environmental pollutants) by producing extracellular enzymes with very low substrate specificity. This makes them suitable for degradation of aromatic compounds, such as PAHs (Valentín et al. 2006; Haritash and Kaushik 2009). Fungal degradation pathway has been reflected in Fig. 10.3 (Catjthaml et al. 2002).

Non-Lignolytic Fungi

According to Reineke (2001), a variety of non-lignolytic fungi have been found to transform polycyclic aromatic hydrocarbons to metabolites that are similar to those produced by mammalian enzymes. Only a few fungi appear to have the ability to catabolize PAHs to CO_2. Non-lignolytic fungi metabolize PAHs by citochrome P450 monoxygenase and epoxide hydrolase-catalysed reactions to form *trans*-dihydrodiols. Other metabolites formed include phenols, quinines and conjugates (Cerniglia and Sutherland 2001).

Non-lignolytic fungi, such as *Cunninghamella elegans* and *Penicillium janthinellum* can metabolize a variety of PAHs to polar metabolites (Potin et al. 2004). Also *Cladosporium sphaerospermum* was able to degrade PAHs in non-sterile soils. *Cunningamella elegans* degraded naphthalene, acenaphthene, anthracene, phenanthrene, benzo[a]pyrene, benzo[a]anthracene, fluoranthene and pyrene (Reineke 2001). Some of these fungi that metabolize polycyclic aromatic hydrocarbons are summarized in Table 10.2, and are included into different classes: Zygomycetes, Ascomycetes, Blastomycetes, Hyphomycetes, and Coelomycetes.

Lignolytic Fungi

Ligninolytic fungi possess an extracellular degradation system which is capable of breaking down lignin (Kirk and Farrell 1987). White-rot fungi can degrade a wide range of organopollutants and the degradative activity is because of the lignin-degradating systems of these fungi (Haritash and Kaushik 2009). Many authors believe that white-rot fungi metabolize PAHs through the extracellular ligninolytic enzymes, including lignin peroxidases, Mn-peroxidase, versatile peroxidase, and laccase. The precise role of these enzymes in PAH degradation has not yet been determined; however, it has been shown that only laccase-producing fungi can mineralize PAHs to CO_2 and H_2O (Pozdnyakova et al. 2010).

Covino et al. (2010b) have conducted a study to assess PAH-degradation capability of *L. tigrinus* strain CBS 577.79 in liquid cultures, to clarify the possible involvement of laccase and MnP in the degradation process and to identify the fungal PAH degradation products. They used two media: malt extract glucose (MEG) and N- limited medium (NKLM). They obtained that Laccase activity was predominant on MEG while Mn-peroxidase (MnP) was preferentially produced in LNKM. The identification of degradation products showed the presence of several PAH derivatives, presumably derived from the action of lignin-modifying enzymes. In 2000, Márquez-Rocha (2000) found that the white rot fungus, *Pleurotus ostreatus*, metabolized four soil adsorbed polycyclic aromatic hydrocarbons: pyrene, anthracene and phenanthrene and were mineralized after 21 d. However, benz[a]pyrene was also oxidized, but not mineralized. They also found that biodegradation was increased when surfactant Tween 40 was added.

A factor that is necessary to take into consideration is the salinity. Valentín et al. (2006), have been studied the application of lignolytic fungi to detoxification

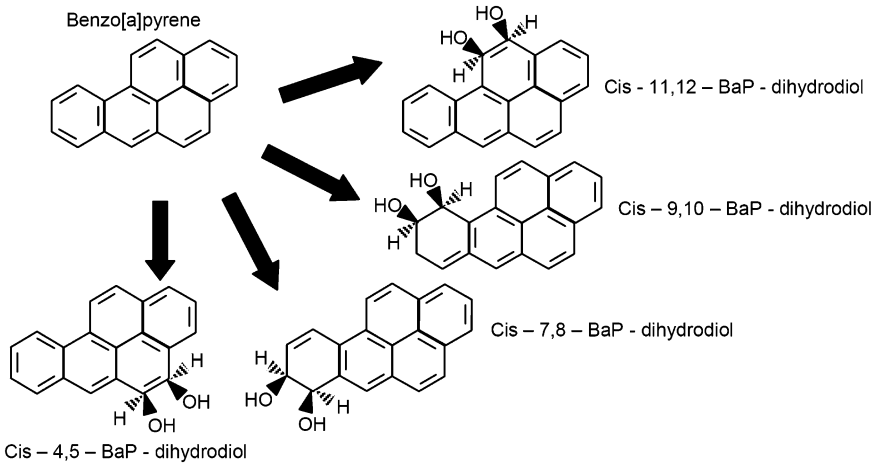


Fig. 10.4 Algal transformation products of benzo[a]pyrene (Redrawn from Juhasz and Naidu 2000)

of marine sites contaminated with PAHs, evaluating the effects of the high salinity associated with coastal areas on fungal growth, production of enzymes and ligninolytic activity. The study was carried out with four PAHs: phenanthrene, fluoranthene, pyrene and chrysene. They found an extensive inhibitory effect on PAH degradation ranging from strong for some fungi to not appreciable for others.

In biosorption context, Chen et al. (2010) carried out an experiment to elucidate biosorption of PAHs to fungal biomass and the relative contributions of biosorption and biodegradation to the total removal of PAHs by white-rot fungi. They studied five PAHs: naphthalene, acenaphthene, fluorene, phenanthrene and pyrene. In this study, the conclusion was that biosorption of PAHs by white-rot fungi is very important process influencing the fate of PAHs in the environment. The partitioning of PAHs into fungal biomass is the primary mechanism of biosorption, and sorption capabilities (Koc, carbon-normalizated partition coefficients) are linearly related to Kow (octanol–water partition coefficients).

Other factor to be taken into consideration is the culture media. Pozdnyakova et al. (2010) have carried out some experiments to study the influence of cultivation conditions on pyrene degradation by the fungus *Pleurotus ostreatus* D1. They found that in Kirk's medium, about 65.6 ± 0.9% of the initial pyrene was metabolized after three weeks, and that in basidiomycetes rich medium, *P. ostreatus* D1 metabolized up to 89.8 ± 2.3% of pyrene within three weeks. In the first case, pyrene-4,5-dihydrodiol was accumulated where as it did not exist in the latter case. Experiments were also conducted to determine the effect of fungal inoculum, biomass and glucose concentrations on fluoranthene, pyrene and chrysene degradation by *Bjerkandera* sp. BOS55 (Valentín et al. 2007). This work showed the capability of the white-rot fungus to degrade PAHs in soil slurry phase

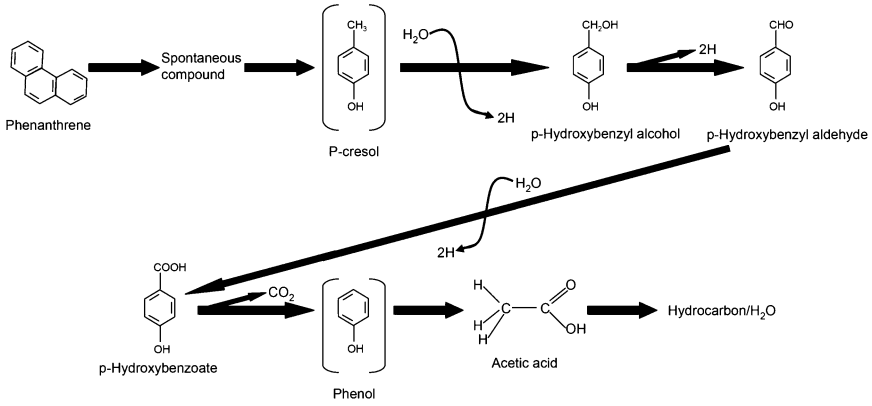


Fig. 10.5 Proposed pathways for anaerobic biotransformation of phenanthrene by sulfate-reducing bacteria (Redrawn from Tesai et al. 2009)

bioreactors. They found that the use of free mycelia seems to be beneficial for the degradative action of the fungus. Thus, degradation of the PAHs was enhanced between 13 and 29% by using mycelium as inoculum compared to fermentation with pellets inoculation. They also found that a small change in the initial glucose concentration in the range 20–3 g/L did not exert a significant effect on PAH degradation while the initial biomass exerted a larger effect on PAH degradation compared to the other two studied parameters. In the study of very concentrated media, Bumpus (1989) demonstrated that *P. chrysosporium* is able to degrade PAHs present in anthracene oil (heavy oil which distils over from coal tar). Analysis by capillary gas chromatography and high-performance liquid chromatography showed that at least 22 PAHs, including all of the most abundant PAH components present in anthracene oil, underwent 70–100% disappearance during 27 days of incubation under nutrient nitrogen–limited media. An experiment was conducted by Kotterman et al. (1994) to optimize the biodegradation of anthracene by *Bjerkandera* sp. with respect to O_2, N, and C. They concluded that the supply of O_2 was the most important factor in the biodegradation of anthracene.

Bioremediation of polluted liquid and solid matrices with lignolytic fungi has been widely studied for last several years. There are some works on comparison of different organisms. A study to assess the PAH-biodegradation potential of *L. tigrinus* CBS 577.79 on real solid matrices derived from a wood treatment plant in view of its possible use in mycoremediation applications was carried out by Covino et al. (2010a). They compared the results with *Irpex lacteus.* They found that *L. tigrinus* was able to colonize and detoxify solid PAH-contaminated matrices under non-sterile conditions. Its growth and degradation performances were invariably higher than those of *I. lacteus.*

Like in other situations, the factor of the aged in the matrix is important. Eggen and Majcherczyk (1998) have studied the degradation and metabolisms of

benzo[a]pyrene in soil inoculated with *P. ostreatus*. Two strains of *P. ostreatus* were used in a mineralization study with [^{14}C]benzo[a]pyrene. They observed that in aged soil contaminated with creosote, *P. ostreatus* removed benzo[a]pyrene most extensively in the very first month. Elimination of spiked ^{14}C-benzo[a]pyrene was higher than benzo[a]pyrene originally present, and 40% removal of the first compound was observed. Although mineralization was low (1%), but there was a significant inoculation effect (with white rot fungi) on mineralization as compared to control.

10.2.1.3 Microalgae and Cyanobacteria

It has been known that Cyanobacteria and eukaryotic algae have the capacity to metabolize PAHs. As Cyanobacteria are the largest and most widely distributed group of photosynthetic prokaryotes on earth (Reuter and Müller 1993), it has been decided to include this group in a specific section, together with microalgae. Figure 10.4 shows an algal biodegradation pathway (Juhasz and Naidu 2000).

Some researchers studied the difference in species of cyanobacteria and micro algae in relation to PAHs degradation. Cerniglia et al. (1980) studied the ability to metabolize naphthalene of nine cyanobacteria, four green microalgae, one red microalgae, and two diatoms. All these organisms oxidized naphthalene under photoautotrophic conditions. Hong et al. (2008) found that the tolerance of *Skeletonema costatum* to phenanthrene and fluoranthene was greater than that of *Nitzschia sp.*, and that the toxic effect of fluoranthene on *S. costatum* and *Nitzschia sp.* was higher than that of phenanthrene. They also found that the accumulation and degradation abilities of *Nitzschia* sp. were higher than those of *S. costatum*. Lei et al. (2007) studied the efficiency of four microalgal species (*Chlorella vulgaris*, *Scenedesmus platydiscus*, *Scenedesmus quadricauda*, and *Selenastrum capricornutum)* to remove fluoranthene, pyrene, and a mixture of fluoranthene and pyrene. They observed that PAH removal was algal species specific and also PAH-dependent. The most effective specie in removing and transforming PAHs was *S. Capricornutum* and the least effective was *C. vulgaris.*

In the field of metabolic pathways, Warshawsky et al. (1995) carried out a study with different culture lamps and found that under gold light, metabolites of Benzo[a]pyrene (BaP) produced by *Selenastrum capricornutum* were the dihydrodiols of which the 11,12-dihydrodiol was the major metabolite. Under white light, at low doses of BaP, the major metabolite was 9,10-dihydrodiol. With increasing dose, the ratio of dihydrodiols to quinones decreased to less than two. Similarly, with increasing light energy output, from gold to white to UV-A in the PAH absorbing region, BaP quinone production was increased. According to Narro et al. (1992a), the marine cyanobacterium *Oscillatoria* sp. strain JCM oxidized naphthalene predominantly to 1-naphthol. Other study of Narro et al. (1992b) showed that under photoautotrophic growth conditions, the marine cyanobacterium *Agmenellum quadruplicatum* PR-6 metabolized phenanthrene to form trans-9,

10-dihydroxy-9,10-dihydrophenanthrene (phenanthrene trans-9,10-dihydrodiol) and 1-methoxyphenanthrene as the major ethyl acetate-extractable metabolites.

Others works were carried out to study the interaction with metals. Ke et al. (2010) investigated the effects of some metals on biosorption and biodegradation of five mixed polycyclic aromatic hydrocarbons, by *Selenastrum capricornutum*. They found that for low molecular weight PAHs, both metal dosage and exposure time had a significant, positive effect on their removal, while for high molecular weight PAHs, the presence of metals did not affect the removal efficiency. Some of the cyanobacteria and microalgae that metabolize polycyclic aromatic hydrocarbons are summarized in Table 10.2.

10.2.2 Anaerobic and Anoxic Conditions

During past two decades, anaerobic biodegradation of aromatic pollutants has been a subject of extensive research (Cao et al. 2009). According to them, some bacteria which are involved in PAHs anaerobic degradation are *Acidovorax* sp., *Bordetella* sp., *Pseudomonas* sp., *Sphingomonas* sp., *Variovorax* sp., *P. stutzeri* and *Vivrio pelagiusrelated*.

Anaerobic degradation is a slower process than aerobic limited by several other factors and present in natural environments and so it cannot be overlooked. For petroleum hydrocarbons, even though aerobic processes are generally used, anaerobic biodegradation is significant under certain circumstances (e.g., O_2-depleted aquifers, oil spilled marshes) (Zhang and Bennett 2005). However, biochemical mechanism of anaerobic biodegradation of PAHs has not yet been elucidated (Haritash and Kaushik 2009). An anaerobic degradation pathway has been shown in Fig. 10.5 (Tesai et al. 2009).

Rockne et al. (2000) showed that highly-enriched, denitrifying cultures could mineralize bicyclic and polycyclic aromatic hydrocarbons. Mineralization was nitrate dependent and was sustainable over several feedings and the cultures produced N_2O, a denitrification product, when was supplied with PAHs as the sole carbon and energy source. Ambrosi et al. (2005) conducted an experiment by adding fluorene, phenanthrene and pyrene to soil samples in order to investigate the anaerobic degradation potential of PAHs under denitrifying conditions. The study was made with the soil alone or enriched with nitrate, as electron acceptor, PAHs and, in some samples, glucose or acetate. The principal variable was the specific PAHs tested, because the molecular conformation prevails over other parameters in controlling the degradation. The next variable in importance was the addition of other carbon sources as different of the PAHs. They concluded that anaerobic biodegradation of PAHs is possible both through fermentative and respiratory metabolism, provided that low molecular weight co-metabolites and suitable electron acceptors (nitrate) are present.

There are also a few studies on co-metabolism. A study carried out by Chang et al. (2008) in mangrove sediments, with phenantrene and pyrene, demonstrates that the anaerobic degradation of PAH was enhanced by the addition of acetate, lactate, pyruvate, sodium chloride, cellulose, or zero-valent iron. However, it was inhibited by the addition of humic acid, di-(2-ethylhexyl) phthalate (DEHP), nonylphenol, or heavy metals. Marine sediment biodegradation was also studied by Kim et al. (2008) who chose lactate as a supplementary carbonaceous substrate to stimulate degradation of PAHs. Results showed that lactate amendment enhanced biodegradation rates of PAHs in the sediments by 4–8 times, and caused a significant shift in archaebacterial community in terms of structure and diversity with time. Li et al. (2009) studied mangrove sediments of Hong-Kong, concretely the vertical distribution and anaerobic biodegradation of polycyclic aromatic hydrocarbons in these sediments. They found close relationships between bacterial population sizes and the concentration of anaerobic electron acceptors. The dominant electron acceptor was sulfate followed by Fe(III). Mn(IV) was at very low concentrations and nitrates were nearly exhausted at sediment depths greater than 6 cm.

The anaerobic treatment of sewage sludge is of great importance as reported by several authors. In the context of anaerobic sludge treatment, it is important to take into consideration the control of sludge quality (particularly its concentration in trace organic pollutants), so as to limit potential risks owing to sludge land application. The anaerobic removal of 13 Polycyclic Aromatic Hydrocarbons (PAHs) was measured in five continuous anaerobic digestors with different feed sludge (Barret et al. 2010), to elucidate if bioavailability and/or co-metabolism limit their biodegradation during the anaerobic digestion of contaminated sludge. In their work, they demonstrated that aqueous PAHs (sum of free and sorbed-to-DCM PAHs) were bioavailable. It was also demonstrated that bioavailability is not the only influencing factor. Indeed, PAHs biodegradation resulted from a combination of bioavailability and co-metabolism. Bernal-Martínez et al. (2005) combined anaerobic digestion and ozonation to remove PAH from urban sludge. The digested sludge with known PAH concentration, was ozonized in a fed batch reactor. The ozonation of anaerobically digested sludge improved the PAH removal rate (61%). An additional enhancement (up to 81%) of the PAH removal rate was obtained by the addition of hydrogen peroxide during ozonation. Similar performances were achieved by the use of surfactants which enhanced PAH bioavailability.

10.3 Environmental Compartments

The spread of chemicals is caused by high production and release rates combined with their relative mobility in air, water, soil and biota, and their stability against biotic and abiotic transformation (Bosma et al. 2001). Highly heterogenic spatial distribution of PAHs in the different compartments depends on several characteristics, i.e. sources location, abundance of emission, the

atmospheric/marine circulation regimen, etc. Therefore, it is of special concern for the study of biodegradation of PAHs in the environmental compartments on which they can be located.

10.3.1 Soils

According to Johnsen et al. (2005), in soil, PAHs are heterogeneously distributed and may be adsorbed on organic particles in small pores which makes them inaccessible to microorganisms, or otherwise occluded by the multitude of solid soil constituents. The spatial distribution of soil microorganisms is also heterogeneous and often autocorrelated at scales from millimetres to tens of metres, and similar scales of heterogeneity apply to geophysical and geochemical parameters (Bengtsson et al. 2010).

The levels of PAHs, like in all compartments, are very heterogeneous from non-polluted areas to high polluted. For example, Nadal et al. (2004) obtained the sum of the 16 PAHs levels between 1002 and 112 ng/g (dry weight) for samples collected near chemical industries and unpolluted sites, respectively, in Tarragona (Spain). Nam et al. (2008) determined PAHs levels from background locations in the UK and Norway. They found that in the UK, the concentrations ranged between 42 and 11200 μg kg^{-1} dry weight, and in Norway, ranged between 8.6 and 1050 μg kg^{-1} dry weight. In urban soils of Beijing (China), Tang et al. (2005) found concentrations ranging from less than 366–27825 ng g^{-1}. In Poland, in arable soils, Maliszewska-Kordybach et al. (2008) obtained that the content of Σ16PAHs ranged from 80–7264 μg kg^{-1}.

Soil is the most extensively studied compartment for PAHs biodegradation. There are different factors that influence degradation in soils, like the presence of aged PAHs, the inoculums, the species presents in soil and the characteristics of the soil.

Related to the aging process, Huesenmann et al. (2004) reported that aged PAHs, that are often thought to be recalcitrant due to bioavailability limitations, may not be so and therefore, may pose a greater risk to environmental receptors than previously thought. Bogan and Sullivan (2003) studied the degradation of phenanthrene and pyrene by *Mycobacterium austroafricanum* strain GTI-23. They studied six different soils and observed that for both, phenanthrene and pyrene, an increase in contact time led to higher sequestration and lower biodegradation, and that TOC content was the most important parameter governing these processes.

In the field of inoculums studies, Arias et al. (2008) worked on the production and fate of PAHs metabolites in soil, as metabolites are potentially more toxic than the parental products. They developed a static soil microcosm system and an analytical methodology for detection of PAHs in soils and their oxidation products. They used this system with a soil contaminated with phenanthrene (as a model PAH) and 1-hydroxy- 2-naphthoic acid, diphenic acid, and phthalic acid (as putative metabolites). They studied this with and without inoculation of strain

Mycobacterium sp. AP1. In inoculated microcosms, 35% of the added phenanthrene was depleted, 19% being recovered as CO_2 and 3% as diphenic acid. The latter, together with other two unidentified metabolites, was accumulated in the soil. Somtrakoon et al. (2008) carried out a study to enhance biodegradation of anthracene in acidic soil by inoculating *Burkholderia* sp. VUN10013 and found that the indigenous microorganisms in the pristine acidic soils have limited ability to degrade anthracene, and bacterial inoculation significantly enhanced anthracene degradation in such acidic soils. Yuan et al. (2002) studied the inoculation of non-local microorganisms in the soil to investigate the differences in biodegradation. They observed that the consortium used was capable of biodegrading five PAHs and found that the remaining PAH amount after 40-day incubation period ranged from 92.6–96.9% in the autoclaved control bottles; from 90.1–94.6% in non-autoclaved bottles and without consortium; from undetectable to 13.5% in non-autoclaved bottles and with PAH-adapted consortium.

Taking into consideration different degrading species and their previous exposure or not to the pollutants, some studies have been carried out. Johnsen and Karlson (2005) estimated the PAH degradation capacity of 13 soils ranging from pristine locations to heavily polluted industrial sites to investigate, if the PAHs degradation capacity depends or not on previous PAH exposure (concretely phenanthrene and pyrene). They found that densities of phenanthrene degraders reflected previous PAH exposure, whereas pyrene degraders were detected only in the most polluted soils. They found that the time to 10% mineralization of added ^{14}C phenanthrene and ^{14}C pyrene was inversely correlated to the initial PAH pollution.

According to the characteristics of the soil, Hwang and Cutright (2004) studied a relationship between soil characteristics and subsequent desorption and biodegradation of pyrene. They found that fraction of expandable clay minerals was correlated to the achievable desorption and biodegradation. It could be possible to that the microbial movement and adhesion to clays turned out to a great extent of the soil-phase biodegradation. The maximum achievable desorptions were 30.2, 10.4, and 1.0 mg/kg for soils which were 1.7, 2.2, and 4.4 wt% of expandable clays (smectite and vermiculite), respectively. The total (soil + water) biodegradation reached to 65, 78.3, and 81.8% of the initial concentration (100 mg/kg) for the three soils, respectively. Nam and Alexander (1998) studied the role of sequestration and bioavailability (of phenanthrene) to the nanoporosity and the hydrophobicity. They found results according to their previous hypothesis, which was that sequestration and reduced bioavailability occur when hydrophobic compounds enter into nanopores having hydrophobic surfaces. Nam and Kim (2002) studied the role of loosely bound humic substances in the bioavailability of aged phenanthrene. They observed that in the humic-mineral fraction occurred major sequestration and, for that reason, humic and fulvic acids may act like a barrier to the biodegradation.

There are several studies of the role of surfactants in enhancing biodegradability of PAHs in soils. Many PAHs sorb strongly onto soil, which limits their availability for microbial degradation. Surfactants have been shown to enhance

desorption and solubilisation of PAHs (Wilson and Jones 1993). Sobish et al. (2000) found that with surfactant combinations T10 and T15 (mixtures of a non-ionic hydrophilic and a non-ionic hydrophobic component) the biodegradation was enhanced. With the addition of T10:1(1%) to a different types of soils, they found in the first one, having an overall low bioavailability of PAH, a reduction of 16 PAHs by 36%, while in other soils they were reduced by 44% and by 90%. Chang et al. (2008) evaluated the effects of chemical interactions on naphthalene biodegradation in the surfactant-soil/water systems. They found that addition nonionic surfactants (TX-100 and Brij35) used as the concentration of monomer or micelle, can influence naphthalene biodegradation. Kim et al. (2001) investigated the effect of non-ionic surfactants on the solubility and biodegradation of PAHs in the soil slurry phase. They found that the PAH solubility was linearly proportional to the surfactant concentration above the critical micelle concentration.

10.3.2 Sediments

Photochemical reactions are not possible in the subsurface, where microbial-mediated transformations constitute the dominant removal mechanism of persistent organic compounds (Bosma et al. 2001). The microbial biodegradation of PAHs in the sediments (like in soils) has been widely studied, due to the presence of different groups of microorganisms that are able to degrade these compounds and due to different PAH levels.

10.3.2.1 Marine Sediments

Because of their low water solubility and hydrophobicity, PAHs in the marine environment rapidly become associated with organic and inorganic suspended particles (Chiou et al. 1998) and they are subsequently deposited into sediments. As PAH solubility decreases with increasing molecular weight, bioaccumulation of PAHs from sediments by marine organisms is generally greater for the lower molecular weight and more water soluble PAHs (Djomo et al. 1996; Porte and Albaigués 1994).

PAH levels in marine sediments are highly heterogeneous depending on geographical location. For example, PAH concentrations as high as 48000 ng/g were found in Venice lagoon (LaRocca et al. 1996), levels between 5700 and 8500 ng/g were found in the Mediterranean littoral of France-Spain (Baumard et al. 1999) and levels of approximately 800 ng/g were found in Todos Santos Bay, Mexico (Macías-Zamora et al. 2002). Shao et al. (2010) carried out an investigation of the deep-sea sub-surface environment, concretely in sub-surface sediments on the Mid-Atlantic Ridge. They found that the total concentration of PAHs was 445 ng/g dry wt sediment, in the sediment 2.7 m beneath the bottom surface at a water depth of 3962 m on the Mid-Atlantic Ridge. The concentrations of phenanthrene and

fluorene were relatively high. In addition, PAH-degrading bacteria were found within the sediments.

In marine sediments, some of the most important factors in PAHs biodegradations are size of inoculum, salinity characteristic of the sediment and pre-exposure of the organisms. There has been growing concern over the mounting concentration of PAHs in the marine environment. One of the most studied areas is the mangrove, important estuarine wetland closely tied to human activities and subject to PAHs contamination (Haritash and Kaushik 2009). Chen et al. (2008) studied the degradation of phenathrene by *Sphingomonas* sp., a bacterial strain isolated from mangrove sediment. They found that in this case, salinity was the most significant factor in biodegradation, followed by inoculum size. They noted that the phenanthrene biodegradation could be best described by the first order rate model, with a kinetic constant of 0.1185 h^{-1}, under the optimal condition (30°C, 15 ‰ salinity, a carbon/nitrogen ratio of 100 : 1 and an inoculum size of 10^6 MPN g^{-1} sediment). They found that the biodegradation was lower with more or less salinity than optimal, and with smaller inoculum size than optimal. Chen et al. (2010) studied the degradation of phenanthrene in mangrove sediments, but with different inocula (*Sphingomonas* sp., a mixture of *Sphingomonas* sp. and *Mycobacterium* sp., and without inoculum), different salinities, different sediment types, and presence of other PAHs. They observed that optimal phenanthrene biodegradation could take place in clay loam sediment slurry at low salinity (5–15‰) with the inoculation of both *Sphingomonas* sp. and *Mycobacterium* sp., and that the presence of other PAHs had little impact in the degradation process.

Tadros and Hughes (1997) investigated the degradation of PAHs by indigenous mixed and pure cultures isolated from coastal sediments. They found two bacterial species degraders of PAHs in their sediments: *Pseudomonas* sp. and *Ochrobactrum* sp. They also found a relationship between bacteria concentration and the degradation of the compounds. The degradations of naphthalene and fluorene were 98.7% and 90.4%, respectively by indigenous mixed microorganisms, both used as sole grown substrate (after 8 days of incubation). Hinga (2003) studied subtidal marine sediments sampled after a fuel oil spill. It was found that the degradation rate of low molecular weight (LMW) polycyclic aromatic hydrocarbons (PAH) was correlated to the sediment total organic carbon (TOC).

10.3.2.2 Freshwater Sediments

Among organic pollutants, polycyclic aromatic hydrocarbons (PAHs) are common in freshwater ecosystems and particularly in river sediments where they accumulate (Quantin et al. 2005).

Jackson and Pardue (1999) investigated the biodegradation of Louisiana "sweet" crude oil in Lousiana's freshwater marshes and found degradation rates of 6.8% per day for the measured polycyclic aromatic hydrocarbon fractions (i.e. naphthalene, methylated naphthalenes, phenanthrene, and methylated phenanthrenes). Yuan et al. (2001) carried out a study in the laboratory to determine

the potential biodegradation of phenanthrene in the river sediment (from Keelung River). They studied seven different sediments, and found half-lives ranged from 0.61 to 5.78 days when the concentration of phenanthrene was 5 μg/g. Moreover, they studied the addition of different substances and found that acetate, pyruvate and yeast extract did not have a significant impact on the biodegradation, but ammonium, sulphate and phosphate had an influence. Quantin et al. (2005) studied the biodegradation of river sediments under oxic and anoxic conditions, and also investigated whether input of fresh organic material (cellulose) could enhance biodegradation. They observed aerobic degradation for the PAHs, but they did not find anaerobic degradation. They observed that total dissipation of 12 PAHs reached a maximum of 78% in the NOM biotic treatment (natural organic matter as the only source of carbon) under aerobiosis after 180 days. Also they found that cellulose addition stimulated both aerobic and anaerobic respiration, but had no effect on PAH dissipation (disappearance).

10.3.3 Water

In water environments, the solubility of PAHs is low, and decreases with increasing molecular weight (solubilities are shown in Table 10.1). In sea water, the solubility of each PAH is less than in fresh water due to salting out effect. PAHs biodegradation studies conducted in the aquatic environment have been carried out mainly with cyanobacteria and algae.

Due to their high hydrophobicity, PAHs tend to interact with non-aqueous phases and natural organic matter also, as a consequence, they are poorly bioavailable for microbial degradation (Buchholz et al. 2007). The biodegradation in water environment has not been studied as widely as in other compartments (like sediments or soils). In this compartment, the biodegradation has been less studied in seawater than in freshwater.

10.3.3.1 Seawater

Like in others compartments, PAHs levels in surface seawater are highly heterogeneous. Witt (2002) found a concentrations of PAHs up to 16,600 pg/L in Central Baltic Sea. In Bahia Blanca estuary (Argentina), levels were found between undetected to more than 4 μg/L (Arias et al. 2009).

Hong et al. (2008) studied the degradation of phenanthrene and fluoranthene by *Skeletonema costatum* and *Nitzschia* sp. (from a mangrove aquatic ecosystem). They found that the microalgal species showed comparable or higher efficiency in the removal of the mixture of these two PAHs than each one singly, suggesting that the presence of one PAH stimulated the degradation of the other. They further noted that in the case of *Nitzschia* sp. the degradation of phenanthrene in single condition was 3.73% and in mixed condition 4.75% and the degradation of

fluoranthene in single condition was 3.33%and in mixed conditions was 3.59%. In the case of *S. costatum*, they observed no significant difference for fluoranthene degradation between single and mixed conditions, but the degradation of phenanthrene in mixed conditions was significantly higher than in single condition (38 and 16%, respectively). Cerniglia et al. (1980) compared the ability of nine cyanobacteria, four green microalgae and two diatoms, some isolated from seawater and some from freshwater to metabolize naphthalene and found that all these organisms oxidized naphthalene under photoautotrophic conditions (between 0.5 and 2.4% in 24 h). Narro et al. (1992b) studied the degradation of phenanthrene by the marine cyanobacterium *Agmenellum quadruplicatum.* They concluded that the marine cyanobacterium *A. quadruplicatum* PR-6 metabolized phenanthrene to form phenanthrene trans-9,10-dihydrodiol and 1-methoxyphenanthrene as the major metabolites. Poeton et al. (1999) studied the biodegradation of PAH by marine bacteria in water, and the effect of solid phase. They found linear relationship between aqueous PAH and biomass concentration. They also found that the coefficients for this model were different with and without sediments, and that the biodegradation was higher in presence of sediments (with sediment present, degradation rates for phenanthrene and fluoranthene were 2.1 to 3.5 and 2.1 to 5.3 times faster, respectively).

10.3.3.2 Freshwater

Levels of different PAHs in freshwater are very heterogeneous. For example, Wang et al. (2009) found in the Three Gorges Reservoir (Yangtze River) levels of total PAHs between 13.8 and 97.2 ng L^{-1}. Fernandes et al. (1997) found PAH level in the Seine River and its estuary, ranging from 4 to 36 ng L^{-1}. Countway et al. (2003) carried out a study in the York River and its estuary, and obtained levels of PAHs between 2.09 and 122.85 ng L^{-1} (with the salinity in these points around 13). Smith et al. (1991) investigated levels of PAHs in waters from three rivers which traverse the largest cities in south-eastern Australia in range of <0.3 to 525 ng L^{-1}.

Ke et al. (2010) studied biodegradation of fluorene, phenanthrene, fluoranthrene, pyrene and benzo[a]pyrene by green freshwater alga *Selenastrum capricornutum*, and the influence of the exposure to metals on the biodegradation process. For the PAHs studied, they only found influence of metal dosage and exposure time on the removal of fluorene and phenanthrene (up to 99 and 87% from the medium in seven days). Warshawsky et al. (1988) also studied *Selenastrum capricornutum* with respect to biodegradation of benzo[a]pyrene. They found that *S. capricornutum* produced cisvicinal dihydrodiols from molecular oxygen via a dioxygenase enzyme pathway. It was the first example for characterization of a dioxygenase pathway in a freshwater alga of a five ring carcinogenic PAH. Lei et al. (2007) investigated the degradation of fluoranthene and pyrene by four freshwater algae: *Chlorella vulgaris, Scenedesmus platydiscus, Scenedesmus quadricauda*, and *Selenastrum capricornutum*. Percentage remaining in medium

after seven days of both PAHs with the different algae was: *Chlorella vulgaris* 23.8%, *Scenedesmus platydiscus* 11.8%, *Scenedesmus quadricauda* 3.4%, and *Selenastrum capricornutum* 2.0%. Xia et al. (2006) studied biodegradation of chrysene, benzo(a)pyrene and benzo(g,h,i)perylene in the Yellow river. As this river is the most large turbid in the world, they studied the effect of suspended sediment on the biodegradation. They found that the biodegradation rates of PAHs increased with the sediment content in the water. For chrysene (with initial concentration of 3.80 μg/L), biodegradation rate constants were 0.053, 0.084 and 0.111 d^{-1}, when the sediment contents were 0, 4 and 10 g/L, respectively).

10.4 Conclusions

The PAH degrading microorganisms from different taxonomic groups (mainly bacteria, microalgae and fungi), produce different metabolites on degradation and carry out degradation in different environmental compartments. In general, PAHs with low molecular weight are more degraded than PAHs with high molecular weight. The compartment most studied is soil, followed by sediment. The water is less studied than soil and sediment, and seawater less than freshwater. In soil and sediment, the main variables are size of inoculum, degrading species, and characteristics of soil/sediment. In the sediment also, salinity is important. In water, some of the factors that influence the biodegradation are degrading species, sediments content in the water and presence of other compounds or other PAHs. In the future, it would be interesting to carry out an extensive study of different compartments (making emphasis on the effect of different variables), especially those compartments which are yet less studied.

References

Ambrosoli R, Petruzzelli L, Luis Minati J, Ajmone Marsan F (2005) Anaerobic PAH degradation in soil by a mixed bacterial consortium under denitrifying conditions. Chemosphere 60: 1231–1236

Anokhina TO, Kochetkov VV, Zelenkova NF, Balakshina VV, Boronin AM (2004) Biodegradation of phenanthrene by *Pseudomonas* bacteria bearing rhizospheric plasmids in model plant-microbial associations. Appl Biochem Microbiol 40:568–572

Arias L, Bauzá J, Tobella J, Vila J, Grifoll M (2008) A microcosm system and an analytical protocol to assess PAH degradation and metabolite formation in soils. Biodegradation 19:425–434

Arias AH, Spetter CC, Freije RH, Marcovecchio JE (2009) Polycyclic aromatic hydrocarbons in water, mussels (Brachidontes sp., Tagelus sp.) and fish (Odontesthes sp.) from Bahía Blanca Estuary, Argentina. Estuarine, Coastal, Shelf Sci 85:67–81

Bamforth SM, Singleton I (2005) Bioremediation of polycyclic aromatic hydrocarbons: current knowledge and future directions. J Chem Technol Biotechnol 80:723–736

Barret M, Carrère H, Delgadillo L, Patureau D (2010) PAH fate during the anaerobic digestion of contaminated sludge: Do bioavailability and/or cometabolism limit their biodegradation? Water Res 44:3797–3806

Baumard P, Budzinski H, Garrigues P, Narbonne JF, Burgeot T, Michel X, Bellocq J (1999) Polycyclic aromatic hydrocarbon (PAH) burden of mussels (*Mytilus* sp.) in different marine environments in relation with sediment PAH contamination, and bioavailability. Marine Environ Res 47:415–439

Bengtsson G, Törneman N, Yang X (2010) Spatial uncoupling of biodegradation, soil respiration, and PAH concentration in a creosote contaminated soil. Environ Pollut 158:2865–2871

Bernal-Martínez A, Carrère H, Patureau D, Delgenès JP (2005) Combining anaerobic digestion and ozonation to remove PAH from urban sludge. Process Biochem 40:3244–3250

Bezalel L, Hadar Y, Fu P, Freeman J, Cerniglia C (1996) Initial oxidation products in the metabolism of pyrene, anthracene, fluorene, and ibenzothiophene by the white rot fungus Pleurotus ostreatus. Appl Environ Microbiol 62:2554–2559

Bogan BW, Sullivan WR (2003) Physicochemical soil parameters affecting sequestration and mycobacterial biodegradation of polycyclic aromatic hydrocarbons in soil. Chemosphere 52:1717–1726

Borde X, Guieysse B, Delgado O, Muñoz R, Hatti-Kaul R, Nugier-Chauvin C, Patin H, Mattiasson B (2003) Synergistic relationships in algal-bacterial microcosms for the treatment of aromatic pollutants. Biores Technol 86:293–300

Bosma T, Harms H, Zehnder A (2001) Biodegradation of xenobiotics in environment and technosphere. In: Beek B (ed) Biodegradation and Persistence. Springer, Berlin

Bouchez M, Blanchet D, Vandecasteele JP (1995) Degradation of polycyclic aromatic hydrocarbons by pure strains and by defined strain associations: inhibition phenomena and cometabolism. Appl Microbiol Biotechnol 43:156–164

Buchholz F, Wick LY, Harms H, Maskow T (2007) The kinetics of polycyclic aromatic hydrocarbon (PAH) biodegradation assessed by isothermal titration calorimetry (ITC). Thermochimica Acta 458:47–53

Bumpus JA (1989) Biodegradation of polycyclic hydrocarbons by Phanerochaete chrysosporium. Appl Environ Microbiol 55:154–158

Cajthaml T, Möder M, Kacer P, Sasek V, Popp P (2002) Study of fungal degradation products of polycyclic aromatic hydrocarbons using gas chromatography with ion trap mass spectrometry detection. J Chromatogr A 974:213–222

Cao B, Nagarajan K, Loh K-C (2009) Biodegradation of aromatic compounds: current status and opportunities for biomolecular approaches. Appl Microbiol Biotechnol 85:207–228

Cerniglia CE (1984) Microbial metabolism of polycyclic aromatic hydrocarbons. In: Allen IL (ed) Advances in applied microbiology. Academic Press, Orlando

Cerniglia CE (1993) Biodegradation of polycyclic aromatic hydrocarbons. Curr Opin Biotechnol 4:331–338

Cerniglia CE, Sutherland JB (2001) Bioremediation of polycyclic aromatic hydrocarbons by ligninolytic and non-ligninolytic fungi. In: de Gadd GM (ed) Fungi in Bioremediation. Cambridge University Press, Cambridge

Cerniglia CE, Gibson DT, Van Baalen C (1980) Oxidation of naphthalene by cyanobacteria and microalgae. J Gen Microbiol 116:495–500

Chang B-V, Chang I, Yuan S (2008) Anaerobic degradation of phenanthrene and pyrene in mangrove sediment. Bull Environ Contam Toxicol 80:145–149

Chen J, Wong MH, Wong YS, Tam NFY (2008) Multi-factors on biodegradation kinetics of polycyclic aromatic hydrocarbons (PAHs) by *Sphingomonas* sp. a bacterial strain isolated from mangrove sediment. Marine Pollut Bull 57:695–702

Chen B, Wang Y, Hu D (2010) Biosorption and biodegradation of polycyclic aromatic hydrocarbons in aqueous solutions by a consortium of white-rot fungi. J Hazard Mater 179:845–851

Chiou CT, Mcgroddy SE, Kile DE (1998) Partition characteristics of polycyclic aromatic hydrocarbons on soils and sediments. Environ Sci Technol 32:264–269

Code of Federal Regulation (1982) Title 40, Appendix A to part 423-126 Priority pollutants. 47 FR 52304

Countway RE, Dickhut RM, Canuel EA (2003) Polycyclic aromatic hydrocarbon (PAH) distributions and associations with organic matter in surface waters of the York River, VA Estuary. Organ Geochem 34:209–224

Covino S, Cvancarová M, Muzikár M, Svobodová K, D'annibale A, Petruccioli M, Federici F, Kresinová Z, Cajthaml T (2010a) An efficient PAH-degrading *Lentinus* (Panus) *tigrinus* strain: Effect of inoculum formulation and pollutant bioavailability in solid matrices. J Hazard Mater 183:669–676

Covino S, Svobodová K, Kresinová Z, Petruccioli M, Federici F, D'annibale A, Cvancarová M, Cajthaml T (2010b) In vivo and in vitro polycyclic aromatic hydrocarbons degradation by *Lentinus* (Panus) *tigrinus* CBS 577.79. Biores Technol 101:3004–3012

Daane LL, Harjono I, Zylstra GJ, Haggblom MM (2001) Isolation and characterization of polycyclic aromatic hydrocarbon-degrading bacteria associated with the rhizosphere of salt marsh plants. Appl Environ Microbiol 67:2683–2691

Daane LL, Harjono I, Barns SM, Launen LA, Palleroni NJ, Haggblom MM (2002) PAH-degradation by *Paenibacillus* spp. and description of Paenibacillus naphthalenovorans sp. nov., a naphthalene-degrading bacterium from the rhizosphere of salt marsh plants. Int J Syst Evol Microbiol 52:131–139

Das K, Mukherjee AK (2007) Crude petroleum-oil biodegradation efficiency of *Bacillus subtilis* and *Pseudomonas aeruginosa* strains isolated from a petroleum-oil contaminated soil from North-East India. Biores Technol 98:1339–1345

Dean-Ross D, Moody J, Cerniglia CE (2002) Utilization of mixtures of polycyclic aromatic hydrocarbons by bacteria isolated from contaminated sediment. FEMS Microbiol Ecol 41:1–7

Djomo JE, Garrigues P, Narbonne JF (1996) Uptake and depuration of polycyclic aromatic hydrocarbons from sediment by the zebrafish (Brachydanio Rerio). Environ Toxicol Chem 15:1177–1181

Eggen T, Majcherczyk A (1998) Removal of polycyclic aromatic hydrocarbons (PAH) in contaminated soil by white rot fungus Pleurotus ostreatus. Int Biodeter Biodegrad 41:111–117

Fernandes MB, Sicre MA, Boireau A, Tronczynski J (1997) Polyaromatic hydrocarbon (PAH) distributions in the Seine river and its estuary. Marine Pollut Bull 34:857–867

Fuchedzhieva N, Karakashev D, Angelidaki I (2008) Anaerobic biodegradation of fluoranthene under methanogenic conditions in presence of surface-active compounds. J Hazard Mater 153:123–127

Haritash AK, Kaushik CP (2009) Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): A review. J Hazard Mater 169:1–15

Hinga KR (2003) Degradation rates of low molecular weight PAH correlate with sediment TOC in marine subtidal sediments. Marine Pollut Bull 46:466–474

Hong Y-W, Yuan D-X, Lin Q-M, Yang T-L (2008) Accumulation and biodegradation of phenanthrene and fluoranthene by the algae enriched from a mangrove aquatic ecosystem. Marine Pollut Bull 56:1400–1405

Huesemann MH, Hausmann TS, Fortman TJ (2004) Does bioavailability limit biodegradation? A comparison of hydrocarbon biodegradation and desorption rates in aged soils. Biodegradation 15:261–274

Hwang S, Cutright TJ (2004) Preliminary exploration of the relationships between soil characteristics and PAH desorption and biodegradation. Environ Int 29:887–894

IARC (2010) Some non-heterocyclic polycyclic aromatic hydrocarbons and some related exposures. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, vol 92. Ed. International Agency for Research on Cancer, Lyon, France

Jackson W, Pardue J (1999) Potential for intrinsic and enhanced crude oil biodegradation in Louisiana's freshwater marshes. Wetlands 19:28–34

Johnsen A, Karlson U (2005) PAH degradation capacity of soil microbial communities: Does it depend on PAH exposure? Microb Ecol 50:488–495

Johnsen AR, Wick LY, Harms H (2005) Principles of microbial PAH-degradation in soil. Environ Pollut 133:71–84

Juhasz AL, Naidu R (2000) Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: a review of the microbial degradation of benzo[a]pyrene. Int Biodeter Biodegrad 45:57–88

Ke L, Luo L, Wang P, Luan T, Tam NF-Y (2010) Effects of metals on biosorption and biodegradation of mixed polycyclic aromatic hydrocarbons by a freshwater green alga Selenastrum capricornutum. Biores Technol 101:6950–6961

Kim IS, Park J-S, Kim K-W (2001) Enhanced biodegradation of polycyclic aromatic hydrocarbons using nonionic surfactants in soil slurry. Appl Geochem 16:1419–1428

Kim M, Bae S, Seol M, Lee J-H, Oh Y-S (2008) Monitoring nutrient impact on bacterial community composition during bioremediation of anoxic PAH-contaminated sediment. J Microbiol 46:615–623

Kirk TK, Farrell RL (1987) Enzymatic "Combustion": The microbial degradation of Lignin. Ann Rev Microbiol 41:465–501

Kotterman MJJ, Heessels E, Jong E, Field JA (1994) The physiology of anthracene biodegradation by the white-rot fungus Bjerkandera sp. strain BOS55. Appl Microbiol Biotechnol 42:179–186

LaRocca C, Conti L, Crebelli R, Crochi B, Iacovella N, Rodriguez F, Turrio-Baldassarri L, DiDomenico A (1996) PAH content and mutagenicity of marine sediments from the Venice Lagoon. Ecotoxicol Environ Safety 33:236–245

Lei A-P, Hu Z-L, Wong Y-S, Tam NF-Y (2007) Removal of fluoranthene and pyrene by different microalgal species. Biores Technol 98:273–280

Li C-H, Zhou H-W, Wong Y-S, Tam NF-Y (2009) Vertical distribution and anaerobic biodegradation of polycyclic aromatic hydrocarbons in mangrove sediments in Hong Kong, South China. Sci Total Environ 407:5772–5779

Lin C, Gan L, Chen Z-L (2010) Biodegradation of naphthalene by strain Bacillus fusiformis (BFN). J Hazard Mater 182:771–777

Macías-Zamora JV, Mendoza-Vega E, Villaescusa-Celaya JA (2002) PAHs composition of surface marine sediments: a comparison to potential local sources in Todos Santos Bay, B.C., Mexico. Chemosphere 46:459–468

Mackay D, Shiu WY, Ma K-C, Lee SC (2006) Polynuclear aromatic hydrocarbons (PAHs) and related aromatic hydrocarbons. Handbook of physical-chemical properties and environmental fate for organic chemicals, 2nd Edition edn. CRC Press, Boca Raton

Mahanty B, Pakshirajan K, Dasu V (2010) Batch biodegradation of PAHs in mixture by *Mycobacterium frederiksbergense:* analysis of main and interaction effects. Clean Technol Environ Pol 12:441–447

Maliszewska-Kordybach B, Smreczak B, Klimkowicz-Pawlas A, Terelak H (2008) Monitoring of the total content of polycyclic aromatic hydrocarbons (PAHs) in arable soils in Poland. Chemosphere 73:1284–1291

Márquez-Rocha FJ, Hernández-Rodríguez VZ, Vázquez-Duhalt R (2000) Biodegradation of soil-adsorbed polycyclic aromatic hydrocarbons by the white rot fungus *Pleurotus ostreatus*. Biotechnol Lett 22:469–472

McElroy AE, Farrington JW, Teal JM (1989) Bioavailability of polycyclic aromatic hydrocarbons in the aquatic environment. In: Varanasi U (ed) Metabolism of Polycyclic Aromatic Hydrocarbons in the Aquatic Environment. CRC Press, Boca Raton, pp 1–40

Nadal M, Schuhmacher M, Domingo JL (2004) Levels of PAHs in soil and vegetation samples from Tarragona County, Spain. Environ Pollut 132:1–11

Nam K, Alexander M (1998) Role of nanoporosity and hydrophobicity in sequestration and bioavailability: Tests with model solids. Environ Sci Technol 32:71–74

Nam K, Kim JY (2002) Role of loosely bound humic substances and humin in the bioavailability of phenanthrene aged in soil. Environ Pollut 118:427–433

Nam JJ, Thomas GO, Jaward FM, Steinnes E, Gustafsson O, Jones KC (2008) PAHs in background soils from Western Europe: Influence of atmospheric deposition and soil organic matter. Chemosphere 70:1596–1602

Narro ML, Cerniglia CE, Van Baalen C, Gibson DT (1992a) Evidence for an NIH shift in oxidation of naphthalene by the marine cyanobacterium *Oscillatoria* sp. strain JCM. Appl Environ Microbiol 58:1360–1363

Narro M, Cerniglia CE, Van Baalen C, Gibson DT (1992b) Metabolism of phenanthrene by the marine cyanobacterium *Agmenellum quadruplicatum* PR-6. Appl Environ Microbiol 58: 1351–1359

Navarro RR, Iimura Y, Ichikawa H, Tatsumi K (2008) Treatment of PAHs in contaminated soil by extraction with aqueous DNA followed by biodegradation with a pure culture of *Sphingomonas* sp. Chemosphere 73:1414–1419

Neff JM (1979) Polycyclic aromatic hydrocarbons in the aquatic environment, sources, fates, and biological effects. Applied Science Publishers Ltd., London

Pagnout C, Frache G, Poupin P, Maunit B, Muller J-F, Férard J-F (2007) Isolation and characterization of a gene cluster involved in PAH degradation in Mycobacterium sp. strain SNP11: Expression in Mycobacterium smegmatis mc2155. Res Microbiol 158:175–186

Poeton TS, Stensel HD, Strand SE (1999) Biodegradation of polyaromatic hydrocarbons by marine bacteria: effect of solid phase on degradation kinetics. Water Res 33:868–880

Porte C, Albaigés J (1994) Bioaccumulation patterns of hydrocarbons and polychlorinated biphenyls in bivalves, crustaceans, and fishes. Arch Environ Contam Toxicol 26:273–281

Potin O, Veignie E, Rafin C (2004) Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by *Cladosporium sphaerospermum* isolated from an aged PAH contaminated soil. FEMS Microbiol Ecol 51:71–78

Pott P (1775) Chirurgical Observations Relative to the Cataract, the Polypus of the Nose, the Cancer of the Scrotum, the Different Kinds of Ruptures, and the Mortification of the Toes and Feet. In: T.J. Carnegy for Hawes L, Clarke W & Collins R (eds) London, pp 63–68

Pozdnyakova N, Nikiforova S, Makarov O, Chernyshova M, Pankin K, Turkovskaya O (2010) Influence of cultivation conditions on pyrene degradation by the fungus *Pleurotus ostreatus* D1. World J Microbiol Biotechnol 26:205–211

Quantin C, Joner EJ, Portal JM, Berthelin J (2005) PAH dissipation in a contaminated river sediment under oxic and anoxic conditions. Environ Pollut 134:315–322

Rehmann L, Prpich GP, Daugulis AJ (2008) Remediation of PAH contaminated soils: application of a solid-liquid two-phase partitioning bioreactor. Chemosphere 73:798–804

Reineke W (2001) Aerobic and anaerobic biodegradation potentials of microorganisms. In: Beek B (ed) Biodegradation and Persistance. Springer, Berlin

Reuter W, Müller C (1993) New trends in photobiology: Adaptation of the photosynthetic apparatus of cyanobacteria to light and CO_2. J Photochem Photobiol B: Biol 21:3–27

Rockne KJ, Chee-Sanford JC, Sanford RA, Hedlund BP, Staley JT, Strand SE (2000) Anaerobic naphthalene degradation by microbial pure cultures under nitrate-reducing conditions. Appl Environ Microbiol 66:1595–1601

Shao Z, Cui Z, Dong C, Lai Q, Chen L (2010) Analysis of a PAH-degrading bacterial population in subsurface sediments on the Mid-Atlantic Ridge. Deep Sea Res Part I: Oceanographic Res Papers 57:724–730

Smith JD, Bagg J, Wrigley I (1991) Extractable polycyclic hydrocarbons in waters from rivers in South-Eastern Australia. Water Res 25:1145–1150

Sobisch T, Heß H, Niebelschütz H, Schmidt U (2000) Effect of additives on biodegradation of PAH in soils. Colloids and Surfaces A: Physicochemical and Eng Aspects 162:1–14

Somtrakoon K, Suanjit S, Pokethitiyook P, Kruatrachue M, Lee H, Upatham S (2008) Enhanced biodegradation of anthracene in acidic soil by inoculated *Burkholderia* sp. VUN10013. Curr Microbiol 57:102–106

Tadros M, Hughes J (1997) Degradation of polycyclic aromatic hydrocarbons (PAHs) by indigenous mixed and pure cultures isolated from coastal sediments. Appl Biochem Biotechnol 63–65:865–870

Tang L, Tang X-Y, Zhu Y-G, Zheng M-H, Miao Q-L (2005) Contamination of polycyclic aromatic hydrocarbons (PAHs) in urban soils in Beijing, China. Environ Int 31:822–828

Tesai J-C, Kumar M, Lin J-G (2009) Anaerobic biotransformation of fluorene and phenanthrene by sulfate-reducing bacteria and identification of biotransformation pathway. J Hazard Mater 164:847–855

Valentín L, Feijoo G, Moreira MT, Lema JM (2006) Biodegradation of polycyclic aromatic hydrocarbons in forest and salt marsh soils by white-rot fungi. Int Biodeter Biodegrad 58: 15–21

Valentín L, Lu-Chau TA, López C, Feijoo G, Moreira MT, Lema JM (2007) Biodegradation of dibenzothiophene, fluoranthene, pyrene and chrysene in a soil slurry reactor by the white-rot fungus *Bjerkandera* sp. BOS55. Process Biochem 42:641–648

Walter U, Beyer M, Klein J, Rehm HJ (1991) Degradation of pyrene by Rhodococcus sp. UW1. Appl Microbiol Biotechnol 34:671–676

Wang J, Bi Y, Pfister G, Henkelmann B, Zhu K, Schramm K-W (2009) Determination of PAH, PCB, and OCP in water from the Three Gorges reservoir accumulated by semipermeable membrane devices (SPMD). Chemosphere 75:1119–1127

Warshawsky D, Radike M, Jayasimhulu K, Cody T (1988) Metabolism of benzo(A)pyrene by a dioxygenase enzyme system of the freshwater green alga *Selenastrum capricornutum*. Biochem Biophys Res Commun 152:540–544

Warshawsky D, Cody T, Radike M, Reilman R, Schumann B, Ladow K, Schneider J (1995) Biotransformation of benzo[a]pyrene and other polycyclic aromatic hydrocarbons and heterocyclic analogs by several green algae and other algal species under gold and white light. Chemico-Biol Interact 97:131–148

Wilson SC, Jones KC (1993) Bioremediation of soil contaminated with polynuclear aromatic hydrocarbons (PAHs): A review. Environ Pollut 81:229–249

Witt G (2002) Occurrence and transport of polycyclic aromatic hydrocarbons in the water bodies of the Baltic Sea. Marine Chem 79:49–66

Xia XH, Yu H, Yang ZF, Huang GH (2006) Biodegradation of polycyclic aromatic hydrocarbons in the natural waters of the Yellow River: Effects of high sediment content on biodegradation. Chemosphere 65:457–466

Xue W, Warshawsky D (2005) Metabolic activation of polycyclic and heterocyclic aromatic hydrocarbons and DNA damage: A review. Toxicol Appl Pharmacol 206:73–93

Yamagiwa K, Ichikawa K (1915) Experimentelle Studie über die Pathogenese der Epithelial-geschwülste. Mitteilungen der Medizinischen Fakultät, Universität Tokyo 15, pp 295–344

Yuan SY, Chang JS, Yen JH, Chang B-V (2001) Biodegradation of phenanthrene in river sediment. Chemosphere 43:273–278

Yuan SY, Shiung LC, Chang BB (2002) Biodegradation of polycyclic aromatic hydrocarbons by inoculated microorganisms in soil. Bull Environ Contam Toxicol 69:66–73

Yutthammo C, Thongthammachat N, Pinphanichakarn P, Luepromchai E (2010) Diversity and activity of PAH-degrading bacteria in the Phyllosphere of ornamental plants. Microb Ecol 59:357–368

Zhang C, Bennett GN (2005) Biodegradation of xenobiotics by anaerobic bacteria. Appl Microbiol Biotechnol 67:600–618

Chapter 11
Biodegradation of Aromatic Pollutants by Ligninolytic Fungal Strains

Tomáš Cajthaml and Kateřina Svobodová

11.1 Introduction

The environmental impact of organic aromatic pollutants mostly of anthropogenic origin is of increasing interest due to their persistency and often of serious effects on the environmental health. Many of these compounds represent industrial chemicals that were deliberately or inadvertently released into the environment. Others belong to undesired chemical by-products, personal care compounds or pharmaceuticals that resist against microbial or chemical processes, either during waste water treatment or in soil. Aromatic compounds are usually chemically resistant due to delocalization of energy and moreover environmental persistence of conjugated compounds has been suggested to be due to the dense clouds of PI-electrons on both sides of the ring structures which make them scarcely susceptible to nucleophilic attack (Johnsen et al. 2005). The resistance is even more pronounced when the structures are substituted with so-called xenophores e.g., halogens (Alexander 1994). Some of the aromatic pollutants, for example polycyclic aromatic hydrocarbons and polychlorinated biphenyls, are characterized by low water solubility and high lipophility, resulting in their accumulation in fatty tissues of living organisms, including humans, and they are found at alarming levels in the food chain. Other compounds with higher water solubility as pharmaceuticals are transported by the movement of fresh waters and those with lipophilic character are often found accumulated in waste water treatment sludge or sediments (Cajthaml et al. 2009a). Semi-volatile or volatile compounds are transported over long distances in the atmosphere. Thus, both humans and

T. Cajthaml (✉) · K. Svobodová
Laboratory of Environmental Biotechnology, Institute of Microbiology, v.v.i., Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague 4, Czech Republic
e-mail: cajthaml@biomed.cas.cz

S. N. Singh (ed.), *Microbial Degradation of Xenobiotics*, Environmental Science and Engineering, DOI: 10.1007/978-3-642-23789-8_11,

environmental organisms are exposed to persistent aromatic pollutants around the world, in many cases for extended periods of time spanning generations, resulting in both acute and chronic toxic effects to both humans and wildlife. Generally, aromatic pollutants are distributed widespread in the environment; however, in most cases, their introduction into the environment can be restricted or minimized. Either they could be removed from sources of actual pollution or decontaminated from aged polluted sites found in many industrial areas across the globe. Decontamination–remediation methods are generally classified as physical, chemical and biological. The first two are referred to as engineering strategies, the latter as bioremediation. Bioremediation is often characterized as cost-effective and environmental friendly approach for the removal of contaminants from the environment. As cleanup tools, various types of organisms are employed, mainly microorganisms or plants. The bioremediation method has been established to exploit mostly bacterial and its limits have been repeatedly reviewed (Alexander 1994). There are several limiting factors that can significantly restrict successful bacterial application for biodegradation of especially aromatic pollutants present in the environment. The bacterial biodegradation of organopollutants is mainly performed by intracellular enzymes with high substrate specificity that results in limited availability of compounds with low water solubility. In bacterial degradation, these compounds are used as sources of carbon and energy, however, the process is active only above a certain concentration threshold. Moreover, genes responsible for organopollutant transformations are very often localized on plasmids. If the bacteria are not exposed to presence of the compounds for a longer time, the activity of the genes easily disappears. However, these impediments can be overcome by the application of fungi for degradation.

11.2 Ligninolytic Basidiomycetes

Various groups of basidiomycetes were studied for the degradation of organopollutants. Among them the most promising group was wood saprophytes belonging to ligninolytic fungi, also known as white rot fungi. Therefore, this group of lignin degrading fungi has received considerable attention for their bioremediation potential. In nature, these fungi colonize lignocellulosic materials, playing an important role in lignin decomposition causing white rot of wood. The enzymes involved in lignin breakdown are extracellular and have low substrate specificity. Lignin is a complex three-dimensional polymer consisting of phenyl propanoid units. The stereo irregularity of lignin makes it very resistant to the attack by enzymes. Besides, lignin macromolecule cannot be absorbed by the microbial cell and hence degraded by intracellular enzymes. Thus, ligninolytic fungi possess very non-specific mechanism for degrading lignin. Initial attack of lignin macromolecule is caused by an extracellular degradative system. This system consists of several enzymes that play a role in the degradation: heme-peroxidases, lignin peroxidase (LiP, E.C. 1.11.1.14), manganese-dependent

peroxidase (MnP, E.C.1.11.1.13) and versatile peroxidases (VP, E.C. 1.11.1.16). Phenol oxidases, such as laccase (Lac, E.C. 1.10.3.2), which is present in many ligninolytic fungi, are also thought to participate in the oxidative degradation of lignin (Camarero et al. 1999; Ruiz-Duenas et al. 2001). In addition, the following enzymes are also associated with lignin breakdown, but are unable to degrade lignin alone: glyoxal oxidase (E.C. 1.2.3.5), superoxide dismutase (E.C. 1.15.1.1), glucose oxidase (E.C. 1.1.3.4), aryl alcohol oxidase (E.C. 1.1.3.7), and cellobiose dehydrogenase (E.C. 1.1.99.18). They produce H_2O_2 required by peroxidases (LiP and MnP) or serve to link lignocellulose degradation pathways (Leonowicz et al. 2001). Due to low specificity of the enzymes, the same mechanism that white rot fungi use to degrade lignin is also involved in degradation of a wide range of pollutants (Pointing 2001). In fact, ligninolytic fungi are the most efficient lignin degraders in nature and are also capable of degrading and, to some extent, mineralizing a wide range of xenobiotics by means of their extracellular enzyme system (Hammel et al. 1986; Bezalel et al. 1996a; Cajthaml et al. 2006). These abilities have been documented for degradation of petroleum hydrocarbons, chlorophenols, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, dioxins and furans, pesticides, herbicides and nitroaromatic explosives (Alexander 1994; Juhasz and Naidu 2000; Rabinovich et al. 2004).

Several papers have been published (Cajthaml et al. 2008; Novotny et al. 1997; Schutzendubel et al. 1999), but they do not establish a direct correlation between the activity of ligninolytic enzymes and degradation of xenobiotic aromatics. Therefore, some authors suggested (Field et al. 1993; Sasek et al. 1993) that other "unidentified" enzymes may be also involved in the oxidation of pollutants. In ligninolytic fungi, similar to other microorganisms (George and VanEtten 2001), P-450 was also found and its participation in the degradation of aromatics was proved (Bezalel et al. 1996c; Cajthaml et al. 2008). More probable explanation seems to be that fungi produce various isoforms of the ligninolytic enzymes that can be partially specific to pollutants (Pozdnyakova et al. 2010; Novotny et al. 2009) and their activity for the pollutant degradation could be influenced by the presence of suitable mediators (Sack et al. 1997; Cañas et al. 2007; Camarero et al. 2008). Despite the fact that the role of ligninolytic enzymes in destruction of aromatic pollutants has not been elucidated completely, the ligninolytic fungi undoubtedly represent powerful prospective tool for bioremediation strategies (Lang et al. 1996; Bhatt et al. 2002; Novotny et al. 2004; Baldrian 2008).

11.3 Biodegradation of Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous compounds, with two or more fused benzene rings arranged either in a linear or a cluster mode, derived from both natural and anthropogenic sources. Several members of this class of compounds have been included among priority pollutants owing to their toxic, mutagenic and carcinogenic properties (Haritash and Kaushik 2009).

The persistence of these compounds in the environment is mainly due to their low solubility in water and stable polycondensed aromatic structure. Hydrophobicity and recalcitrance of PAHs to microbial degradation generally increase as the molecular weight increases. Besides being toxic to animals, some PAHs with four or more benzene rings, such as benzo[a]anthracene, chrysene and benzo[a]pyrene, have been shown to be carcinogenic (Bezalel et al. 1996a). In the last few decades, different approaches to PAHs biodegradation have been investigated and white rot basidiomycetes have attracted significant interest since the pioneering studies with *Phanerochaete chrysosporium* (Hammel et al. 1986). Later on, several other fungal species have been demonstrated to metabolize PAHs significantly under model liquid culture conditions or in the soil and the most studied and efficient strains of ligninolytic fungi are *P. chrysosporium*, *Trametes versicolor*, *Pleurotus ostreatus*, *Bjerkandera adusta*, *Irpex lacteus* and *Panus tigrinus* (Pointing 2001; Novotny et al. 2009; Baldrian 2008; Covino et al. 2010). It was proved that these fungi can oxidize PAHs under in vitro conditions using Lac and ligninolytic peroxidases (Majcherczyk et al. 1998; Eibes et al. 2006; Baborova et al. 2006). In addition, the use of either natural or synthetic mediators was described (Sack et al. 1997; Johannes and Majcherczyk 2000; Cañas and Camarero 2010; Camarero et al. 2008). Besides, the extracellular degradation pathway, cytochrome P-450 monooxygenase and epoxide hydrolase in the initial degradation have been proved to be active in the degradation (Bezalel et al. 1997). Using ^{14}C-labeled compounds, it was proved that ligninolytic fungi are able to mineralize PAHs completely to carbon dioxide (Bezalel et al. 1996b; Wolter et al. 1997). However, intermediates arisen from ring-cleavage reactions that were triggered by fungi are hardly described. Previously, Hammel et al. (1991) showed that *P. chrysosporium* was able to decompose anthracene into phthalic acid that was identified as ring-fission product. Bezalel et al. (1996c) presented the mechanism of 2,2′-diphenic acid production from phenanthrene. These authors suggested that cytochrom P-450 of *P. ostreatus* was responsible for the attack on phenanthrene enabling further ring opening reactions. Moen and Hammel (1994) reported formation of 2,2′-diphenic acid from phenanthrene after lipid peroxidation by MnP. Majcherczyk and co-workers found several ring-cleavage products of acenaphthylene and acenaphthene after incubation with Lac as well as a laccase-mediator system of *T. versicolor* (Johannes et al. 1998; Majcherczyk et al. 1998). In a later study (Cajthaml et al. 2002), the degradation of phenanthrene, anthracene, fluoranthene and pyrene by liquid culture of *I. lacteus* (degradation extent after 50 days: 67–95%) provided several metabolites that pointed to ring-cleavage processes during degradation. Structures of some of the compounds suggested involvement of both enzymatic systems, P-450 and ligninolytic peroxidases (Fig. 11.1).

Later, in vitro experiments were carried out using MnP from the same fungus and proved that this enzyme is able to degrade the representatives of PAHs (Baborova et al. 2006). Major degradation products of anthracene were identified and the results showed a new role of MnP in PAH degradation by *I. lacteus* when a ring fission product was detected 2-(2′-Hydroxybenzoyl)-benzoic acid. A pathway similar to anthracene has been also published for benzo(a)anthracene resulting

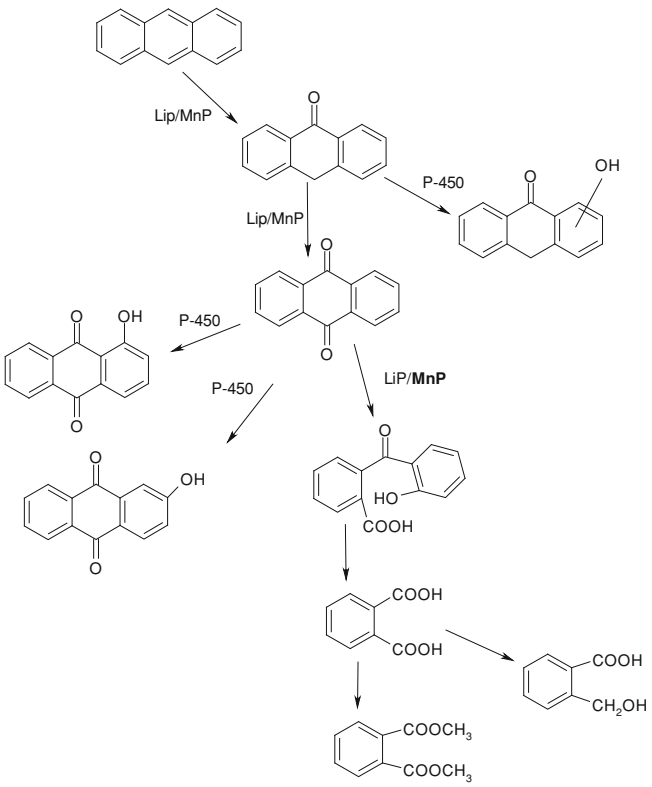


Fig. 11.1 Pathway proposed for anthracene degradation by *I. lacteus* with indication of possible enzyme involvement

(Table 11.1) in phthalic acid via several intermediates possessing two aromatic rings e.g., 1,4-naphthalenedione, 1,4-naphthalenediol and 1,2,3,4-tetrahydro-1-hydroxynaphthalene (Cajthaml et al. 2006).

Similar findings were documented also in case of *P. tigrinus* when the best results were obtained in shaking cultures where PAHs were degraded by 91 and 97% in complex maltextract-glucose media and nitrogen limited mineral media, respectively, within 4 weeks. In stationary cultures, on the contrary, the degradation was never higher than 50%. This fungus was also able to decompose aromatic structures and a set of various products after ring-cleavages were detected (Covino et al. 2010). The presence of some degradation products (e.g., hydroxylated derivatives of anthrone and phenanthrene 9,10-dihydrodiol) suggested the possible involvement of cytochrome P-450 monooxygenous and epoxide hydrolase system, the active form of which was found in 7-day-old cultures on the complex rich media. In vitro experiments showed that the MnP from *P. tigrinus* had a wider PAH substrate range and higher oxidation ability than the laccase produced by the same strain.

Table 11.1 Structural suggestion and mass spectral characteristics of BaA metabolites

Structural suggestion	MW (CI)	m/z of fragment ions (relative intensity)
Benz(a)anthracene-7,12-dione[a]	258	258 (100), 230 (41), 202 (47.2), 174 (4.5), 150 (4.6)
1-tetralone[a]	146	146 (75.9), 131 (13), 118 (100), 104 (3.4), 90 (26.9), 77 (8.7)
1,2,3,4-tetrahydro-1-hydroxynaphthalene[a]	148	148 (8.4), 147 (20.1), 130 (100), 119 (42.4), 105 (20.2), 91 (25), 77 (5.9)
4-hydroxy-1-tetralone	162	162 (18.6), 145 (17), 134 (68.2), 115 (15.8), 105 (100), 77 (24.1), 51 (15.8)
4-hydroxy-1-tetralone-TMS	234	219 (100), 189 (8.8), 115 (10.2)
Phthalic anhydride[ab]	148	148 (2.3), 104 (100), 76 (41.2), 50 (20.4)
Phthalic acid di-TMS[a]	310	310 (3.7), 295 (57.6), 265 (6.4), 221 (27.5), 193 (3.8), 147 (100), 73 (53.1)
Monomethyl phthalic acid[a]	163	163 (15.4), 149 (60.7),136 (14.2), 104 (100), 92 (19.5), 76 (96.7)
Monomethyl phthalic acid-TMS[a]	252	252 (2.2), 237 (100),163 (50),133 (7.5), 89 (77.7)
Phthalide[a]	134	134 (12.7), 105 (100), 77 (40.9), 51 (9.0)
Dimethyl phthalic acid[a]	194	194 (3.0), 163 (100), 133 (15.8), 77 (9.7)
Naphthalene-1,4-dione[a]	158	158 (100), 130 (38.9), 104 (62.9), 102 (60.5), 76 (45.1)
1,4-dihydroxynaphthalene[a]	160	160 (85.4), 131 (21.5), 104 (100), 76 (41.5)
1,2-naphthalic anhydride[b]	198	198 (80.1), 154 (87.9), 126 (100)

[a] Structures were later identified with authentic standard
[b] Dehydrated form of the metabolite

P. ostreatus, well known as oyster mushroom, has been many times described as efficient degrader of PAHs, as well (Bezalel et al. 1996b; Wolter et al. 1997). Grown in the presence of several PAHs and their analogues (benzo[a]pyrene, pyrene, fluorene, phenanthrene, anthracene), it was able to metabolize and in some cases to mineralize them. Among the metabolites identified were: phenanthrene trans-9,10-dihydrodiol and 2,2′-diphenic acid from phenanthrene, pyrene trans-4,5-dihydrodiol from pyrene and anthracene trans-1,2-dihydrodiol and 9,10-anthraquinone from anthracene. For instance, the fungus was shown to be able to decompose phenanthrene, anthracene and pyrene by 50, 92 and 35%, respectively, in bran flakes media in 5 days (Pickard et al. 1999). Schutzendubel et al. (1999) found that during 3 days of incubation, *B. adusta* removed 56 and 38% of fluorene and anthracene, while *P. ostreatus* degraded 43 and 60% of these compounds; other PAHs were degraded to a lower extent. Except for anthracene in cultures of *P. ostreatus*, all PAHs were removed uniformly during the cultivation time but fluorene and anthracene were degraded faster than other PAHs in basidiomycete's rich media. The detected intermediates were mostly keto compounds.

11.4 Biodegradation of Polychlorinated Biphenyls

Polychlorinated biphenyls belong to persistent organic pollutants. Although their production was stopped long time ago, they still persist in the environment and represent a serious environmental problem. The aromatic "double-ring" structure may be substituted with one to ten chlorine atoms. Although there are 209 individual compounds designated as congeners, but only 130 could be found in commercial PCB mixtures. The toxicity of such molecules is directly correlated to both number and position of the chlorine substituents. The coplanar PCBs (not substituted at the ring positions ortho-) tend to have dioxin-like properties, and generally are among the most toxic congeners. It has been demonstrated that PCBs are highly toxic to humans and animals, although their effects become apparent only after long-term exposure. The high resistance of these toxic compounds requires drastic conditions for decomposition, either high temperatures or chemical reagents, both very expensive processes. Hence, PCB contaminated material is disposed off at special sites to avoid exposure to human population. Alternatively to thermal and physico-chemical methods, bioremediation gains more public interest and acceptance as an effective and economic strategy for the removal of persistent toxic pollutants. PCBs can be degraded by aerobic bacteria via several pathways, however, the most important is co-metabolic process operated by biphenyl catabolic enzymes (Furukawa and Fujihara 2008). Unfortunately, this pathway leads to the formation of chlorobenzoic acids that tend to accumulate as dead-end products, since PCB degrading bacteria are not able to grow on these substrates (Kobayashi et al. 1996). The build up of these metabolites in the growth medium may result in a feed-back inhibition and impede or slow down PCB biotransformation (Adebusoye et al. 2008). Therefore, it is worthwhile to look for other microorganisms that are able to degrade PCBs.

Ligninolytic basidiomycetes have been also documented to be able to degrade PCBs. Besides *P. chrysosporium*, several other white-rot fungi: e.g., *P. ostreatus* (Beaudette et al. 1998; Kubatova et al. 2001), *Coriolopsis polyzona* (Vyas et al. 1994; Novotny et al. 1997), *T. versicolor* (Beaudette et al. 1998; Cloete and Celliers 1999). *B. adusta* (Beaudette et al. 1998), *Lentinus* (*Lentinula*) *edodes* (Sasek et al. 1993; Ruiz-Aguilar et al. 2002) are also known to metabolize PCBs. Mineralization of PCBs by *P. chrysosporium* was first reported by Eaton (1985) who found 8% mineralization of 0.3 ppm ^{14}C-Aroclor 1254 to $^{14}CO_2$ in a nitrogen-limited culture after 5 week incubation. Thomas et al. (1992) demonstrated mineralization of 2-chlorobiphenyl and 2,2′,4,4′-tetrachlorobiphenyl to $^{14}CO_2$. After 32-day incubation, only 1% of tetrachlorobiphenyl was mineralized, but 40% was found incorporated into fungal biomass. Dietrich et al. (1995) found that low chlorinated PCB congeners are degraded significantly, whilst higher chlorination becomes a limiting factor. After 28 days of incubation in liquid

media, the degradation of 4,4′-dichlorobiphenyl was recorded 11% mineralization, in contrast, there was negligible mineralization of the 3,3′,4,4′–tetrachorobiphenyl and 2,2′,4,4′,5,5′-hexachlorobiphenyl. Evidence for substantial degradation of Arochlor 1242 (60.9%), 1254 (30.5%), and 1260 (17.6%) after 30-day incubation was presented by Yadav et al. (1995). Contrary to an earlier observation in N-limited media, these authors found degradation also in high N-medium, and the most extensive in malt extract medium. Using congener-specific analysis, they documented that the degree of degradation is affected by chlorine number, but not by their position on the biphenyl ring. Based on the congener non-specificity and the fact that the degradation takes place also under non-ligninolytic conditions, these workers had a doubt in the role of ligninolytic enzymes, and supposed that the degradation might be due to free radical attack. These results were confirmed by Krcmar and Ulrich (1998) who described degradation of PCB mixture under non-ligninolytic conditions, but not under ligninolytic ones. Krcmar et al. (1999) performed an experiment where they observed in vitro degradation of PCB mixtures containing low and highly chlorinated biphenyls using intact mycelium, crude extracellular liquid and the enriched MnP and LiP. A decrease in PCB concentration during a 44 h treatment with mycelium (74%) or crude extracellular liquid (60%) was observed. In contrast, MnP and LiP isolated from the extracellular liquid did not catalyze any degradation. There are only a few publications describing possible pathways of PCB degradation and clarifying an involvement of fungal enzymes in the degradation. Kamei et al. (2006a) published a work about degradation of PCBs by the white-rot fungus *Phlebia brevispora* that was shown to degrade PCBs with the formation of methoxylated, p-dechlorinated and p-methoxylated metabolites. They performed another experiment where cultures of *Phanerochaete* sp. were contaminated with 4,4′-Dichlorobiphenyl (4,4′-DCB) and its metabolites and the metabolic pathway was partially elucidated by the identification of metabolites, namely 2-hydroxy-4,4′-DCB and 3-methoxy-4, 4′-DCB, 4-chlorobenzoic acid, 4-chlorobenzaldehyde, 4-chlorobenzyl alcohol, and 4-hydroxy-3,4′-DCB (Kamei et al. 2006b). Biodegradation of PCB 9 using the white rot fungus *T. versicolor* was studied by Koller et al. (2000). The cultivation took 4 weeks and reduction was recorded about 80% of the initial concentration and the formed metabolites were dichlorobenzenes, chlorophenols and alkylated benzenes. De et al. (2006) indicated that nitrate reductase gene of *P. chrysosporium* could be involved in dechlorination of hexachlorobiphenyl (PCB-153) under non-ligninolytic condition, when the nitrate reductase enzyme and its cofactor, molybdenum, were found to mediate reductive dechlorination of PCBs even in aerobic condition. Tungsten, a competitive inhibitor of this enzyme, was found to suppress this dechlorination and chlorine release assay provided further evidence for nitrate reductase mediated dechlorination of PCB. In conclusion, the ability of white rot fungi to degrade PCBs was proven, however, the enzymatic background and mechanism of the attack is still to be explored.

11.5 Fungal Biodegradation of Endocrine Disrupting Compounds

Endocrine-disrupting compounds (EDCs) are a group of environmental pollutants known for their negative influence, particularly on aquatic organisms. EDCs tend to accumulate in aquatic organisms and also get adsorbed by sediments and on particles in the aquatic environment. These compounds mimic or antagonize the effects of endogenous hormones and hence, alter the synthesis and metabolism of natural hormones, or modify hormone receptor levels, disrupting endocrine and reproductive functions which ultimately affect the health of humans and wildlife.

Many of these chemicals have been released into the environment during the past a few decades. For example, endocrine disruption by pesticides caused an increase in salmon smolt mortality, gonad malformations in American alligators, and a decline in the seal population in the Baltic Sea (McKinlay et al. 2008). Typical EDCs of anthropogenic origin with estrogen-like action include 4-nonylphenols (NPs), bisphenol A (BPA), and 17a-ethinylestradiol (EE2) (Fig. 11.2). NPs mainly occur in the environment as degradation products of nonylphenol-polyethoxylates, which are used widely as non-ionic surfactants in many industrial applications. BPA is a raw material of some polycarbonates and epoxy resins and it is widely used as food packaging material. The compound leaches also from dental materials and it was also found in waste landfill leachates. Synthetic estrogens, such as EE2, are used as oral contraceptives. Non-metabolized EE2 and its conjugates are excreted into wastewater. During sewage treatment, EE2 is released from the corresponding conjugates by hydrolysis and reaches the environment.

Some of the latest works have focused on EDCs degradation by ligninolytic fungi as reviewed by Asgher et al. (2008). An association of compound degradation with fungal ligninolytic enzyme system was suggested in these works. However, ligninolytic fungi were found a highly efficient in removal of EDCs in aqueous media and soils using both lignin-modifying enzymes and non lignin-modifying-enzyme systems (Cabana et al. 2007b). In addition to degradation, most of the works also emphasized on changes in endocrine-disrupting activity of chemicals during the degradation. For that, sensitive and easy-to-perform in vitro bioassays were developed during the last one decade (Svobodova and Cajthaml 2010).

Purified laccase from *Trametes villosa* efficiently degraded BPA under in vitro conditions without requirement of mediators for the electron transfer (Fukuda et al. 2001). Structural analysis of the BPA reaction products indicated that the oligomers of BPA were formed as the result of successive oxidation-condensation (Fukuda et al. 2004). The presence of oligomers fragments, each with phenol molecules, suggested the occurrence of cleavage of the formed oligomers to release 4-isopropenylphenol. Both the soluble and insoluble fractions of the BPA reaction products had than no estrogenic activity even at high concentrations (Fukuda et al. 2004). MnP, laccase, and the laccase-mediator systems of other

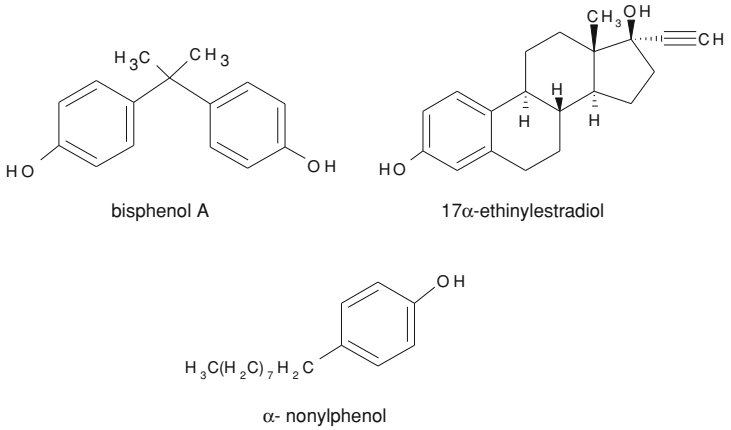


Fig. 11.2 Structures of anthropogenic EDCs

ligninolytic fungi are also effective in removing the estrogenic activities of BPA, NP, 17 β-estradiol (E2), and EE2 with production of high molecular weight oligomeric metabolites through radical polymerization mechanism and formation of C–C and C–O bonds (Tsutsumi et al. 2001; Suzuki et al. 2003; Lee et al. 2005). Removal of NP and BPA is associated with the production of laccase by *T. versicolor* and *Bjerkandera* sp. BOL13 (Soares et al. 2005, 2006). The enhanced biocatalytic elimination of NP, BPA and triclosan by *C. polyzona* by the addition of ABTS (Cabana et al. 2007a) also suggested the involvement of laccase/mediator system.

The biodegradation efficiency and estrogenic activity reduction of dibuthylphthalate were characterized in the white rot fungi *P. chrysosporium*, *T. versicolor* and *Daldinia concentrica* (Lee et al. 2004). Fungi showed resistance towards the chemical and an induction of laccase in *T. versicolor* was observed during the dibuthylphthalate degradation. In the study of Cajthaml et al. (2009b), 4-n-nonylphenol, technical 4-nonylphenol, BPA, EE2, and triclosan were biodegraded by liquid cultures of eight ligninolytic fungal strains (*I. lacteus* 617/93, *B. adusta* 606/93, *P. chrysosporium* ME 446, *Phanerochaete magnoliae* CCBAS 134/I, *P. ostreatus* 3004 CCBAS 278, *T. versicolor* 167/93, *Pycnoporus cinnabarinus* CCBAS 595, and *Dichomitus squalens* CCBAS 750). The results enabled comparison of EDCs degradation by various fungal strains and also showed that under the used conditions, the fungi were able to degrade the EDCs within 14 days of cultivation with exception of *B. adusta* and *P. chrysosporium* in the case of triclosane and BPA, respectively. *I. lacteus* and *P. ostreatus* were found to be most efficient EDC degraders with their degradation efficiency exceeding 90 or 80%, respectively, in 7 days. Both fungi degraded technical 4-nonylphenol, BPA, and EE2 below the detection limit within first 3 days of incubation. In general, estrogenic activities assayed with a recombinant yeast test decreased

with advanced degradation (Cajthaml et al. 2009b). However, in case of *I. lacteus*, *P. ostreatus*, and *P. chrysosporium*, the yeast assay showed a residual estrogenic activity (28–85% of initial) in EE2 cultures. Estrogenic activity in *B. adusta* cultures temporally increased during degradation of technical 4-nonylphenol, suggesting a production of endocrine-active intermediates. Attention was paid also to the effects of EDCs on the ligninolytic enzyme activities of the different fungal strains to evaluate their possible stimulation or suppression of activities during the biodegradation processes (Cajthaml et al. 2009b).

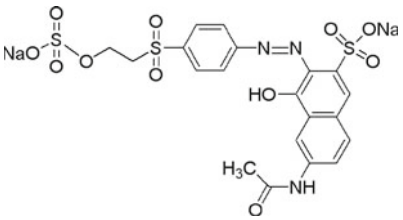
Liquid medium experiments with *Cephalosporium aphidicola* and *Cunninghamella elegans* were focused on identifying microbial transformation metabolites of oral contraceptives, EE2 and norethisterone (Choudhary et al. 2004). Transformation of norethisterone by *C. aphidicola* led to an oxidized metabolite, EE2, in this work, while the transformation of EE2 by *C. elegans* yielded several metabolites, 19-nor-17α-pregna-1,3,5 (10)-trien-20-yne-3,4,17*β*-triol, 19-nor-17α-pregna-1,3,5 (10)-trien-20-yne-3,7α,17*β*-triol, 19-nor-17α-pregna-1,3,5 (10)-trien-20-yne-3,11α,17*β*-triol, 19-nor-17α-pregna-1,3,5 (10)-trien-20-yne-3,6*β*,17*β*-triol and 19-nor-17α-pregna-1,3,5 (10)-trien-20-yne-3,17*β*-diol-6*β*-methoxy. These metabolites were structurally characterized on the basis of spectroscopic techniques.

To scale up the degradation process, the laccase from the fungus *C. polyzona* was immobilized through the formation of cross-linked enzyme aggregates using polyethylene glycol and glutaraldehyde as a cross-linking agent (Cabana et al. 2007c). Enzyme preparations were then tested for their capacity to eliminate NP, BPA, and triclosan in a fluidized bed reactor which could remove all three EDCs from 5 mg/l solution. Another type of bioreactor operating with *T. versicolor* pellets was then used for E2 and EE2 continuous degradation (Blanquez and Guieysse 2008). In this study, E2 and EE2 were completely removed from solutions at volumetric rates of 0.16 and 0.09 mg l^{-1} h^{-1}.

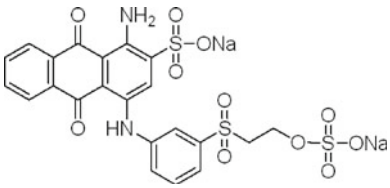
11.6 Fungal Decolorization and Degradation of Synthetic Dyes

Inefficiencies in industrial dyeing processes result in large amounts of synthetic dyes being directly lost to the wastewaters and consequently to the water environment. The amount of dye lost is dependent on the chemical structure of the dye and varies from 2% loss (basic dyes) to 50% loss (reactive dyes). Synthetic dyes represent a group of structurally different chemicals and are normally divided into azo, nitro, nitroso, diarylmethane, triarylsmethane, xanthene, anthraquinoid, acridine, cyanine, quinone-imine, pthalocyanine, and thiazole dyes. Figure 11.3 describes structures of selected group representatives.

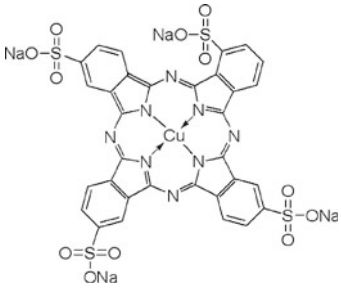
Since the first study on the fungus *P. chrysosporium* (Paszczynski et al. 1991; Pastigrigsby et al. 1992), ligninolytic fungi have been shown to degrade various synthetic dyes. Fungal decolorization and degradation of textile dyes has been



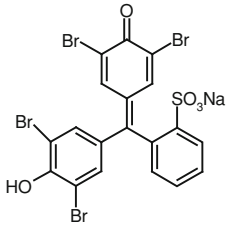
Reactive Orange 16 (RO16), azo dye



Remazol Brilliant Blue R (RBBR), anthraquinoid dye



Copper II phthalocyanine



Bromophenol Blue sodium salt, triarylmethane dye

Fig. 11.3 Structures of representatives of synthetic dyes

recently reviewed in several papers (Pointing 2001; McMullan et al. 2001; Kaushik and Malik 2009). The involvement of lignin-degrading enzymes in dye degradation process has been confirmed using purified enzyme activities (Pointing 2001; Wesenberg et al. 2003; Husain 2010). An enzymatic degradation has attracted much interest and approaches like using various redox mediators were undertaken to enhance the range of substrates and efficiency of degradation (Husain and Husain 2008).

Despite all efforts, the mechanism of fungal-mediated dye decolorization and the role of ligninolytic enzymes in the process under in vivo conditions are still disputed. Mechanisms, such as sorption to the fungal biomass, the participation of free radicals, and lipid peroxides can also be involved in dye degradation by ligninolytic fungi. This chapter describes the key achievements in the study of synthetic dye degradation by white rot fungi and mechanisms involved.

Several mechanisms can be responsible for dye decolorization capacity of white rot fungi. In a study with *T. versicolor*, the initial adsorption of synthetic dyes on fungal mycelium happened in the first hour of contact of the dye with the mycelium (Wang and Yu 1998). The affinity of fungal hyphae to the dyes was shown to depend on the structure of individual dyes. An efficient decolorization of 17 diverse dyes due to a sorption of dyes to fungal cells was also observed in *Cunninghamella polymorfa* cultures (Sugimori et al. 1999). Dye molecule biosorption onto cell surface appears to be quick and often completes in a few hours.

Amino, carboxyl, thiol and phosphate groups present in the fungal cell wall are responsible for binding dye molecules (Kaushik and Malik 2009). Fu and Viraraghavan (2002) studied biosorption of four dyes using *Aspergilus niger* biomass. In case of Basic Blue 9, carboxyl and amino groups were found to be the main binding sites while in biosorption of Acid Blue 29, only amino group was a major site and electrostatic attraction was believed to be the primary mechanism.

There are also several reports on dye adsorption performance of raw chitosan (Srinivasan and Viraraghavan 2010). In particular, it has been found to posses high removal capacity for anionic dyes. The chemistry of dye molecules and fungal biomass/its components in an aqueous solution is further influenced by initial pH of dye solution, temperature, ionic strength, and initial dye concentration.

Various biogenic radicals produced by fungi may also participate in the xenobiotic degradation, like reactive oxygen species produced by fungi when they grow on wood (Hammel et al. 2002). In addition, MnP of ligninolytic fungi was demonstrated to peroxidize unsaturated fatty acids resulting in the formation of peroxyl lipid radicals (Kapich et al. 1999; Watanabe et al. 2001). The involvement of lipid peroxidation was considered to play an important role in decolorization of the azo dye Reactive Red 120, using purified MnP from *P. chrysosporium* (Harazono et al. 2003).

Most studies are focused on the evidence of involvement of ligninolytic enzyme activities in the dye decolorization process, namely laccases, MnPs, versatile and lignin peroxidases (Wesenberg et al. 2003; Husain 2010). A new and unique dye-decolorizing peroxidase DyP has been recently identified from *P. ostreatus* (Faraco et al. 2007) and other fungi (Shimokawa et al. 2009). They oxidize non-phenolic lignin compounds and high-redox potential dyes (Liers et al. 2010).

White rot fungi capable of dye degradation often differ in their ligninolytic enzyme pattern and enzyme regulation (Hatakka 1994; Palma et al. 2000). The difference between the dye decolorization ability of *P. chrysosporium* and *Pleurotus sajor-caju* was thus correlated to the ligninolytic enzyme production by the two fungi (Chagas and Durrant 2001). Similarly, Kapdan et al. (2000) compared the dye decolorization capacities of *P. chrysosporium* and *T. versicolor.* 19 fungal isolates were then screened to decolorize the reactive dye blue-BF-R (Dos Santos et al. 2004). In these cases, the different patterns of dye decolorization could be explained by differences in the enzymatic system of the fungi tested.

Since the first study, it is known that dye decolorization capacity of white rot fungi depends on the culture conditions because they affect fungal physiology and the expression and the activity of the ligninolytic enzymes (Dosoretz and Grethlein 1991). Agitation of fungal cultures suppresses ligninolytic activity of *P. chrysosporium* (Faison and Kirk 1985). On the other hand, static liquid cultures of *P. chrysosporium*, *Bjerkandera* sp. BOSS55, and *T. versicolor* did not decolorize tested dyes beyond some mycelial sorption, whereas agitated cultures of *P. chrysosporium* and *T. versicolor* were able to decolorize most of these dyes (Swamy and Ramsay 1999b). The superior performance of the agitated cultures was ascribed to an increased oxygen transfer between the cells and the culture

medium due to agitation. This difference between the two culture types, however, can not be generalized for all fungi and dyes.

Next, the effect of nutrient supplementation on dye decolorization by white rot fungi *P. chrysosporium* and *Coriolopsis gallica* was also studied by comparing decolorization of an artificial textile-effluent in N-rich and N-limited conditions (Robinson et al. 2001). In *T. versicolor* cultures, a low nitrogen concentration in the culture medium was essential for effective decolorization of the phthalocyanine dye Everzol Turquoise Blue G (Kapdan et al. 2000). The requirement for nitrogen supplementation for *T. versicolor* was also demonstrated during Amaranth decolorization (Swamy and Ramsay 1999a). However, the regulation processes of dye decolorization may also vary among fungi. Like nutrient supplementation, other stresses may also affect enzyme production by white rots and consequently their dye decolorization ability. For example, the addition of Cu^{2+} ions increased laccase activity in *T. versicolor* (Lorenzo et al. 2006). Similarly, the addition of the dye Bromophenol Blue altered the profile of *I. lacteus* MnP forms, which led consequently to the changes in dye decolorization capability of enzyme preparations (Susla and Svobodova 2008).

In the study of degradation of structurally diverse dyes by *P. chrysosporium, Phanerochaete sordida,* and *Bjerkandera* sp. BOS55, the decolorization was correlated to MnP activity produced by these fungi (Moreira et al. 2000). Also phthalocyanine dyes, Remazol Turquoise Blue G133, Everzol Turquoise Blue, and Heligon Blue S4, were found to be metabolized by ligninolytic enzymes, which resulted in dye decolorization, formation of free copper ions and phthalocyanine ring breakdown (Conneely et al. 2002). In *P. chrysosporium* cultures, laccase activity could be responsible for the production of an early metabolite to cause break-up of phthalocyanine ring structure. MnP is also believed to be involved in the release of Cu^{2+} from the phthalocyanine. The phthalocyanine ring is then further metabolized to give phthalimides (Fig. 11.4) (Conneely et al. 2002). Similarly, *B. adusta* was shown to degrade sulfonated phthalocyanine dyes, giving sulfophthalimide as a major metabolite (Heinfling-Weidtmann et al. 2001). Sulfophthalimides were shown to be further degraded or even mineralized by aerobic bacteria from activated sludge (Reemtsma and Jakobs 2001). These results illustrate on the molecular level a cooperation of white rot fungi and bacteria in mineralizing structurally complex phthalocyanine dyes.

I. lacteus, immobilized on polyurethane foam and pine wood, has been shown to efficiently degrade various types of synthetic dyes (Kasinath et al. 2003; Tavčar et al. 2006). It was used for degradation of RO16 and identification of dye degradation products by LC–MS (Svobodova et al. 2007). A pathway for the dye degradation was suggested which was in accordance with the mechanism of azo dye oxidation by fungal laccases (Chivukula and Renganathan 1995) and similar to Acid Orange 7 degradation by *P. ostreatus* (Lu and Hardin 2006).

Disperse Orange 3, 4-(4-nitrophenylazo)aniline degradation by *P. ostreatus* was also investigated and the generated metabolites were identified as 4-nitroaniline, 4-nitrobenzene, 4-nitrophenol, and 4-nitroanisole with the last one being the major metabolite, when 4-nitrophenol was incubated with *P. ostreatus* (Zhao et al. 2006).

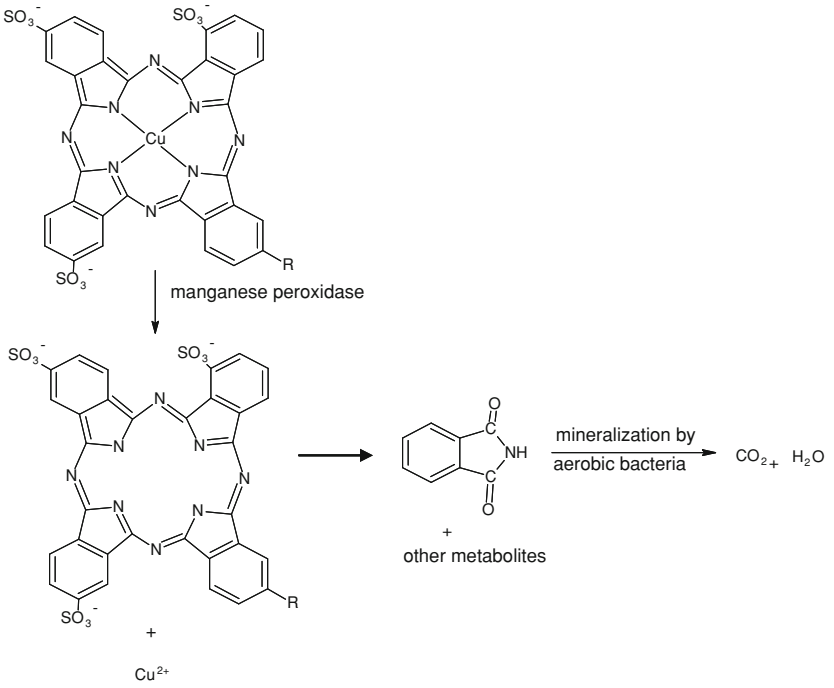


Fig. 11.4 Proposed degradation pathway for copper phthalocyanine dyes using *P. chrysosporium* and aerobic bacteria

Biodegradation products from model azo dyes Orange II, Acid Orange 8, Food Yellow 3, and 4-[(4-hydroxyphenyl)azo]-benzenesulfonic acid during *P. ostreatus* degradation were also determined (Zhao et al. 2007). Results showed that the tested dyes were degraded quickly in the fungal cultures with major products being 4-hydroxy-benzenesulfonic acid, 3-methyl-4-hydroxy-benzenesulfonic acid, benzenesulfonic acid, 1,2-naphthoquinone-6-sulfonic acid and 3-methyl-benzenesulfonic acid.

P. chrysosporium LiP is known to oxidize various sulfonated azo dyes resulting in the formation of benzoquinones and sulfophenyl hydroperoxides. The degradation mechanism involves two enzyme-catalyzed one-electron oxidations of the phenolic ring of an azo dye producing a carbonium ion and followed by several non-enzymatic reactions (Chivukula et al. 1995). Moreover, according to the kinetic data obtained from Crocein Orange G decolorization, the oxidation of azo dyes by *P. chrysosporium* LiP appeared to follow Michaelis–Menten kinetics (Ollikka et al. 1998). MnP activity of this fungus also decolorized synthetic dyes in vitro (Moldes et al. 2003). Poly R-478 and Crystal Violet decolorization data also indicated a typical Michaelis–Menten kinetic model for the reactions. Podgornik et al. (2001) demonstrated that the two *P. chrysosporium* peroxidases, LiP and MnP, were capable of Indigo Carmine decolorization. MnP transformed Indigo

Carmine into a yellow final product through formation and degradation of a red intermediate. Shin (2004) correlated decolorization of a dyeing industry wastewater to MnP and non-specific peroxidases detected in static *I. lacteus* cultures. Purified MnPs of *I. lacteus* were then shown to decolorize several synthetic dyes under in vitro conditions (Shin et al. 2005; Svobodova et al. 2006).

B. adusta and *P. eryngii* MnP isoenzymes are known to be different from those of *P. chrysosporium*. These enzymes were also shown to decolorize azo and phthalocyanine dyes in Mn^{2+}-independent reactions because Mn^{2+} present in the reaction mixture acted as a non-competive inhibitor of the dye oxidation (Heinfling et al. 1998). Relatively high loads of azo dyes (1500 mg l^{-1}) were also effectively decolorized using MnP isolated from *Bjerkandera* sp. BOS55 (Mielgo et al. 2003).

There are also many reports on the dye decolorization potential of laccases isolated from different white rot fungi (Wong and Yu 1999; Husain 2006). Six products were formed in the oxidation of 2,6-dimethoxy derivative of 4-(4′-sulfophenylazo)-2,6-dimethylphenol by laccase from *Pyricularia oryzae* and three of them were identified as sulfophenylhydroperoxide, 4-hydroxybenzenesulfonic acid, and 2,6-dimethoxybenzoquinone (Chivukula and Renganathan 1995). Since similar products were also identified during LiP-catalyzed azo dye oxidation, a mechanism similar to that of LiP-catalyzed oxidation was suggested to be involved in the laccase oxidation of azo dyes.

In *T. versicolor* cultures, laccase activity was shown to be responsible for decolorization of azo, anthraquinone, and indigo dyes (Wong and Yu 1999). The addition of various redox mediators can further extend the already broad dye decolorization capacity of fungal laccases as demonstrated in the studies on decolorization of the recalcitrant anthraquinone dye Remazol Brilliant Blue R with a commercial laccase (Soares et al. 2001a, b). Since then several redox mediators have been reported in the literature in connection with laccases, manganese peroxidases, and lignin peroxidases (Husain and Husain 2008).

While studying various dye classes, disazo dyes were shown to be decolorized by fungal laccases (Schliephake et al. 2000; Soares et al. 2002). Their decolorization was reported to be improved by the addition of violuric acid and N-hydroxybenzotriazole (Soares et al. 2001b). The decolorization of an anthraquinone dye, SN4R, by *P. ostreatus* laccase was improved by 90% with 2,2-azino-di-(3-ethylbenzothiazolinone-6-sulfonic acid) addition as the mediator (Hou et al. 2004). Decolorization of RO16 and Remazol Brilliant Blue R dyes by *I. lacteus* cultures was also shown to be connected with the action of a mycelium-bound, laccase-like enzyme activity. Despite of low stability the enzyme isolated from fungal mycelium, decolorized chemically different synthetic dyes in vitro (Svobodova et al. 2008).

T. villosa laccase was used for direct azo dye degradation (Zille et al. 2005a, b). Reaction product analysis showed the formation of phenolic compounds during the oxidation process as well as laccase catalysed polymerization of reaction products during long-term batch decolorization processes. Demethylation of Acid Orange 52 by *T. modesta* laccase and ultrasound treatment was also reported (Tauber et al. 2005).

11.7 Biodegradation of Other Relevant Aromatic Pollutants

Polychlorinated dibenzo-p-dioxins (PCDDs) are recognized as environmental hazards due to their acute toxicity to animals and humans. PCCDs have been identified in paper pulp mill effluents and in ash generated by a variety of combustion processes. Since these compounds are degraded very slowly by soil microflora, they are of major concern for environmental impact. The capability of *P. chysosporium* to degrade and also mineralize this type of compounds was demonstrated in 1985 (Bumpus et al. 1985), but the metabolism was not elucidated. Later, the metabolism of a model compound, 2,7-dichlorodibenzo-p-dioxin, by *P. chrysosporium* was elucidated, the oxidation products were characterized, and the involvement of fungal ligninolytic system was documented (Valli et al. 1992). Several other white rot fungi were studied for degradation of dibenzo-p-dioxins and dibenzofurans and several strains of *Plebia* spp., *Trametes* sp., *Irpex* sp., *Pleurotus pulmonarius* for hydroxylate and methoxylate PCDDs bio degradation (Pinedo-Rivilla et al. 2009).

White-rot fungi were also shown that these organisms are able to degrade explosives that are found as recalcitrant pollutants at abandoned military areas. *P. sordida* was demonstrated as efficient TNT degrader (Donnelly et al. 1997). Another white rot basidiomycete *Phlebia radiata* was shown to transform and mineralize the first identified reduction product of TNT–4-hydroxylamino-2,6,-dinitrotoluene (Van Aken et al. 1999). While screening for TNT mineralizing fungi, *Nematoloma frowardii* was selected as one of the best degraders. Involvement of MnP in the TNT conversion and mineralization was documented as well as mineralization of amino-dinitrotoluenes (Scheibner et al. 1997; Scheibner and Hofrichter 1998) Royal Demolition Explosive (RDX) was also degraded by white-rot fungi, showing that the removal efficiency in wastewater could reach 87% under optimum conditions (Lin et al. 2006; Gao et al. 2005).

P. chrysosporium was shown to perform complete degradation of meta- and para-chlorophenol in 15 days (Perez et al. 1997). The degradation ability of *P. chrysosporium* depends on the position of substituent chlorine, and the ortho-position is the preferred attack position. 2,6-dichlorophenol (2,6-DCP) was degraded in 3 days by *P. chrysosporium* at the same rate in nitrogen limited and unlimited media (Wu et al. 1996). Of four species, *P. sajor-caju* showed the highest 2,4-DCP mineralization rate of 2% after 15 days (Rodriguez et al. 2004). Several *Phanerochaete* species (*P. chrysorhiza*, *P. laevis*, *P. sanguinea*, *P. filamentosa*, *P. sordida*) were included in a screening for sensitivity to pentachlorophenol (PCP) and ability to degrade this pollutant in soil and *P. sordida* due to its resistance to PCP, was successfully used in soil remediation trials (Lamar and Dietrich 1990). *T. versicolor* has been used in bioremediation research because of its strong extracellular laccase production, high resistance to PCP and also due to fact that contrary to *P. chrysosporium* that metabolises PCP to more toxic chloroanisoles, *T. versicolor* produces only insignificant amounts of this intermetabolite (Lamar et al. 1993). Besides, it was shown that organochlorine

fraction of paper bleaching process was selectively attacked by *T. versicolor*, its laccase rapidly dechlorinated a large number of polychlorinated phenols and guaiacols (Roy-Arcand and Archbald 1991; Iimura et al. 1996). The degradation capacity of the fungus and its laccase was extensively studied with chlorophenols (Alleman et al. 1995; Ricotta et al. 1996; Grey et al. 1998). Furthermore, *B. adusta*, *T. versicolor* and *Trametes* sp. were studied in the degradation of pentachlorophenol (Yemendzhiev et al. 2008; Rubilar et al. 2007; Ford et al. 2007).

11.8 Conclusion

White rot fungi represent a group of microorganisms with a great potential for application to bioremediation. Their ability to decompose a wide range of organic pollutants was documented and their unique enzymatic systems possess special properties that cause direct decomposition of aromatics to less complex structures. Bioremediation by fungi has drawn less attention in the past decades since most bioremediation research has focused mainly on the use of bacteria. Nevertheless, fungi have received considerable attention in recent years for their bioremediation potential which is attributed to the enzymes they produce. In addition, fungi have advantages over bacteria, such as fungal active colonization of contaminated soil. Successful application of these fungi for remediation purposes could be strongly constrained by many environmental and physiological factors, such as soil colonization, nutritional requirements, physiology of their enzyme production, pollutant bioavailability and interactions with indigenous microflora. However, the most important and prerequisite quality of fungi is their ability to decompose efficiently targeted pollutants. From the environmental perspective, an important aspect of the bioremediation process lies in full understanding of biodegradation mechanisms, in order to ensure that the produced metabolites of pollutants are less harmful then their parent compounds. Therefore, further research in this field is needed that will increase our knowledge of the suitability of the fungi for practical bioremediation applications for complete mineralization of toxic wastes released by various anthropogenic activities.

References

Adebusoye SA, Picardal FW, Ilori MO, Amund OO (2008) Influence of chlorobenzoic acids on the growth and degradation potentials of PCB-degrading microorganisms. World J Microbiol Biotechnol 24:1203–1208

Alexander M (1994) Biodegradation and bioremediation. Academic Press, San Diego

Alleman BC, Logan BE, Gilbertson RL (1995) Degradation of pentachlorophenol by fixed films of white rot fungi in rotating tube bioreactors. Water Res 29:61–67

Asgher M, Bhatti HN, Ashraf M, Legge RL (2008) Recent developments in biodegradation of industrial pollutants by white rot fungi and their enzyme system. Biodegradation 19:771–783

Baborova P, Moder M, Baldrian P, Cajthamlova K, Cajthaml T (2006) Purification of a new manganese peroxidase of the white-rot fungus *Irpex lacteus*, and degradation of polycyclic aromatic hydrocarbons by the enzyme. Res Microbiol 157:248–253

Baldrian P (2008) Wood-inhabiting ligninolytic basidiomycetes in soils: ecology and constraints for applicability in bioremediation. Fungal Ecol 1:4–12

Beaudette LA, Davies S, Fedorak PM, Ward OP, Pickard MA (1998) Comparison of gas chromatography and mineralization experiments for measuring loss of selected polychlorinated biphenyl congeners in cultures of white rot fungi. Appl Environ Microbiol 64:2020–2025

Bezalel L, Hadar Y, Fu PP, Freeman JP, Cerniglia CE (1996a) Initial oxidation products in the metabolism of pyrene, anthracene, fluorene and dibenzothiophene by the white rot fungus *Pleurotus ostreatus*. Appl Environ Microbiol 62:2554–2559

Bezalel L, Hadar Y, Cerniglia CE (1996b) Mineralization of polycyclic aromatic hydrocarbons by the white-rot fungus *Pleurotus ostreatus*. Appl Environ Microbiol 62:292–295

Bezalel L, Hadar Y, Fu PP, Freeman JP, Cerniglia CE (1996c) Metabolism of phenanthrene by the white-rot fungus *Pleurotus ostreatus*. Appl Environ Microbiol 62:2547–2553

Bezalel L, Hadar Y, Cerniglia CE (1997) Enzymatic mechanisms involved in phenanthrene degradation by the white-rot fungus *Pleurotus ostreatus*. Appl Environ Microbiol 63:2495–2501

Bhatt M, Cajthaml T, Sasek V (2002) Mycoremediation of PAH-contaminated soil. Folia Microbiol 47:255–258

Blanquez P, Guieysse B (2008) Continuous biodegradation of 17 beta-estradiol and 17 alpha-ethynylestradiol by *Trametes versicolor*. J Hazard Mater 150:459–462

Bumpus JA, Tien M, Wright D, Aust SD (1985) Oxidation of persistent environmental pollutants by a white rot fungus. Science 228:1434–1436

Cabana H, Jiwan JLH, Rozenberg R, Elisashvili V, Penninckx M, Agathos SN, Jones JP (2007a) Elimination of endocrine disrupting chemicals nonylphenol and bisphenol A and personal care product ingredient triclosan using enzyme preparation from the white rot fungus *Coriolopsis polyzona*. Chemosphere 67:770–778

Cabana H, Jones JP, Agathos SN (2007b) Elimination of endocrine disrupting chemicals using white rot fungi and their lignin modifying enzymes: a review. Eng Life Sci 7:429–456

Cabana H, Jones JP, Agathos SN (2007c) Preparation and characterization of cross-linked laccase aggregates and their application to the elimination of endocrine disrupting chemicals. J Biotechnol 132:23–31

Cajthaml T, Möder M, Kačer P, Sasek V, Popp P (2002) Study of fungal degradation products of polycyclic aromatic hydrocarbons using gas chromatography with ion trap mass spectrometry detection. J Chromatogr A 974:213–222

Cajthaml T, Erbanova P, Vaclav S, Moeder M (2006) Breakdown products on metabolic pathway of degradation of benz[a]anthracene by a ligninolytic fungus. Chemosphere 64:560–564

Cajthaml T, Erbanova P, Kollmann A, Novotny C, Sasek V (2008) Mougin C Degradation of PAHs by ligninolytic enzymes of *Irpex lacteus*. Folia Microbiol 53:289–294

Cajthaml T, Kresinova Z, Svobodova K, Sigler K, Rezanka T (2009a) Microbial transformation of synthetic estrogen 17 alpha-ethinylestradiol. Environ Pollut 157:3325–3335

Cajthaml T, Kresinova Z, Svobodova K, Moeder M (2009b) Biodegradation of endocrine-disrupting compounds and suppression of estrogenic activity by ligninolytic fungi. Chemosphere 75:745–750

Camarero S, Sarkar S, Ruiz-Duenas FJ, Martinez MJ, Martinez AT (1999) Description of a versatile peroxidase involved in natural degradation of lignin that has both Mn-peroxidase and lignin-peroxidase substrate binding sites. J Biol Chem 274:10324–10330

Camarero S, Cañas AI, Nousiainen P, Record E, Lomascolo A, Martínez J, Martíneza T (2008) P-Hydroxycinnamic acids as natural mediators for laccase oxidation of recalcitrant compounds. Environ Sci Technol 42:6703–6709

Cañas AI, Camarero S (2010) Laccases and their natural mediators: biotechnological tools for sustainable eco-friendly processes. Biotechnol Adv 28:694–705

Cañas AI, Alcalde M, Plou F, Martinez MJ, Martinez AT, Camarero S (2007) Transformation of polycyclic aromatic hydrocarbons by laccase is strongly enhanced by phenolic compounds present in soil. Environ Sci Technol 41:2964–2971

Chagas EP, Durrant LR (2001) Decolorization of azo dyes by *Phanerochaete chrysosporium* and *Pleurotus sajorcaju*. Enzyme Microb Technol 29:473–477

Chivukula M, Renganathan V (1995) Phenolic azo dye oxidation by laccase from *Pyricularia oryzae*. Appl Environ Microbiol 61:4374–4377

Chivukula M, Spadaro JT, Renganathan V (1995) Lignin peroxidase catalyzed oxidation of sulfonated azo dyes generates novel sulfophenyl hydroperoxides. Biochemistry 34:7765–7772

Choudhary MI, Musharraf SG, Ali RA, Atif M, Atta UR (2004) Microbial transformation of antifertility agents, norethisterone and 17 alpha-ethynylestradiol. Zeitschrift fur Naturforschung Section B-A J Chem Sci 59:319–323

Cloete TE, Celliers L (1999) Removal of aroclor 1254 by the white rot fungus *Coriolus versicolor* in the presence of different concentrations of Mn(IV) oxide. Int Biodeterior Biodegrad 44:243–253

Conneely A, Smyth WF, McMullan G (2002) Study of the white-rot fungal degradation of selected phthalocyanine dyes by capillary electrophoresis and liquid chromatography. Anal Chim Acta 451:259–270

Covino S, Svobodova K, Kresinova Z, Petruccioli M, Federici F, D'Annibale A (2010) In vivo and in vitro polycyclic aromatic hydrocarbons degradation by *Lentinus* (*Panus*) tigrinus CBS 577.79. Bioresour Technol 101:3004–3012

De S, Perkins M, Dutta SK (2006) Nitrate reductase gene involvement in hexachlorobiphenyl dechlorination by *Phanerochaete chrysosporium*. J Hazard Mater B135:350–354

Dietrich D, Hickey WJ, Lamar R (1995) Degradation of 4,4′-dichlorobiphenyl, 4,3′,4,4′-tetrachlorobiphenyl, and 2,2′,4,4′,5,5′-hexachlorobiphenyl by the white rot fungus *Phanerochaete chrysosporium*. Appl Environ Microbiol 61:3904–3909

Donnelly KC, Chen JC, Huebner HJ, Brown KW, Autenrieth RL, Bonner JS (1997) Utility of four strains of white-rot fungi for the detoxification of 2,4,6-trinitrotoluene in liquid culture. Environ Toxicol Chem 16:1105–1110

Dosoretz CG, Grethlein HE (1991) Physiological aspects of the regulation of extracellular enzymes of *Phanerochaete chrysosporium*. Appl Biochem Biotechnol 28–9:253–265

Dos Santos AZ, Neto JMC, Tavares CRG, Da Costa SMG (2004) Screening of filamentous fungi for the decolorization of a commercial reactive dye. J Basic Microbiol 44:288–295

Eaton DC (1985) Mineralization of polychlorinated biphenyls by *Phanerochaete chrysosporium*: a ligninolytic fungus. Enzyme Microb Technol 7:194–196

Eibes G, Cajthaml T, Moreira MT, Feijoo G, Lema JM (2006) Enzymatic degradation of anthracene, dibenzothiophene and pyrene by manganese peroxidase in media containing acetone. Chemosphere 64:408–414

Faison BD, Kirk TK (1985) Factors involved in the regulation of a ligninase activity in *Phanerochaete chrysosporium*. Appl Environ Microbiol 49:299–304

Faraco V, Piscitelli A, Sannia G, Giardina P (2007) Identification of a new member of the dye-decolorizing peroxidase family from *Pleurotus ostreatus*. World J Microbiol Biotechnol 23:889–893

Field JA, de Jong E, Costa GF, de Bont JAM (1993) Screening for ligninolytic fungi applicable to the biodegradation of xenobiotics. Trends Biotechnol 11:44–49

Ford CI, Walter M, Northcott GL, Di Hong J, Cameron KC, Trower T (2007) Fungal inoculum properties: extracellular enzyme expression and pentachlorophenol removal by New Zealand Trametes species in contaminated field soils. J Environ Qual 36:1749–1759

Fu YZ, Viraraghavan T (2002) Dye biosorption sites in *Aspergillus niger*. Bioresour Technol 82:139–145

Fukuda T, Uchida H, Takashima Y, Uwajima T, Kawabata T, Suzuki M (2001) Degradation of bisphenol a by purified laccase from *Trametes villosa*. Biochem Biophys Res Comm 284:704–706

Fukuda T, Uchida H, Suzuki M, Miyamoto H, Morinaga H, Nawata H, Uwajima T (2004) Transformation products of bisphenol A by a recombinant *Trametes vilosa* laccase and their estrogenic activity. J Chem Technol Biotechnol 79:1212–1218

Furukawa K, Fujihara H (2008) Microbial degradation of polychlorinated biphenyls. Biochemical and molecular features. J Biosci Bioeng 105:433–449

Gao X, Zhang X, Zhou S (2005) Biodegradation of RDX wastewater by white rot fungi. Jiangsu Huagong 33:56–58

George HL, VanEtten HD (2001) Characterization of pisatin-inducible cytochrome P450s in fungal pathogens of pea that detoxify the pea phytoalexin pisatin. Fungal Genet Biol 33:37–48

Grey R, Hofer C, Schlosser D (1998) Degradation of 2-chlorophenol and formation of 2-chloro-1,4-benzoquinone by mycelia and cell-free crude culture liquids of Trametes versicolor in relation to extracellular laccase activity. J Basic Microbiol 38:371–382

Hammel KE, Kalyanaraman B, Kirk TK (1986) Oxidation of polycyclic aromatic hydrocarbons and dibenzo[p]-dioxins by *Phanerochaete chrysosporium*. J Biol Chem 261:16948–16952

Hammel KE, Green B, Wen ZG (1991) Ring fission of anthracene by eukaryote. Proc Natl Acad Sci USA 88:10605–10608

Hammel KE, Kapich AN, Jensen KA, Ryan ZC (2002) Reactive oxygen species as agents of wood decay by fungi. Enzyme Microb Technol 30:445–453

Harazono K, Watanabe Y, Nakamura K (2003) Decolorization of azo dye by the white-rot basidiomycete *Phanerochaete sordida* and by its manganese peroxidase. J Biosci Bioeng 95:455–459

Haritash AK, Kaushik CP (2009) Biodegradation aspects of Polycyclic Aromatic Hydrocarbons (PAHs): a review. J Hazard Mater 169:1–15

Hatakka A (1994) Lignin-modifying enzymes from selected white-rot fungi-production and role in lignin degradation. FEMS Microbiol Rev 13:125–135

Heinfling A, Martinez MJ, Martinez AT, Bergbauer M, Szewzyk U (1998) Transformation of industrial dyes by manganese peroxidases from *Bjerkandera adusta* and *Pleurotus eryngii* in a manganese-independent reaction. Appl Environ Microbiol 64:2788–2793

Heinfling-Weidtmann A, Reemtsma T, Storm T, Szewzyk U (2001) Sulfophthalimide as major metabolite formed from sulfonated phthalocyanine dyes by the white-rot fungus *Bjerkandera adusta*. FEMS Microbiol Lett 203:179–183

Hou HM, Zhou JT, Wang J, Du CH, Yan B (2004) Enhancement of laccase production by Pleurotus ostreatus and its use for the decolorization of anthraquinone dye. Process Biochem 39:1415–1419

Husain Q (2006) Potential applications of the oxidoreductive enzymes in the decolorization and detoxification of textile and other synthetic dyes from polluted water: a review. Crit Rev Biotechnol 26:201–221

Husain Q (2010) Peroxidase mediated decolorization and remediation of wastewater containing industrial dyes: a review. Rev Environ Sci Biotechnol 9:117–140

Husain M, Husain Q (2008) Applications of redox mediators in the treatment of organic pollutants by using oxidoreductive enzymes: a review. Crit Rev Environ Sci Technol 38:1–42

Iimura Y, Hartikainen P, Tatsumi K (1996) Dechlorination of tetrachloroguaiacol by laccase of white rot basidiomycete *Coriolus versocolor*. Appl Microbiol Biotechnol 45:434–439

Johannes C, Majcherczyk A (2000) Natural mediators in the oxidation of polycyclic aromatic hydrocarbons by laccase mediator systems. Appl Environ Microbiol 66:524–528

Johannes C, Majcherczyk A, Huttermann A (1998) Oxidation of acenaphthene and acenaphthylene by laccase of *Trametes versicolor* in a laccase-mediator system. J Biotechnol 61:151–156

Johnsen AR, Wick LY, Harms H (2005) Principles of microbial PAH-degradation in soil. Environ Pollut 133:71–84

Juhasz AL, Naidu R (2000) Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: a review of the microbial degradation of benzo[a]pyrene. Int Biodeterior Biodegrad 45:57–88

Kamei I, Sonoki S, Haraguchi K, Kondo R (2006a) Fungal bioconversion of toxic polychlorinated biphenyls by white-rot fungus *Phlebia brevispora*. Appl Microbiol Biotechnol 73:932–940

Kamei I, Kogura R, Kondo R (2006b) Metabolism of 4,4′-dichlorobiphenyl by white-rot fungi *Phanerochaete chrysosporium* and *Phanerochaete* sp. MZ142. Appl Microbiol Biotechnol 72:566–575

Kapdan I, Kargi F, McMullan G, Marchant R (2000) Comparison of white-rot fungi cultures for decolorization of textile dyestuffs. Bioprocess Eng 22:347–351

Kapich AN, Jensen KA, Hammel KE (1999) Peroxyl radicals are potential agents of lignin biodegradation. Febs Lett 461:115–119

Kasinath A, Novotny C, Svobodova K, Patel KC, Sasek V (2003) Decolorization of synthetic dyes by *Irpex lacteus* in liquid cultures and packed-bed bioreactor. Enzyme Microb Technol 32:167–173

Kaushik P, Malik A (2009) Fungal dye decolourization: recent advances and future potential. Environ Int 35:127–141

Kobayashi K, Katayama-Hirayama K, Tobita S (1996) Isolation and characterization of microorganisms that degrade 4-chlorobiphenyl to 4-chlorobenzoic acid. J Gen Appl Microbiol 42:401–410

Koller G, Moder M, Czihal K (2000) Peroxidative degradation of selected PCB: a mechanistic study. Chemosphere 41:1827–1834

Krcmar P, Ulrich R (1998) Degradation of polychlorinated biphenyl mixtures by the lignin-degrading fungus *Phanerochaete chrysosporium*. Folia Microbiol 43:79–84

Krcmar P, Kubatova A, Votruba J, Erbanova P, Novotny C, Sasek V (1999) Degradation of polychlorinated biphenyls by extracellular enzymes of *Phanerochaete chrysosporium* produced in a perforated plate bioreactor. World J Microbiol Biotechnol 15:237–242

Kubatova A, Erbanova P, Eichlerova I, Homolka L, Nerud F, Sasek V (2001) PCB congener selective biodegradation by the white rot fungus *Pleurotus ostreatus* in contaminated soil. Chemosphere 43:207–215

Lamar RT, Dietrich DM (1990) In situ depletion of pentachlorophenol from contaminated soil by *Phanerochaete* spp. Appl Environ Microbiol 56:3093–3100

Lamar RT, Evans JW, Glaser JA (1993) Solid-phase treatment of a pentachlorophenol-contaminated soil using lignin-degrading fungi. Environ Sci Technol 27:2566–2571

Lang E, Nerud F, Novotna E, Zadražil F, Martens R (1996) Production of ligninolytic exoenzymes and ^{14}C-pyrene mineralization by *Pleurotus* sp in lignocellulose substrate. Folia Microbiol 41:489–493

Lee SM, Koo BW, Lee SS, Kim MK, Choi DH, Hong EJ, Jeung EB, Choi IG (2004) Biodegradation of dibutylphthalate by white rot fungi and evaluation on its estrogenic activity. Enzyme Microb Technol 35:417–423

Lee SM, Koo BW, Choi JW, Choi DH, An BS, Jeung EB, Choi IG (2005) Degradation of bisphenol A by white rot fungi, *Stereum hirsutum* and *Heterobasidium insulare*, and reduction of its estrogenic activity. Biol Pharm Bull 28:201–207

Leonowicz A, Cho NS, Luterek J, Wilkolazka A, Wojtas-Wasilewska M, Matuszewska A, Hofrichter M, Wesenberg D, Rogalski J (2001) Fungal laccase: properties and activity on lignin. J Basic Microb 41:185–227

Liers C, Bobeth C, Pecyna M, Ullrich R, Hofrichter M (2010) DyP-like peroxidases of the jelly fungus *Auricularia auricula-judae* oxidize nonphenolic lignin model compounds and high-redox potential dyes. Appl Microbiol Biotechnol 85:1869–1879

Lin J, Tang W, Lin J, Zhou S, Zheng P (2006) Degradation of royal demolition explosive by white-rot-fungi. Zhongguo Jishui Paishui 22:74–77

Lorenzo M, Moldes D, Sanroman MA (2006) Effect of heavy metals on the production of several laccase isoenzymes by *Trametes versicolor* and on their ability to decolourise dyes. Chemosphere 63:912–917

Lu YP, Hardin I (2006) Analysis of sulfonated azo dyes degraded by white rot fungus *Pleurotus ostreatus*. AATCC Rev 6:31–36

Majcherczyk A, Johannes C, Huttermann A (1998) Oxidation of polycyclic aromatic hydrocarbons (PAH) by laccase of *Trametes versicolor*. Enzyme Microb Technol 22:335–341

McKinlay R, Plant JA, Bell JNB, Voulvoulis N (2008) Endocrine disrupting pesticides: implications for risk assessment. Environ Int 34:168–183

McMullan G, Meehan C, Conneely A, Kirby N, Robinson T, Nigam P, Banat IM, Marchant R, Smyth WE (2001) Microbial decolourisation and degradation of textile dyes. Appl Microbiol Biotechnol 56:81–87

Mielgo I, Lopez C, Moreira MT, Feijoo G, Lema JM (2003) Oxidative degradation of azo dyes by manganese peroxidase under optimized conditions. Biotechnol Prog 19:325–331

Moen MA, Hammel KE (1994) Lipid-peroxidation by the manganese peroxidase of *Phanerochaete chrysosporium* is the basis for phenanthrene oxidation by the intact fungus. Appl Environ Microbiol 60:1956–1961

Moldes D, Couto SR, Cameselle C, Sanroman MA (2003) Study of the degradation of dyes by MnP of *Phanerochaete chrysosporium* produced in a fixed-bed bioreactor. Chemosphere 51:295–303

Moreira MT, Mielgo I, Feijoo G, Lema JM (2000) Evaluation of different fungal strains in the decolourisation of synthetic dyes. Biotechnol Lett 22:1499–1503

Novotny C, Vyas BRM, Erbanova P, Kubatova A, Sasek V (1997) Removal of PCBs by various white rot fungi in liquid culture. Folia Microbiol 42:136–140

Novotny C, Svobodova K, Erbanova P, Cajthaml T, Kasinath A, Lang E, Sasek V (2004) Ligninolytic fungi in bioremediation: extracellular enzyme production and degradation rate. Soil Biol Biochem 36:1545–1551

Novotny C, Cajthaml T, Svobodova K, Susla M, Sasek V (2009) Irpex lacteus, a white-rot fungus with biotechnological potential–review. Folia Microbiol 54:375–390

Ollikka P, Harjunpaa T, Palmu K, Mantsala P, Suominen I (1998) Oxidation of crocein orange G by lignin peroxidase isoenzymes kinetics and effect of H_2O_2. Appl Biochem Biotechnol 75:307–321

Palma C, Martinez AT, Lema JM, Martinez MJ (2000) Different fungal manganese-oxidizing peroxidases: a comparison between *Bjerkandera* sp and Phanerochaete chrysosporium. J Biotechnol 77:235–245

Pastigrigsby MB, Paszczynski A, Goszczynski S, Crawford DL, Crawford RL (1992) Influence of aromatic-substitution patterns on azo dye degradability by Streptomyces spp and *Phanerochaete chrysosporium*. Appl Environ Microbiol 58:3605–3613

Paszczynski A, Pasti MB, Goszczynski S, Crawford DL, Crawford RL (1991) New approach to improve degradation of recalcitrant azo dyes by Streptomyces spp and *Phanerochaete chrysosporium*. Enzyme Microb Technol 13:378–384

Perez RB, Benito GG, Miranda MP (1997) Chlorophenol degradation by *Phanerochaete chrysosporium*. Bioresour Technol 60:207–213

Pickard MA, Roman R, Tinoco R, Vazquez-Duhalt R (1999) Polycyclic aromatic hydrocarbon metabolism by white rot fungi and oxidation by *Coriolopsis gallica* UAMH 8260 laccase. Appl Environ Microbiol 65:3805–3809

Pinedo-Rivilla C, Aleu J, Collado IG (2009) Pollutants biodegradation by fungi. Curr Org Chem 13:1194–1214

Podgornik H, Poljansek I, Perdih A (2001) Transformation of indigo carmine by *Phanerochaete chrysosporium* ligninolytic enzymes. Enzyme Microb Technol 29:166–172

Pointing SB (2001) Feasibility of bioremediation by white-rot fungi. Appl Microbiol Biotechnol 57:20–33

Pozdnyakova NN, Nikiforova SV, Turkovskaya OV (2010) Influence of PAHs on ligninolytic enzymes of the fungus *Pleurotus ostreatus* D1. Cent Eur J Biol 5:83–94

Rabinovich ML, Bolobova AV, Vasilchenko LG (2004) Decomposition of natural aromatic structures and xenobiotics by fungi. Appl Biochem Microbiol 40:5–23

Reemtsma T, Jakobs J (2001) Concerted chemical and microbial degradation of sulfophthalimides formed from sulfophthalocyanine dyes by white rot fungi. Environ Sci Technol 35:4655–4659

Ricotta A, Unz RF, Bollag J-M (1996) Role of laccase in degradation of pentachlorophenols. Bull Environ Contam Toxicol 57:560–567

Robinson T, Chandran B, Nigam P (2001) Studies on the decolourisation of an artificial textile-effluent by white-rot fungi in N-rich and N-limited media. Appl Microbiol Biotechnol 57:810–813

Rodriguez E, Nuero O, Guillen F, Martinez AT, Martinez MJ (2004) Degradation of phenolic and non-phenolic aromatic pollutants by four Pleurotus species: the role of laccase and versatile peroxidase. Soil Biol Biochem 36:909–916

Roy-Arcand L, Archbald FS (1991) Direct dechlorination of chlorophenolic compounds by laccase from *Trametes* (*Coriolus*) *versicolor*. Enzyme Microb Technol 13:194–203

Rubilar O, Feijoo G, Diez C, Lu-Chau TA, Moreira MT, Lema JM (2007) Biodegradation of pentachlorophenol in soil slurry culturesby *Bjerkandera adusta* and *Anthracophyllum discolor*. Ind Eng Chem Res 46:6744–6751

Ruiz-Aguilar GML, Fernandez-Sanchez JM, Rodriguez-Vazquez R, Poggi-Veraldo H (2002) Degradation by white rot fungi of high concentrations of PCB extracted from a contaminated soil. Adv Environ Res 6:559–568

Ruiz-Duenas FJ, Camarero S, Perez-Boada M, Martinez M, Martinez AT (2001) A new versatile peroxidise from *Pleurotus*. Biochem Soc Trans 29:116–122

Sack U, Hofrichter M, Fritsche W (1997) Degradation of polycyclic aromatic hydrocarbons by manganese peroxidise of *Nematoloma frowardii*. FEMS Microbiol Lett 152:227–234

Sasek V, Volfova O, Erbanova P, Vyas BRM, Matucha M (1993) Degradation of PCBs by white rot fungi, methylotrophic and hydrocarbon utilizing yeasts and bacteria. Biotechnol Lett 15:521–526

Scheibner K, Hofrichter M (1998) Conversion of aminonitrotoluenes by fungal manganese peroxidase. J Basic Microbiol 38:51–59

Scheibner K, Hofrichter M, Herre A, Michels J, Fritsche W (1997) Screening for fungi intensively mineralizing 2,4,6-trinitrotoluene. Appl Microbiol Biotechnol 47:452–457

Schliephake K, Mainwaring DE, Lonergan GT, Jones IK, Baker WL (2000) Transformation and degradation of the disazo dye Chicago Sky Blue by a purified laccase from *Pycnoporus cinnabarinus*. Enzyme Microb Technol 27:100–107

Schutzendubel A, Majcherczyk A, Johannes C, Huttermann A (1999) Degradation of fluorene, anthracene, phenanthrene, fluoranthene, and pyrene lacks connection to the production of extracellular enzymes by *Pleurotus ostreatus* and *Bjerkandera adusta*. Int Biodeterior Biodegrad 43:93–100

Shimokawa T, Shoda M, Sugano Y (2009) Purification and characterization of two DyP isozymes from *Thanatephorus cucumeris* Dec 1 specifically expressed in an air-membrane surface bioreactor. J Biosci Bioeng 107:113–115

Shin KW (2004) The role of enzymes produced by white-rot fungus *Irpex lacteus* in the decolorization of the textile industry effluent. J Microbiol 42:37–41

Shin KS, Kim YH, Lim JS (2005) Purification and characterization of manganese peroxidase of the white-rot fungus *Irpex lacteus*. J Microbiol 43:503–509

Soares GMB, Costa-Ferreira M, de Amorim MTP (2001a) Decolorization of an anthraquinone-type dye using a laccase formulation. Bioresour Technol 79:171–177

Soares GMB, de Amorim MTP, Costa-Ferreira M (2001b) Use of laccase together with redox mediators to decolourize Remazol Brilliant Blue R. J Biotechnol 89:123–129

Soares GMB, Amorim MTP, Hrdina R, Costa-Ferreira M (2002) Studies on the biotransformation of novel disazo dyes by laccase. Process Biochem 37:581–587

Soares A, Jonasson K, Terrazas E, Guieysse B, Mattiasson B (2005) The ability of white-rot fungi to degrade the endocrine-disrupting compound nonylphenol. Appl Microbiol Biotechnol 66:719–725

Soares A, Guieysse B, Mattiasson B (2006) Influence of agitation on the removal of nonylphenol by the white-rot fungi *Trametes versicolor* and *Bjerkandera* sp. BOL 13. Biotechnol Lett 28:139–143

Srinivasan A, Viraraghavan T (2010) Decolorization of dye wastewaters by biosorbents: a review. J Environ Manag 91:1915–1929

Sugimori D, Banzawa R, Kurozumi M, Okura I (1999) Removal of disperse dyes by the fungus *Cunninghamella polymorpha*. J Biosci Bioeng 87:252–254

Susla M, Svobodova K (2008) Effect of various synthetic dyes on the production of manganese-dependent peroxidase isoenzymes by immobilized *Irpex lacteus*. World J Microbiol Biotechnol 24:225–230

Suzuki K, Hirai H, Murata H, Nishida T (2003) Removal of estrogenic activities of 17 beta-estradiol and ethinylestradiol by ligninolytic enzymes from white rot fungi. Water Res 37:1972–1975

Svobodova K, Cajthaml T (2010) New in vitro reporter gene bioassays for screening of hormonal active compounds in the environment. Appl Microbiol Biotechnol 88:839–847

Svobodova K, Erbanova P, Sklenar J, Novotny C (2006) The role of Mn-dependent peroxidase in dye decolorization by static and agitated cultures of *Irpex lacteus*. Folia Microbiol 51:573–578

Svobodova K, Senholdt M, Novotny C, Rehorek A (2007) Mechanism of reactive orange 16 degradation with the white rot fungus *Irpex lacteus*. Process Biochem 42:1279–1284

Svobodova K, Majcherczyk A, Novotny C, Kues U (2008) Implication of mycelium-associated laccase from *Irpex lacteus* in the decolorization of synthetic dyes. Bioresour Technol 99:463–471

Swamy J, Ramsay JA (1999a) Effects of glucose and NH4+ concentrations on sequential dye decoloration by *Trametes versicolor*. Enzyme Microb Technol 25:278–284

Swamy J, Ramsay JA (1999b) The evaluation of white rot fungi in the decoloration of textile dyes. Enzyme Microb Technol 24:130–137

Tauber MM, Guebitz GM, Rehorek A (2005) Degradation of azo dyes by laccase and ultrasound treatment. Appl Environ Microbiol 71:2600–2607

Tavčar M, Svobodova K, Kuplenk J, Novotny C, Pavko A (2006) Biodegradation of azo dye RO16 in different reactors by immobilized *Irpex lacteus*. Acta Chim Slov 53:338–343

Thomas DR, Carswell KS, Georgiou G (1992) Mineralization of biphenyl and PCBs by the white rot fungus *Phanerochaete chrysosporium*. Biotechnol Bioeng 40:1395–1402

Tsutsumi Y, Haneda T, Nishida T (2001) Removal of estrogenic activities of bisphenol A and nonylphenol by oxidative enzymes from lignin-degrading basidiomycetes. Chemosphere 42:271–276

Valli K, Warishi H, Gold MH (1992) Degradation of 2,7-dichlorodibenzo-p-dioxin by the lignin-degrading basidiomycete Phanerochaete chrysosporium. J Bacteriol 174:2131–2137

Van Aken B, Godefroid LM, Peres CM, Naveau H, Agathos SN (1999) Mineralization of 14C-U ring labeled 4-hydroxylamino-2,6-dinitrotoluene by manganese-dependent peroxidase of the white-rot basidiomycete Phlebia radiata. J Biotechnol 68:159–169

Vyas BRM, Sasek V, Matucha M, Bubner M (1994) Degradation of 3,3′,4,4′-tetrachlorobiphenyl by selected white rot fungi. Chemosphere 28:1127–1134

Wang YX, Yu J (1998) Adsorption and degradation of synthetic dyes on the mycelium of *Trametes versicolor*. Water Sci Technol 38:233–238

Watanabe T, Shirai N, Okada H, Honda Y, Kuwahara M (2001) Production and chemiluminescent free radical reactions of glyoxal in lipid peroxidation of linoleic acid by the ligninolytic enzyme, manganese peroxidase. Eur J Biochem 268:6114–6122

Wesenberg D, Kyriakides I, Agathos SN (2003) White-rot fungi and their enzymes for the treatment of industrial dye effluents. Biotechnol Adv 22:161–187

Wolter M, Zadrazil F, Martens R, Bahadir M (1997) Degradation of eight highly condensed polycyclic aromatic hydrocarbons by *Pleurotus* sp. Florida in solid wheat straw substrate. Appl Microbiol Biotechnol 48:398–404

Wong YX, Yu J (1999) Laccase-catalyzed decolorization of synthetic dyes. Water Res 33:3512–3520

Wu F, Ozaki H, Terashima Y, Imada T, Ohkouchi Y (1996) Activities of ligninolytic enzymes of the white rot fungus, *Phanerochaete chrysosporium* and its recalcitrant substance degradability. Water Sci Technol 34:69–78

Yadav JS, Quensen JF, Tiedje JM, Reddy CA (1995) Degradation of polychlorinated biphenyl mixtures (Aroclor 1242, 1254, and 1260) by the white rot fungus Phanerochaete chrysosporium as evidenced by congener-specific analysis. Appl Environ Microbiol 61:2560–2565

Yemendzhiev H, Gerginova M, Krastanov A, Stoilova I, Alexieva Z (2008) Growth of *Trametes versicolor* on phenol. J Ind Microbiol Biotechnol 35:1309–1312

Zhao XH, Hardin IR, Hwang HM (2006) Biodegradation of a model azo disperse dye by the white rot fungus *Pleurotus ostreatus*. Int Biodeterior Biodegrad 57:1–6

Zhao XH, Lu YP, Phillips DR, Hwang HM, Hardin IR (2007) Study of biodegradation products from azo dyes in fungal degradation by capillary electrophoresis/electrospray mass spectrometry. J Chromatogr A 1159:217–224

Zille A, Gornacka B, Rehorek A, Cavaco-Paulo A (2005a) Degradation of azo dyes by *Trametes villosa* laccase over long periods of oxidative conditions. Appl Environ Microb 71:6711–6718

Zille A, Munteanu FD, Gubitz GM, Cavaco-Paulo A (2005b) Laccase kinetics of degradation and coupling reactions. J Mol Catal B-Enzymatic 33:23–28

Chapter 12
Microbial Degradation of Dye-Containing Wastewater

Pijush Kanti Mondal and Brajesh Chauhan

12.1 Introduction

Rapid industrialization and urbanization cause environmental pollution and release several xenobiotic compounds into the environment. Many of them have been listed as priority pollutants by the United States Environment Protection Agency (http://www.epa.gov) due to their toxicity and persistence in nature. Dyes are used by a large number of industrial processes, most notably textile, leather, plastics, food, pharmaceuticals and paints manufacturing industries. Release of these compounds into the environment is undesirable, not only because of their colour, but also because many dyes and their breakdown products are toxic, mutagenic and carcinogenic in nature (Dos Santos et al. 2007) and have negative impact on the living organisms.

The most problematic industries in the terms of dyes release to the environment in the form of wastewater are dyes manufacturing and the dyeing industry. The uncontrolled release of these compounds in the environment causes severe problems. Since they are designed to be chemically and photolytically stable, they are highly persistent in the natural environments. For instance, the half-life of hydrolised reactive blue 19 is about 46 years at pH 7 and 25°C (Hao et al. 2000). In fact, most of the commercially used dyes are xenobiotic in nature, because they do not exist as natural products and therefore, contain structural elements that cannot be synthesized biochemically (Stolz 2001; Rieger et al. 2002).

P. K. Mondal (✉)
Environmental Research Laboratory, Department of Applied Chemistry, Faculty of Engineering and Technology, Aligarh Muslim University, Aligarh, UP 202 002, India
e-mail: pijushamu@gmail.com

B. Chauhan
Department of Civil Engineering, Faculty of Engineering and Technology, Aligarh Muslim University, Aligarh, UP 202 002, India

S. N. Singh (ed.), *Microbial Degradation of Xenobiotics*, Environmental Science and Engineering, DOI: 10.1007/978-3-642-23789-8_12,

An incident was reported where 15 workers were working in distilling 2-naphthylamine plant (Chung and Cerniglia 1992). Long term of exposure to 2-naphthylamine caused all of these 15 workers to develop bladder cancer. Over the years, epidemiological studies have shown the evidence that long-term occupational exposure to certain aromatic amines, such as benzidine, 4-aminobiphenyl and 2-naphthylamine, that are used in dye industries increases the risk of developing cancer.

Different physical, chemical and biological techniques can be applied to remove dyes from the wastewater. These techniques include adsorption, coagulation–flocculation, reverse osmosis, oxidation, photodegradation, membrane filtration and microbial degradation. Each technology has its technical and economical limitations (Table 12.1). Most physico-chemical dye removal methods have drawbacks because they are expensive, have limited versatility and are greatly interfered by other wastewater constituents, and/or generate waste products that must be handled (Hao et al. 2000; Robinson et al. 2001). Alternatively, biological treatment may present an environmental-friendly methods that are becoming increasingly efficient and cost-effective for the remediation of dye-contaminated wastewater.

Microorganisms have the ability to adapt to a variety of environmental conditions. Their versatility makes them useful for various biotechnological applications. One such application is the use of microorganisms for removal of dyes from the environment which is called bioremediation. Biodegradation is the process by which organic substances (like dyes) are broken down by other living organisms. Biological dye removal techniques are based on microbial biotransformation of dyes. As dyes are designed to be stable and long-lasting colorants, they are usually not easily biodegraded. Dyes can be degraded aerobically (with oxygen) or anaerobically (without oxygen). A term related to biodegradation is biomineralisation, in which dyes are converted into minerals.

A number of research papers have reported the results of microbial biodegradation treatment of dye containing wastewaters. This chapter summarizes the results of those research studies and discusses the feasibility of microbial treatment for the bioremediation of dyes from wastewater.

12.2 Aerobic Degradation of Dyes

Most common aerobic process is activated sludge process. Activated sludge is a process dealing with the treatment of sewage and dyes containing wastewaters (Beun et al. 1999). The combination of raw sewage (or industrial wastewater) and biological mass is commonly known as mixed liquor. In all activated sludge plants, once the sewage (or industrial wastewater) has received sufficient treatment, excess mixed liquor is discharged into settling tanks and the treated supernatant is run off to undergo further treatment before discharge. A part of the settled material, the sludge, is returned to the head of the aeration system to re-seed the new sewage (or industrial wastewater) entering the tank. This fraction of the floc is called return activated sludge (RAS). It involves a series of steps- primary

Table 12.1 Current physical, chemical and biological technology for color removal

Various methods	Advantages	Disadvantages
Adsorption	Good removal of a wide variety of dyes	Adsorbent requires regeneration or disposal. Expensive capital investment, long residence times, low adsorption capacity, frequent and expensive regeneration
Membrane technologies	Removes all dye types and efficiency is better than other	Concentrated sludge production and very expensive
Advanced oxidation/ photodegradation	Rapid process	High energy costs and unwanted byproduct released
Coagulation/ flocculation, Reverse osmosis	Economically feasible	High sludge production rate
Microbial biodegradation	It is inexpensive and more environmental friendly. Good removal of a wide variety of dyes. Efficient COD and BOD reduction	Long residence times, may require nutrients, very large aeration tanks, lagoons, land areas, many toxic compounds not removed, variable color removal
Electrolysis	Not totally mineralized	High power source is required

treatment, secondary treatment and in some cases a tertiary treatment may also be used. The effluent from one tank becomes the influent for the other.

12.2.1 Aerobic Treatment

Conventional activated sludge treatment of wastes is often an effective and highly economic system for reducing organic pollutants in wastewater. A fair amount of research has been conducted assessing the viability of using activated sludge to treat textile effluents (Zissi et al. 1997). However, aerobic treatment of azo dye wastes has been proven ineffective in most cases, hence, a typical method of treatment is used today. Because aerobic microbes cannot reduce azo linkages, their ability to destroy dye chromogens is less than anaerobic bacterium. However, aerobic sludges have been successfully used to stabilize dye metabolites (Brown and Laboureur 1983a).

Pagga and Brown (1986) conducted a study on 87 commercial dye stuffs. Some of the dye stuffs were in a technically pure form, while others were in a sales form containing organic substances such as wetting agents. The exact class of each dye was not given. The tests were performed in a reactor designed to simulate the conditions of adapted activated sludge wastewater treatment plant. The samples were tested for color and dissolve organic carbon (DOC) removal following 42 days of treatment. Pagga and Brown (1986) concluded that as expected from their structures and function, dyestuffs are most unlikely to biodegrade in short-term aerobic tests. They further indicated that the primary mechanism for removal of dyes in activated sludge systems might occur by adsorption onto the cell walls. Also, they concluded that DOC removal was possible in an aerobic environment, but did not always correlate with decolorization. Degradation of non-dye molecules in the dye solution may be responsible for this reduction, as destruction of the chromogen is not generally observed.

In an earlier study by Brown and Laboureur (1983b), the aerobic biodegradability of aniline, *o*-toludine, *p*-anisidine, *p*-phenetidine, *o*-dianisidine, and 3,3′-dichlorobenzidine, was investigated. These compounds are all lipophilic aromatic amines and possible by-products of azo dyes. Because many aromatic compounds are non-biodegradable in anaerobic environments and are not hydrophilic, they can accumulate in the adipose tissues of organisms. Many aromatics have been identified as possible carcinogens, which find their release into the environment. Brown and Laboureur (1983a) indicated that azo dyes might be broken down to their intermediate structures in the reductive environment, but were not amenable to further degradation by anaerobes.

Brown and Laboureur (1983b) concluded that aniline, *p*-anisidine, *p*-phenetidine and *o*-toludine were readily biodegradable by aerobes, while *o*-dianisidine and 3,3-dichlorobenzidine were inherently non-biodegradable. They suggested that these compounds could be stabilized if released into the environment or directly from a dyehouse into a conventional wastewater treatment plant.

Shaul et al. (1991) conducted a study on 18 dyes to determine their fate in the activated sludge process. Of these dyes, 15 were acid azo dyes and three were direct azo dyes. The dyes were spiked into pilot-scale treatment systems, and effluent and sludge samples were collected. High performance liquid chromatography (HPLC) was used to analyze the samples. Mass balance calculations were preformed to determine the amount of the dye in the sludge and in the effluent. Eleven of the dyes passed through the activated sludge system substantially untreated, four were significantly absorbed onto the sludge, and three were apparently biodegraded.

Loyd (1992) also performed activated sludge treatment tests on two textile wastewaters. The first was a textile dyeing and finishing process water that contained reactive navy 106, and the second was a municipal wastewater consisting predominately of textile effluents. Both effluents were fed to laboratory -scale activated sludge reactors. The reactor effluents were analyzed for ADMI color and total organic carbon (TOC) removal, and also for toxicity. He concluded that aerobic treatment of the azo dye wastewaters caused significant biodegradation with minimal decolorization, but the biodegradation did not include the azo dyestuffs. Again one would presume that the removal of organic compounds, such as wetting agents and other process additives resulted in the TOC loss. Toxicity tests indicated LC50 values falling outside of the 100% effluent concentration standard. However, he indicated that toxicity was slightly reduced following the aerobic treatment.

Furthermore, Zissi and Lyberatos (2000) investigated the biological oxidation of *p*-aminoazobenzene (*p*AAB) by *Bacillus subtilis*. This was carried out in batch experiments using a suspension medium supplemented with glucose, ammonium chloride, and *p*AAB under sterile conditions. Cellular growth rates and inhibition, glucose utilization, *p*AAB degradation, and by -product formation were critically observed. The results indicated that *B. subtilis* could co-metabolize *p*AAB in the presence of glucose, breaking the N=N double bond and produced aniline and *p*-phenylenediamine. However, an evidence was found that suggested *p*AAB was inhibitory to microbial growth, and that glucose was the growth-limiting substrate. The degradation of the dye was the direct result of an oxygen-insensitive azo reductase enzyme found to be present in the soluble fraction of the biomass. This enzyme was also synthesized independently of the presence of *p*AAB.

The previous researchers have suggested that aerobic biodegradation of most azo dyes is not very effective. Hence, conventional activated sludge systems are not adequate for treating azo dye wastewaters. Evidence on the contrary, the aerobic biodegradation of azo dye intermediates is possible and is perhaps an effective treatment process for stabilizing these compounds after anaerobic reduction.

An important property of textile dyes that they are resistant to oxidation. A garment saturated with water or perspiration and well inoculated with microorganisms is an excellent culture medium. The above criterion clarifies why many commercial dyes are recalcitrant to oxidative microbial breakdown and, therefore, existing forms of aerobic wastewater treatment are not usually effective against dye house effluent.

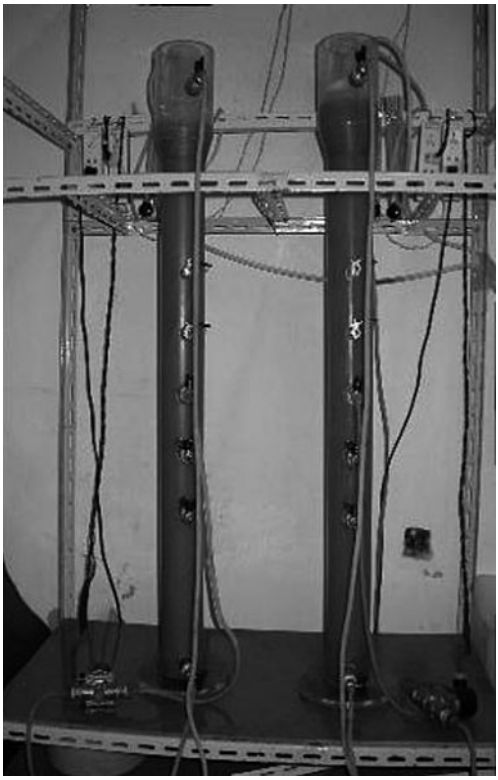


Fig. 12.1 Photo of a laboratory scale sequential batch reactor (SBR)

12.2.2 Activated Sludge Process

The activated sludge process is a wastewater treatment method in which the carbonaceous organic matter of wastewater provides an energy source for the production of new cells for a mixed population of microorganisms in an aquatic aerobic environment. The microbes convert carbon into cell tissue and oxidize the dyes finally to carbon dioxide and water. In addition, a limited number of microorganisms may exist in activated sludge that obtains energy by oxidizing ammonia nitrogen to nitrate nitrogen in the process known as nitrification.

Aerobic granulation is a novel biotechnique for coloured wastewater treatment developed in SBR. Granulation is a process of microbial cell self-immobilization, resulting in a cell-structured shape characterized by dense biomass. Similar to anaerobic granules, aerobic granules have a number of advantages over conventional bioflocs, such as a round and compact structure, good settling ability, high biomass retention and ability to withstand high organic loading rate (Liu et al. 2006). Aerobic granulation by SBR has been demonstrated for the treatment of a wide variety of wastewaters, including industrial wastewater, nutrient-rich and toxic wastewaters (Fig. 12.1).

Bacteria constitute the majority of microorganisms present in the activated sludge. Bacteria that require organic compounds for their supply of carbon and energy (heterotrophic bacteria) predominate, but bacteria that use inorganic compounds for cell growth (autotrophic bacteria) also occur in proportion to concentrations of carbon and nitrogen. Both aerobic and anaerobic bacteria may exist in the activated sludge, but the preponderance of species are facultative, able to live either in the presence of or lack of dissolved oxygen.

Fungi, rotifers, and protozoans are also residents of activated sludge. The latter microorganisms are represented largely by ciliated species, but flagellated protozoan and amoebae may also be present. Protozoans serve as indicators of the activated sludge condition, and ciliated species are instrumental in removing *Escherichia coli* from sewage. Additionally, viruses of human origin may be found in the raw sewage influent, but a large percentage appears to be removed by the activated-sludge process.

The success of the activated-sludge process is dependent upon establishing a mixed community of microorganisms that will remove and consume organic waste material, that will aggregate and adhere in a process known as bio-flocculation, and that will settle in such a manner so as to produce a concentrated sludge (return activated sludge, or RAS) for recycling. Any of several types of activated sludge solids separations problems indicates an imbalance in the biological component of this process. In the ideal "healthy" system, filamentous organisms grow within a floc (a large aggregate of adherent, or floc-forming, microorganisms, such as bacteria) and give it strength, with few filaments protruding out into the surrounding bulk solution. In such a system, there is no interference with the compaction and settling rates of the activated sludge prior to its recycling.

Nevertheless, many researches have demonstrated partial or complete biodegradation of dyes by pure and mixed cultures of bacteria, fungi and algal species.

12.3 Anaerobic Degradation of Dyes

Anaerobic treatment means treatment in a closed container in absence of air. Upflow anaerobic sludge blanket reactor (UASB) is most popular among anaerobic treatment processes. For anaerobic process, much more efficient reactors have been developed for UASB reactors (Fig. 12.2). In some anaerobic (Lettinga et al. 1980, 1993) and anoxic process like UASB, granular sludge was developed for high organic load wastewater treatment.

12.3.1 Anaerobic Treatment

Anaerobic reduction of dyes using microbial sludges can be an effective and economic treatment process for removing color from dye house effluents. Previous studies have demonstrated the ability of anaerobic bacteria to reductively cleave

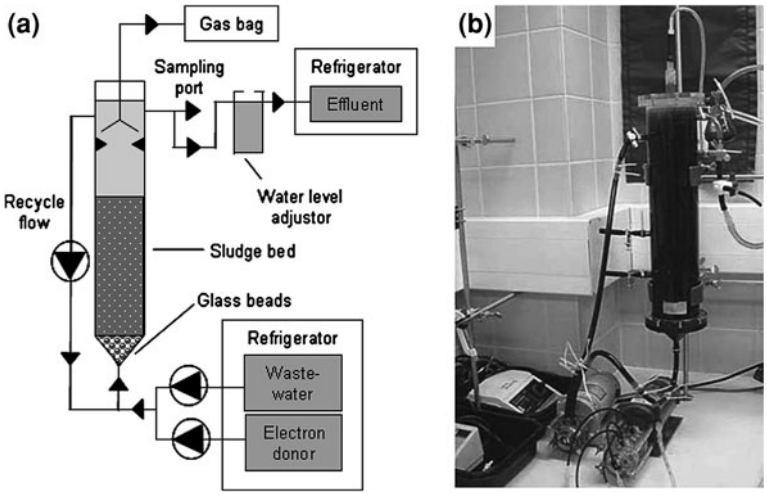


Fig. 12.2 A schematic diagram (**a**) and photo (**b**) of a laboratory scale upflow anaerobic sludge blanket reactor (UASB)

the azo linkages in reactive dyes (Chung et al. 1978; Brown and Laboureur 1983a; Brown and Hamburger 1987; Ganesh 1992; Loyd 1992; Razo-Flores et al. 1997; Chinwetkitvanich et al. 2000). Although this effectively alters the chromogen and destroys the observed color of the dye, many aromatic groups are not susceptible to anaerobic reduction. However, there is an evidence that some azo dye metabolites may be fully stabilized in the anaerobic environments (Razo-Flores et al. 1997).

Chung et al. (1978) conducted a study on the degradability of seven azo dyes using intestinal and other major anaerobes. The studies were carried out using isolated strains of bacteria in suspended cell mediums containing different azo dyes. Although the dyes studied were not fiber-reactive dyes, their findings showed that the reduction of azo compounds could be accomplished by intestinal and other major anaerobes. Furthermore, the presence of aromatic intermediates was also detected in measurable amounts for each dye. Toxicity tests were not conducted, but some of the intermediates had been previously reported to be mutagenic.

In a three-part research series, Brown and Laboureur (1983a, b) studied the degradability of various azo dyes in both anaerobic and aerobic systems. In the first study, Brown and Laboureur (1983b) investigated the anaerobic degradability of 22 commercial dyes. Of the dyes studied, four monoazo and six diazo dyes showed substantial biodegradation, while two polyazo dyes showed moderate to variable reductions.

Later, Brown and Hamburger (1987) conducted a study on 14 azo dyes subjected to anaerobic sludge digestion followed by aerobic treatment. This study focused on both the reduction of the dye molecules as well as the production and subsequent degradation of dye metabolites. Brown and Hamburger's results

confirmed the findings of earlier research, showing decolorization of the azo dyes. Confirming the cleavage of azo linkages, the production of metabolites was also observed, at less than theoretical concentrations. Speculation was made as to why these concentrations were low, but no conclusive evidence was provided. They do indicate that based on the yield of metabolites, further dye reduction in anaerobic environments is in general, neither rapid nor appreciable.

Razo-Flores et al. (1997) investigated the fate of mordant orange1 (MO1) and azodisalicylate (ADS) under methanogenic conditions using continuous UASB reactors. Their research focused on the reduction of by-products, 5-aminosalicylic acid (5-ASA) and 1,4-phenylenediamine. Co-substrates, volatile fatty acid (VFA) or glucose, were also fed to the reactors in order to supply the reducing equivalents needed for the reduction of the azo bonds. The results of this study demonstrated the ability of an anaerobic consortium to completely mineralize some azo dye compounds. The experiments were conducted using two reactor series. In the first reactor, only MO1 and VFA were fed for a 217-day period. Data from this period showed high decolorization, and high consent ratio of 5-ASA and 1,4-phenylenediamine. In the second reactor, MO1 was fed for 217 days, which was followed by ADS, with and without glucose, for a period of 340 days. Data from this reactor showed high decolorization throughout testing, but a lower concentration of 5-ASA. Razo-Flores et al. (1997) observed the complete mineralization of ADS with and without a co-substrate, indicating the possibility for aromatic amine destruction in methanogenic environments. The compound, 1,4-phenylenediamine was not observed to degrade in either test reactor, indicating the specificity of aromatic amine utilization by anaerobes.

The studies were conducted by Loyd (1992), Ganesh (1992) on the anaerobic reduction of textile mill effluents and the azo dyes Reactive Black 5 and Navy 106 were investigated, respectively. In both cases, laboratory scale anaerobic reactors were used for dye degradation. The results of Loyd (1992), Ganesh (1992) were similar; both observed good decolorization with minimal nutrient removal. These findings support the earlier studies found in the literature. While high decolorization of textile effluents was often achieved in the anaerobic environments, poor TOC and nitrogen removals were usually observed.

Chinwetkitvanich et al. (2000) performed a study on various reactive dye bath effluents. They examined the effect of co-substrate and initial color concentrations on fiber-reactive dye reduction efficiencies in UASB reactors. Five different experiments were conducted using a variation of red, blue, and black dye synthetic wastewaters and also real dye house effluents composed of red, blue, and black dyes. Their results showed that with the addition of a co-substrate, such as tapioca, increased reduction efficiencies could be achieved. However, at high level of tapioca addition, no enhancement was observed. Furthermore, Chinwetkitvanich et al. (2000) concluded that higher initial color concentrations might be deleterious to acid forming bacteria, resulting in a lower dye removal. Additionally, the authors suggest that sulfate-reducing bacteria might out-compete other anaerobic microorganisms for available organic carbon, but contribute minimally to

decolorization. This could serve to limit the reduction equivalents necessary for dye degradation.

The interest in the use of anaerobic treatment process can be explained by considering the advantages and disadvantages of this process. The principal advantages and disadvantages of anaerobic treatment are listed as follows:

12.3.1.1 Advantages

- Less energy requirement since aeration is not required.
- Less biological sludge production.
- Fewer nutrients required.
- Methane production, a potential energy source.
- Smaller reactor volume required.
- Elimination of off-gas air pollution.
- Rapid response to substrate addition after long periods without feeding.

12.3.1.2 Disadvantages

- Longer start-up time to develop necessary biomass inventory.
- Requires alkalinity addition.
- Requires further treatment with an aerobic treatment process to meet discharge requirements.
- Biological nitrogen and phosphorus removal is not possible.
- Much more sensitive to the adverse effects of lower temperatures on reaction rates.
- More susceptible due to toxic substances.
- Potential for production of odor and corrosive gases.

12.4 Bacterial Biodegradation

For general evaluation of dye biodegradability, the dyes chemical structures, rather than their application classes, should be considered. Investigations on bacterial dye biotransformation have been so far focused on the most abundant chemical class, i.e. the azo dyes.

12.4.1 Azo Dyes

The electron withdrawing nature of the azo linkages obstructs the susceptibility of azo dye molecules to oxidative reactions (Fewson 1988). Therefore, azo dyes generally resist aerobic bacterial biodegradation (Pagga and Taeger 1994). Only bacteria with specialized azo dye reducing enzymes were found to degrade azo dyes under fully aerobic conditions. In contrast, breakdown of azo linkages by reduction under anaerobic conditions is much less specific. This anaerobic reduction implies decolourisation as the azo dyes are converted to usually colorless but potentially harmful aromatic amines. Aromatic amines are generally not further degraded under anaerobic conditions. Anaerobic treatment may, therefore, be considered merely as the first stage of the complete degradation of azo dyes. The second stage involves conversion of the produced aromatic amines. For several aromatic amines, this can be achieved by biodegradation under aerobic conditions. Combined anaerobic and aerobic bacterial biodegradation of azo dyes, as well as its applications in wastewater treatment processes, will be further discussed later. However, bacterial biodegradation of non-azo dyes has received little attention so far.

12.4.2 Anthraquinone Dyes

Anthraquinone dyes may possibly be aerobically degraded analogous to anthraquinone (Meulenberg et al. 1997) or anthraquinone-2-sulphonate (Seignez et al. 1996). At least, it has been demonstrated that three bacterial strains could grow with the anthraquinone dye acid blue (Ramakrishna and Viraraghavan 1997) as sole source of energy (Walker and Weatherley 2000). Under anaerobic conditions, the transformation of anthraquinone dyes is presumably limited to reduction of quinone to hydroquinone, a reaction that reverses once the molecule is again exposed to oxygen (Pasti and Crawford 1990; Seignez et al. 1996). Some anthraquinone dyes have been observed to be removed from the water phase by formation of an ‘insoluble pigment’ under anaerobic conditions. This is in line with the observation that electrochemical reduction of an anthraquinone dye increases its adsorptive properties (DeFazio and Lenley 1999).

12.4.3 Triphenylmethane Dyes

Aerobic decolourisation of triphenylmethane dyes has been demonstrated repeatedly by several workers (Azmi et al. 1998; Sani and Banerjee 1999; Sarnaik and Kanekar 1999; Yatome et al. 1991; Yatome et al. 1993). It is also stated that these dyes resist degradation in activated sludge systems (Jank et al. 1998). Under anaerobic conditions, the transformation of triphenylmethane dyes is presumably

limited to reversible reactions like the reduction of malachite green (basic green 4) to leucomalachite green (Henderson et al. 1997). Phtalocyanine dyes are probably not biodegradable. Reversible reduction and decolourisation occur under anaerobic conditions (Nigam et al. 1996; Beydilli et al. 1998).

It has been noted that the brief overview in this section did not include dye degradation by Streptomyces and other actinomycetes, i.e. bacteria that produce extracellular oxidative enzymes like white-rot fungi. These extracellular oxidative enzymes are relatively non-specific enzymes catalysing the oxidation of a variety of dyes (Ball et al. 1989; McMullan et al. 2001; Zhou and Zimmermann 1993).

12.5 Fungal Biodegradation

Lignin-degrading fungi, white-rot fungi, can degrade a wide range of aromatics. This property is mainly due to the relatively non-specific activity of their lignolytic enzymes, such as lignin peroxidase, manganese peroxidase and laccase. The reactions catalysed by these extracellular enzymes are oxidation reactions, e.g. lignin peroxidase catalyses the oxidation of non-phenolic aromatics, whereas manganese peroxidase and laccase catalyse the oxidation of phenolic compounds (McMullan et al. 2001). The degradation of dyes by white-rot fungi was first reported in 1983 (Glenn and Gold 1983) and since then has been the subject of many research activities. An exhaustive review on this aspects was published by Fu et al. (2002). Virtually, all dyes from all chemically distinct groups are prone to fungal oxidation, but there are large differences between fungal species with respect to their catalyzing power and dye selectivity. A clear relationship between dye structure and fungal dye biodegradability has not been established so far (Fu and Viraraghavan 2001). Fungal degradation of aromatic structures is a secondary metabolic event that starts when nutrients (C, N and S) become limiting (Kirk and Bartlett 1987).

Therefore, while the enzymes are optimally expressed under starving conditions, supplementation of energy substrates and nutrients are necessary for propagation of the cultures. Other important factors for cultivation of white-rot fungi and expression of lignolytic activity are the availability of enzyme co-factors and the pH of the environment. A stable operation condition for continuous fungal bioreactors for the treatment of synthetic dye solutions has been achieved (Zhang et al. 2007). Application of white-rot fungi for the removal of dyes from textile wastewater faces many problems. As wastewater is not the natural environment of white-rot fungi, the enzyme production may be unreliable (Robinson et al. 2001) and the biomass growth and retention in bioreactors will be a matter of concern (Stolz 2001). Treatment of large wastewater volumes may be difficult, as extraction and concentration of dyes prior to fungal treatment, may be necessary (Nigam et al. 2000). Furthermore, the low optimum pH for lignin peroxidase (4.5-5) requires extensive acidification of the usually highly alkaline textile wastewater which causes inhibition of other useful microorganisms like bacteria. Moreover, other wastewater constituents, especially aromatics, may interfere with fungal dye degradation (Stolz 2001).

12.6 Algal Biodegradation

The use of algae for the degradation of dyes is mentioned in only a few reports and this has been achieved by *Chlorella* (Acuner and Dilek 2004), *Oscillateria* (Jinqi and Houtian 1992) and *Spirogyra* (Mohan et al. 2002) species. All these reports used azo dyes. Jinqi and Houtian (1992), Acuner and Dilek (2004) mentioned the reduction of the azo bond as the decolourising mechanism. Mohan et al. (2002) attributed decolourisation to biosorption followed by bioconvertion and biocoagulation. Furthermore, they stated that the formed amines can be totally mineralized. Jinqi and Houtian (1992) also stated that some of the tested azo compounds could be used as sole sources of carbon and nitrogen by the algae. In open wastewater treatment systems, especially in (shallow) stabilization ponds, algae may, therefore, contribute to the removal of azo dyes and aromatic amines from the water phase.

12.7 Enzymatic Biodegradation

In the studies of biological degradation of dyes, an effort has been made in order to identify, isolate and test the enzymes responsible for the decolourisation. In the case of extracellular fungal enzymes, like manganese and lignin peroxidases and laccases or cytosolic azoreductases from bacteria, this has been achieved by several groups (Dass and Reddy 1990; Ghosh et al. 1992; Spadaro and Renganathan 1994; Heinfling et al. 1998; Rafii and Coleman 1999; Schliephake et al. 2000; Campos et al. 2001; Blümel and Stolz 2003; Ryan et al. 2003). The application of enzyme preparations shows considerable benefits over the use of microorganisms. Commercial preparations can be easily standardized, for accurate dosage. The application is simple and can be rapidly modified according to the character of the dye or dyes to be removed (Forgacs et al. 2004).

Nevertheless, the use of whole cells rather than isolated enzymes is advantageous, because costs of purification are extremely high and the cell offers protection from the harsh process environment to the enzymes. Also, degradation is often carried out by a number of enzymes working sequentially and not by one single enzyme (Pearce et al. 2003).

12.8 Biodegradation of Dyes by Combined Anaerobic–Aerobic Bioreactor

Under anaerobic conditions, azo dyes are readily cleaved via a four-electron reduction at the azo linkage generating aromatic amines (Brown and Laboureur 1983b; Brown and Hamburger 1987). Biodegradation of dyes has been the subject

of a large number of research activities and several review articles have been published on this aspect (Walker 1970; Levine 1991; Chung and Cerniglia 1992; Bumpus 1995; Banat et al. 1996; Delee et al. 1998; McMullan et al. 2001; Stolz 2001; Pearce et al. 2003; Forgacs et al. 2004). Especially, anaerobic azo dye reduction has been thoroughly investigated and most researchers agree that it is a non-specific and presumably extracellular process, in which reducing equivalents from either biological or chemical source are transferred to the dye.

The bacterial metabolism of azo dyes is initiated in most cases by a reductive cleavage of the azo bond, which results in the formation of colorless aromatic amines. Anaerobic reduction of the azo dyes is relatively easy to achieve, but the products have been found to be biorecalcitrant under anaerobic conditions (Stolz 2001). This observation coupled with the fact that many aromatic amines are completely degraded under aerobic conditions, has led to the conclusion that anaerobic–aerobic systems might be effective in achieving the complete biodegradation of azo dyes (Supaka et al. 2004).

Azo dyes are generally persistent under aerobic conditions (Isik and Sponza 2004a). However, under anaerobic conditions, they undergo facile reductive fission, yielding colorless aromatic amine compounds (Stolz 2001) which generally require aerobic conditions for their biodegradation (Ekici et al. 2001; Sponza and Isiki 2002). Azo dyes require an anaerobic and an aerobic phase for their complete biodegradation. Combined anaerobic and aerobic treatment is, therefore, the most logical strategy for the complete removal of azo dyes from wastewater.

The combined anaerobic and aerobic degradation has been studied for the conversion of azo dyes by numerous researchers (O'Neill et al. 2000; Rajaguru et al. 2000; Supaka et al. 2004). However, in most cases a clear evidence for a complete biodegradation was not found, mainly due to a lack of proof for mineralization of the aromatic amines. In most experiments, only the decolorization and organic load were measured while additional examination for the mineralization of the aromatic amines. Only few references are available in which there exists real proof of mineralization of an azo dye by a bacterial co-culture under sequential anaerobic/aerobic batch conditions (Albuquerque et al. 2005).

12.9 Factors Affecting on Microbial Degradation of Dyes

Due to the highly variable nature of biological treatment systems and especially textile effluents, there are a number of factors that may affect the biodegradation rate of dyes. The researchers have discussed various problems associated with dye biodegradation that may or may not be anticipated or remedied. Non-dye related parameters, such as temperature, pH, HRT, dissolved oxygen or nitrate concentrations, type and source of reduction equivalents, bacterial consortium, and cell permeability can all affect the biodegradation of dyes and textile effluents. Dye related parameters, such as class and type of dye (i.e. azo, triphenylmethane, reactive dyes), reduction metabolites, dye concentration, dye side-groups, and

organic dye additives could also affect the biodegradability of dye containing wastewaters.

The wastewater pH can affect the proper functioning of both anaerobic and aerobic organisms. Wuhrmann et al. (1980) also investigated the effect of pH on dye reduction rates, but was unable to conclusively establish a relationship. However, they stated that an exponential increase in the decolorization rate was observed by decreasing the pH, but this relationship depended on the dye being tested. Loyd (1992) observed an indirect increase in the rate of decolorization of Navy-106, with decreased pH values in the anaerobic batch tests. However, no statistical data was performed to verify this result.

12.9.1 Dye Structure

Different color removal efficiencies were investigated according to dye structure. Normally, colour of triphenylmethane and azo dyes was removed with high efficiency in synthetic wastewater treatment. In real wastewater treatment, the efficiency was slightly low because of presence of various unwanted compounds. The total mineralization of dyes was dependent on dye structure. If the reduced aromatic amines are unsulphonated, then aerobic biodegradation was found very efficient (Tan 2001).

12.9.2 Hydraulic Retention Time

The effect of Hydraulic Retention Time (HRT) was positively correlated to the degradation efficiency. The steady state results were obtained at a gradually rising HRT. The overall degradation of dye and co-substrate was enhanced, as HRT increased up to a certain level, but the enhancement became lower immediately, if the HRT increased at a high rate (Isik and Sponza 2004b; Albuquerque et al. 2005).

12.9.3 Biomass Concentration

Biomass concentration was measured in terms of volatile suspended solid (VSS). The sludge was quantified gravimetrically by weighing the oven-dried samples at 105°C for 24 h. Oven-dried solid samples were scrapped out from the supports and ignited at 550°C for 2 h to estimate the volatile solid (VSS) content. Several studies reported that when biomass concentration (g/l) decreases, solid retention time will decrease and total degradation efficiency falls (Lourenco et al. 2000). Systems with a higher biomass retention capacity (e.g. upflow anaerobic reactors)

might, therefore, be better suited for azo dye decolorization than systems with a lower biomass retention capacity (e.g. SBR systems). The higher Reactive Black 5 color removal efficiency of an UASB reactor (Sponza and Isik 2002) in comparison to those of the anaerobic phases of SBR systems, operated with similar dye concentrations and reaction times (Luangdilok and Panswad 2000; Panswad and Luangdilok 2000; Lourenco et al. 2001; Panswad et al. 2001).

12.9.4 Dye Concentration

Dye concentration is an another factor that may play a vital role in the color removal process. Hence, large variations in dye concentrations have been applied in the reactor studies. In several cases, the applied dye concentrations exceed the 10–250 mg/l range of normal concentrations in dye house effluents (O'Neill et al. 1999). High dye concentrations may negatively affect the anaerobic color removal efficiency, either by exceeding the reactor's biological azo dye reduction capacity or by causing toxicity to the anaerobic biomass. Investigations with different dye concentrations usually reported higher net color removal efficiencies at lower dye concentrations (Seshadri et al. 1994; Luangdilok and Paswad 2000; O'Neill et al. 2000; Rajaguru et al. 2000; Cruz and Buitron 2001; Kapdan and Oztekin 2003; Sponza and Isik 2005), even though the amount of dye reduced per unit of time may increase with increasing dye concentrations, e.g. as reported by Cruz and Buitron (2001).

12.9.5 Co-Metabolism and Inhibition

Biodegradation is defined as the biologically catalyzed redox reaction in complexity of chemical compounds (Alexander 1994). It is based on two processes, growth and co-metabolism. In case of growth, organic pollutants are used as sole carbon and energy source. This process results in complete degradation (mineralization) whereas in co-metabolism, the metabolism of an organic compound occurred in presence of a growth substrate which is used as the primary carbon and energy source (Alexander 1994). The importance of co-metabolism is not limited to the accumulation of biochemical products. Co-metabolism has also been used for the detection and demonstration of specific enzyme action. The accumulation of end products allowed the isolation and identification of metabolites and the determination of specific bonds of substituted which were cleaved by the co-metabolic process. A universally accepted definition of co-metabolism has proven elusive, but there is a general compromise that it describes the metabolism of a non-growth substrate in which no apparent benefit is accrued by the metabolizing organism (Wackett 1996).

Microbial aggregation into compact aerobic granules offers additional benefits, such as protection against inhibition and resistance to chemical toxicity (Jiang et al. 2004). Aerobic granules have high resistance to toxic compounds due to their compact structure (Bergsma-Vlami et al. 2005). Degradation efficiency with granules increases with an increase in substrate concentration up to a certain limit and then decreases. Performance of the reactor also decreases with further increase in substrate concentration due to inhibition of microbial growth by substrate concentration itself as reported by Kargi and Eker (2005). The flocs reported by Sahinkaya and Dilek (2007) have a high degradation rate at low concentration, but at high concentration they were also inhibited leading to a decrease in the degradation rate.

12.9.6 Dye Toxicity

Toxicity to microorganisms affecting the transformation is obviously an important factor governing the fate and behaviour of a chemical compound in the natural environment. Dye toxicity is defined as the % inhibition of microbial growth at a particular substrate concentration. However, genotoxicity is defined as the gene mutation, chromosomal alteration or DNA damage caused by the substrate. In case of azo dyes, polyazo dyes is most toxic than others due to steric, electronic and hydrodrophobic effects of the substituents.

12.10 Recent Advances

In recent years, the researchers have taken up various methods which can enhance the process of dye removal by wild-type of bioreactor or by using various isolated species. Combining compound analysis with toxicity measurements will deliver insight in the size, composition, and potential harm of the recalcitrant fraction, thereby providing the information needed to judge whether discharge can be allowed or whether tertiary treatment (e.g. advanced oxidation, adsorption, coagulation) will have to be applied.

Additionally the biodegradation of dyes is a process relevant to the treatment of wastewaters from textile industries which are frequently heavily coloured and resistant to conventional wastewater treatment processes. The design and operation of a continuous reactor with yeasts to study the effect of several parameters in its operation, namely pH, temperature and presence of salts and other textile additives, would undoubtly reveal more information about the system. This would allow the development of a pilot treatment plant to apply in a textile industry.

Another possible development of this work would be to go on exploring the capability of other yeasts as bioremediation agents of textile dyes, particularly the basidiomycetes isolated from contaminated soil in the beginning of these studies.

Also oxidative capabilities can be also explored which will be eventually useful for the degradation of other types of dyes.

12.11 Conclusion

Microbial biodegradation is a tool to convert the dyes to less hazardous/non-hazardous forms with less input of chemicals, energy, and time. It is an approach to degrade/remove pollutants in an eco-friendly manner. Over the last years, because of stringent legislation aiming at the protection of the environment, several systems for dye removal have been developed and explored. Dye containing wastewater can be remediated using the microorganisms—algae, bacteria, and fungi individually or in combination. In this chapter, although a large number of lab-scale studies have been described for decolourization of synthetic dye solutions through various biodegradation processes, there is a need to generate relative performance data on industrial effluents. According to the concept of biodegradation of azo dyes, combined anaerobic–aerobic treatment holds a high promise as a method to completely remove azo dyes from wastewater. However, the results of the reactor studies also reveal some limitations, both with respect to azo dye reduction and to the fate of aromatic amines. However, need to assess the extent of mineralization of aromatic amines, as many amines can undergo autoxidation, leading to the formation of soluble recalcitrant polymers, which may be highly toxic. Nevertheless, more research should also be conducted on the probable routes for the safe and eco-friendly way of handling the residues and by-products obtained after decolourization process.

Acknowledgments The authors are thankful to the Aligarh Muslim University, Aligarh (India) for providing research facilities and also thankful to the UGC (University Grants Commission) for providing financial assistance.

References

Acuner E, Dilek FB (2004) Treatment of tectilon yellow 2G by *Chlorella vulgaris*. Process Biochem 39:623–631

Albuquerque MGE, Lopes AT, Serralheiro ML, Novais JM, Pinheiro HM (2005) Biological sulphate reductionand redox mediator effects on azo dye decolourisation inanaerobic–aerobic sequencing batch reactors. Enz Microb Technol 36(5–6):790–799

Alexander M (1994) Biodegradation and bioremediation. Academic Press, San Diego

Azmi W, Sani RK, Banerjee UC (1998) Biodegradation of triphenylmethane dyes. Enzyme Microbiol Technol 22:185–191

Ball AS, Betts WB, McCarthy AJ (1989) Degradation of lignin-related compounds by actinomycetes. Appl Environ Microbiol 55:1642–1644

Banat IM, Nigam P, Singh D, Marchant R (1996) Microbial decolorization of textile-dye-containing effluents: a review. Biores Technol 58(3):217–227

Bergsma-Vlami M, Prins ME, Staats M, Raaijmakers JM (2005) Assessment of genotypic diversity of antibiotic-producing *Pseudomonas* species in the rhizosphere by denaturing gradient gel electrophoresis. Appl Environ Microbiol 71:993–1003

Beun JJ, Hendriks A, van Loosdrecht, Morgenroth MCM, Wilderer PAE, Heijnen JJ (1999) Aerobic granulation in a sequencing batch reactor. Water Res 33:2283–2290

Beydilli MI, Pavlostathis SG, Tincher WC (1998) Decolorization and toxicity screening of selected reactive azo dyes under methanogenic conditions. Water Sci Technol 38:225–232

Blümel S, Stolz A (2003) Cloning and characterization of the gene coding for the aerobic azoreductase from *Pigmentiphaga kullae* K24. Appl Microbiol Biotechnol 62:186–190

Brown D, Hamburger B (1987) The degradation of dyestuffs: Part Ill-investigations of their ultimative biodegradability. Chemosphere 16:1539–1553

Brown D, Laboureur P (1983a) The degradation of dyestuffs: Part I. Primary biodegradation under anaerobic conditions. Chemosphere 12(3):397–404

Brown D, Laboureur P (1983b) The aerobic biodegradability of primary aromatic amines. Chemosphere 12(3):405–414

Bumpus JA (1995) Microbial degradation of azo dyes. Prog Ind Microbiol 32:157–176

Campos R, Kandelbauer A, Robra KH, Cavaco-Paulo A, Gubitz GM (2001) Indigo degradation with purified laccases from *Trametes hirsuta* and *Sclerotium rolfsii*. J Biotechnol 89:131–139

Chinwetkitvanich S, Tuntoolvest M, Panswad T (2000) Anaerobic decolorization of reactive dyebath effluents by a two-stage UASB system with tapioca as a co-substarte. Water Res 34(8):2223–2232

Chung KT, Cerniglia CE (1992) Mutagenicity of azo dyes: structure-activity relationships. Mutat Res 277(3):201–220

Chung KT, Fulk GE, Egan M (1978) Reduction of azo dyes by intestinal anaerobes. Appl Environ Microbiol 35(3):558–562

Cruz A, Buitron G (2001) Biodegradation of Disperse Blue 79 using sequenced anaerobic/aerobic biofilters. Water Sci Technol 44(4):159–166

Dass SB, Reddy CA (1990) Characterization of extracellular peroxidases produced by acetate-buffered cultures of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. FEMS Microbiol Lett 57:221–224

DeFazio SAK, Lemley AT (1999) Electrochemical treatment of acid dye systems: sodium meta-bisulfite addition to the Andco system. J Environ Sci Heal A 34:217–240

Delee W, O'Neill C, Hawkes FR, Pinheiro HM (1998) Anaerobic treatment of textile effluents: a review. J Chem Technol Biotechnol 73(4):323–335

Dos Santos AB, Cervantes FJ, van Lier JB (2007) Review paper on current technologies for decolourisation of textile wastewaters: perspectives for anaerobic biotechnology. Biores Technol 98(12):2369–2385

Ekici P, Leupold G, Parlar H (2001) Degradability of selected azo dye metabolites in activated sludge systems. Chemosphere 44:721–728

Fewson CA (1988) Microbial metabolism of mandelate: a microsom of diversity. FEMS Microbiol Rev 54:85–110

Forgacs E, Cserhati T, Oros G (2004) Removal of synthetic dyes from wastewaters: a review. Environ Intl 30(7):953–971

Fu Y, Viraraghavan T (2001) Fungal decolorization of dye wastewaters: a review. Biores Technol 79:251–262

Fu L, Wen X, Xu L, Qian Y (2002) Removal of copper-phthalocyanine dye from wastewater by acclimated sludge under anaerobic or aerobic conditions. Process Biochem 37:1151–1156

Ganesh R (1992) Fate of azo dyes in sludges. Masters thesis, Virginia Polytechnic Institute and State University, pp 193

Ghosh DK, Mandal A, Chaudhuri J (1992) Purification and partial characterization of two azoreductases from *Shigella dysenteriae* type 1. FEMS Microbiol Lett 77:229–233

Glenn J, Gold MH (1983) Decolorization of several polymeric dyes by the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Appl Environ Technol 45:1741–1747

Hao OJ, Kim H, Chang PC (2000) Decolorization of wastewater. Crit Rev Environ Sci Technol 30(4):449–505

Heinfling A, Martinez MJ, Martinez AT, Bergbauer M, Szewzyk U (1998) Transformation of industrial dyes by manganese peroxidases from *Bjerkandera adusta* and *Pleurotus eryngii* in a manganese-independent reaction. Appl Environ Microbiol 64:2788–2793

Henderson AL, Schmitt TC, Heinze, TM, Cerniglia CE (1997) Reduction of malachite green to leucomalachite green by intestinal bacteria. Appl Environ Microbiol 63:4099–4101, http://www.epa.gov

Isik M, Sponza DT (2004a) Decolorization of azo dyes under batch anaerobic and sequential anaerobic/aerobic conditions. J Environ Sci Health. Part A—Toxic/Hazard Subst Environ Eng 39(4):1107–1127

Isik M, Sponza DT (2004b) Monitoring of toxicity and intermediates of C.I. Direct Black 38 azo dye through decolorization in an anaerobic/aerobic sequential reactor system. J Hazard Mater 114(1–3):29–39

Jank M, Koser H, Lucking F, Martienssen M, Wittchen S (1998) Decolorization and degradation of Erioglaucine (acid blue 9) dye in wastewater. Environ Technol 19:741–747

Jiang HL, Tay JH, Maszenan AM, Tay STL (2004) Bacterial diversity and function of aerobic granules engineered in a sequencing batch reactor for phenol degradation. Appl Environ Microbiol 70:6767–6775

Jinqi L, Houtian L (1992) Degradation of azo dyes by algae. Environ Pollut 75:273–278

Kapdan IK, Oztekin R (2003) Decolorization of textile dyestuff Reactive Orange 16 in fed-batch reactor under anaerobic condition. Enzyme Microb Technol 33(2–3):231–235

Kargi F, Eker S (2005) Removal of 2,4-dichlorophenol and toxicity from synthetic wastewater in a rotating perforated tube biofilm reactor. Process Biochem 40:2105–2111

Kirk JH, Bartlett PC (1987) Nonclinical *Pseudomonas aeruginosa* mastitis in a dairy herd. J Am Vet Med Assoc 184:671

Lettinga G, van Velsen AFM, Hobma SM, de Zeeuw W, Klapwifk A (1980) Use of upflow sludge blanket (USB) reactor concept for biological wastewater treatment, especially for anaerobic treatment. Biotech Bioeng 22:699–734

Lettinga G, Deman A, Van Der Last ARM, Wiegant W, Van Knippenberg K, Frijns J, Van Bueren JCL (1993) Anaerobic treatment of domestic sewage and wastewaters. Water Sci Technol 27(9):67–73

Levine WG (1991) Metabolism of azo dyes: implication for detoxification and activation. Drug Metab Rev 23(3–4):253–309

Liu J, Qian L, Wessells RJ, Bidet Y, Jagla K, Bodmer R (2006) Hedgehog and RAS pathways cooperate in the anterior–posterior specification and positioning of cardiac progenitor cells. Dev Biol 290(2):373–38

Lourenco ND, Novais JM, Pinheiro HM (2000) Reactive textile dye colour removal in a sequencing batch reactor. Water Sci Technol 42(5–6):321–328

Lourenco ND, Novais JM, Pinheiro HM (2001) Effect of some operational parameters on textile dye biodegradation in a sequential batch reactor. J Biotechnol 89(2–3):163–174

Loyd KC (1992) Anaerobic/Aerobic degradation of a textile dye wastewater. Masters Thesis, Virginia Polytechnic Institute and State University, p 184

Luangdilok W, Paswad T (2000) Effect of chemical structures of reactive dyes on color removal by an anaerobic–aerobic process. Water Sci Technol 42(3–4):377–382

McMullan G, Meehan C, Conneely A, Nirby N, Robinson T, Nigam P, Banat IM, Marchant R, Smyth WF (2001) Mini review: microbial decolourisation and degradation of textile dyes. Appl Microbiol Biotechnol 56(1–2):81–87

Meulenberg R, Rijnaarts HHM, Doddema HJ, Field JA (1997) Partially oxidized polycyclic aromatic hydrocarbons show an increased bioavailability and biodegradability. FEMS Microbiol Lett 152:45–49

Mohan SV, Roa CN, Prasad KK, Karthikeyan J (2002) Treatment of simulated reactive yellow 22 (Azo) dye effluents using *Spirogyra* species. Waste Manag 22:575–582

Nigam P, Banat IM, Singh D, Marchant R (1996) Microbial process for the decolorization of textile effluent containing azo, diazo and reactive dyes. Process Biochem 31:435–442

Nigam P, Armour G, Banat IM, Singh D, Marchant R (2000) Physical removal of textile dyes and solid state fermentation of dye-adsorbed agricultural residues. Biores Technol 72:219–226

O'Neill C, Hawkes FR, Hawkes DL, Lourenco ND, Pinheiro HM, Delee W (1999) Colour in textile effluents-sources, measurement, discharge consents and simulation: a review. J Chem Technol Biotechnol 74(11):1009–1018

O'Neill C, Hawkes FR, Hawkes DW, Esteves S, Wilcox SJ (2000) Anaerobic-aerobic biotreatment of simulated textile effluent containing varied ratios of starch and azo dye. Water Res 34(8):2355–2361

Pagga U, Brown D (1986) The degradation of dyestuffs: part II. Behaviour of dyestuffs in aerobic biodegradation tests. Chemosphere 15(4):479–491

Pagga UM, Taeger K (1994) Development of a method for adsorption of of dyestuffs on activated sludge. Water Res 28:1051–1057

Panswad T, Luangdilok W (2000) Decolorization of reactive dyes with different molecular structures under different environmental conditions. Water Res 34(17):4177–4184

Panswad T, Iamsamer K, Anotai J (2001) Decolorisation of azo-reactive dye by polyphosphate and glycogen-accumulating organisms in an anaerobic-aerobic sequencing batch reactor. Biores Technol 76:151–159

Pasti MB, Crawford DL (1990) Isolation of microorganisms able to reductively transform aromatic compounds and their relevance to coal liquefaction. Can J Microbiol 37:902–907

Pearce CI, Lloyd JR, Guthrie JT (2003) The removal of colour from textile wastewater using whole bacterial cells: a review. Dyes Pigm 58(3):179–196

Rafii F, Coleman T (1999) Cloning and expression in *Escherichia coli* of an azoreductase gene from *Clostridium perfringens* and comparison with azoreductase genes from other bacteria. J Basic Microbiol 39:29–35

Rajaguru P, Kalaiselvi K, Palanivel M, Subburam V (2000) Biodegradation of azo dyes in a sequential anaerobic-aerobic system. Appl Microbiol Biotechnol 54:268–273

Ramakrishna KR, Viraraghavan T (1997) Dye removal using low cost adsorbents. Water Sci Technol 36:189–196

Razo-Flores E, Luijten M, Donlon B, Lettinga G, Field J (1997) Biodegradation of selected azo dyes under methanogenic conditions. Water Sci Technol 36(6–7):65–72

Rieger PG, Meier HM, Gerle M, Vogt U, Groth T, Knackmuss HJ (2002) Xenobiotics in the environment: present and future strategies to obviate the problem of biological persistence. J Biotechnol 94:101–123

Robinson T, Chandran B, Nigam P (2001) Studies on the production of enzymes by white-rot fungi for the decolorization of textile dyes. Enzyme Microb Technol 29:575–579

Ryan U, Xiao L, Read C, Zhou L, Lal AA, Pavlasek I (2003) Identification of novel cryptosporidium genotypes from the Czech Republic. Appl Environ Microbiol 69(7):4302–4307

Sahinkaya E, Dilek FB (2007) Modelling chlorophenols degradation in a sequencing batch reactors with instantaneous feed-effect of 2,4-DCP presence on 4-CP degradation kinetics. Biodegradation 18:427–437

Sani RK, Banerjee UC (1999) Decolorization of triphenylmethane dyes and textile and dye-stuff effluent by *Kurthia* sp. Enzyme Microbiol Technol 24:433–437

Sarnaik S, Kanekar P (1999) Biodegradation of methyl violet by *Pseudomonas mendocina* MCM B-402. Appl Microbiol Biotechnol 52:251–254

Schliephake K, Mainwaring DE, Lonergan GT, Jones IK, Baker WL (2000) Transformation and degradation of the disazo dye Chicago Sky Blue by a purified laccase from *Pycnoporus cinnabarinus*. Enzyme Microbiol Technol 27:100–107

Seignez C, Adler N, Suard JC, Peringer P (1996) Aerobic and anaerobic biodegradability of 1-anthraquinone sulphonate. Appl Microbiol Biotechnol 45:719–722

Seshadri S, Bishop PL, Agha AM (1994) Anaerobic/aerobic treatment of selected azo dyes in wastewater. Waste Manag 14(2):127–137

Shaul GM, Holdsworth TJ, Dempsey CR, Dostal KA (1991) Fate of water soluble azo dyes in the activated sludge process. Chemosphere 22:107–119

Spadaro JT, Renganathan V (1994) Peroxidase-catalyzed oxidation of azo dyes: mechanism of disperse Yellow 3 degradation. Arch Biochem Biophys 312:301–307

Sponza DT, Isik M (2002) Decolorization and azo dye degradation by anaerobic/aerobic sequential process. Enzyme Microb Technol 31(1–2):102–110

Sponza DT, Isik M (2005) Reactor performances and fate of aromatic amines through decolorization of direct black 38 dye under anaerobic/aerobic sequentials. Process Biochem 40(1):35–44

Stolz A (2001) Basic and applied aspects in the microbial degradation of azo dyes. Appl Microbiol Biotechnol 56(1–2):69–80

Supaka N, Juntongjin K, Damronglerd S, Delia M-L, Strehaiano P (2004) Microbial decolorization of reactive azo dyes in a sequential anaerobic-aerobic system. Chem Eng J 99(2):169–176

Tan NCG (2001) Integrated and sequential anaerobic/aerobic biodegradation of azo dyes. PhD Thesis, Agro technology and Food Sciences, Sub-department of Environmental Technology, Wageningen University, Wageningen, The Netherlands

Wackett LP (1996) Co-metabolism: is the emperor wearing any clothes? Curr Opin Biotechnol 7:321–325

Walker R (1970) The metabolism of azo compounds: a review of the literature. Food Cosmetics Toxicol 8(6):659–676

Walker GM, Weatherley LR (2000) Biodegradation and biosorption of acid anthraquinone dye. Environ Pollut 108:219–223

Wuhrmann K, Mechsner K, Kappeler T (1980) Investigation on rate-determining factors in the microbial reduction of azo dyes. Europe J Appl Microbiol Biotechnol 9:325–338

Yatome C, Ogawa T, Matsui M (1991) Degradation of crystal violet by *Bacillus subtilis*. J Environ Sci Eng 26:75–88

Yatome C, Yamada S, Ogawa T, Matsui M (1993) Degradation of crystal violet by *Nocardia corallina*. Appl Microbiol Biotechnol 38:565–569

Zhang XY, Liu YX, Yan KL, Wu HJ (2007) Decolorization of anthraquinone-type dye by bilirubin oxidase-producing nonligninolytic fungus *Myrothecium* sp. IMER1. J Biosci Bioeng 104:104–110

Zhou WC, Zimmermann W (1993) Decolorization of industrial effluents containing reactive dyes by actinomycetes. FEMS Microbiol Lett 107:157–162

Zissi U, Lyberatos G (2000) Partial Degradation of p-aminoazobenzene by a defined mixed culture of *Bacillus subtilis* and *Stenotrophomonas maltophilia*. Biotechnol Bioeng 72(1):49–54

Zissi U, Lyberatos G, Pavlous S (1997) Biodegradation of p-aminobenzene by *Bacillus subtilis* under aerobic conditions. J Ind Microbiol Biotechnol 19:49–55

Chapter 13
Bacterial Degradation of Petroleum Hydrocarbons

A. Rajasekar, S. Maruthamuthu, Y. P. Ting, R. Balasubramanian and Pattanathu K. S. M. Rahman

13.1 Introduction

The petroleum refining industry is one of the largest manufacturing industries in the world. After Western Europe and the USA, Latin America has the highest petroleum refining capacity in the world. Huge investments are made each year on capital equipment, their modernization and maintenance, including prevention and treatment of microbial contamination. The major microbial problem in the petroleum industry is contamination of stored products which can lead to loss of product quality, formation of sludge and deterioration of pipework and storage tanks, both in the refinery and at the end-user (Groysman 2010; Rajasekar et al. 2010; Rajasekar and Ting 2010). Reports of such microbial contamination have increased substantially in recent years (Hill 1987; Hill and Hill 1993; Rajasekar

A. Rajasekar (✉) · R. Balasubramanian
Department of Civil and Environmental Engineering,
National University of Singapore, 1 Engineering Drive 2,
Singapore 117576, Singapore
e-mail: ceera@nus.edu.sg; rajasekar.aruliah@gmail.com

S. Maruthamuthu
Corrosion Protection Division, Biocorrosion Group,
Central Electrochemical Research Institute, Karaikudi 630 006, India

A. Rajasekar · Y. P. Ting
Department of Chemical and Biomolecular Engineering,
National University of Singapore, 4 Engineering Drive,
Singapore 117576, Singapore

P. K. S. M. Rahman (✉)
Chemical and Bioprocess Engineering Group,
School of Science and Engineering, Teesside University,
Middlesbrough, Tees Valley, TS13BA, UK
e-mail: p.rahman@tees.ac.uk

S. N. Singh (ed.), *Microbial Degradation of Xenobiotics*,
Environmental Science and Engineering, DOI: 10.1007/978-3-642-23789-8_13,

et al. 2008), probably due to increasing demand for diesel fuel and high quality gasolines and jet fuels (Batts and Fathomi 1991; Gaylarde et al. 1999). Obuekwe and Westlake (1987) reported the occurrence of bacteria in Pembina crude oil pipeline system and their potential role in corrosion process. Crude oil stream from the Pembina fields of North Central Alberta, Canada, contained a relatively high detectable load of bacteria. The oil and produced water contained aerobic and anaerobic microorganisms capable of producing sulphides from sulphates and sulphite, and ferrous ions from ferric compounds. The ability to produce sulphur and Fe(II) in solution is considered very important in corrosion phenomenon in the pipeline system. Apart from sulphate reducers (*Desulfovibrio* spp.), the organisms found in the crude oil system and capable of generating corrosive environment were mainly members of the Genus *Pseudomonas*. In the present chapter, the role of microbes in biodegradation of petroleum hydrocarbon in petroleum transporting pipelines and storage tanks has been extensively discussed for understanding of the microbes in fuel-water environment.

13.2 Water Contamination in Fuel Storage Tanks/Transporting Pipeline

Microorganisms may enter the fuel from the soil, air, polluted wash water, contaminated pipelines, or from the biofilm present on the tank walls, if the latter have not been sufficiently well cleaned (Rajasekar et al. 2010). Even in the best-kept tanks, microbial contamination is an occasional problem. Microorganisms are usually present in the fuel, but good housekeeping (removal of water and use of biocides) minimizes their growth. Nevertheless, reports of microbial growth in fuel tanks have increased in the last few years (Hill and Hill 1993; Rajasekar et al. 2007d) and the holding of strategic reserves for long periods has always been problematic (Fass et al. 1986; Groysman 2005). The most important requirement for microbial growth in fuels is water which is invariably present for the following reasons:

- water dissolved in the fuel can condense on tank walls,
- moisture in the air can enter through floating tank lids or other vents,
- poorly designed tanks do not drain efficiently, and
- water may be added as ballast (on ships) or to purge the delivery system.

In oil pipelines, water can also stratify at the bottom of the line in low lying areas because of topography (Fig. 13.1), if the velocity is less than that required to entrain water and sweep it through the pipeline system. Factors, such as nutrients (nitrite, phosphate, etc.), oxygen, chloride and bacteria are the causative factors for internal biodegradation in petroleum transporting pipeline. In a stagnant water situation, the availability of chloride, sulphate in contaminated water and microbes determine the biodegradation rate of internal pipeline and thus influence the corrosion process (Maruthamuthu et al. 2005; Rajasekar et al. 2005, 2007a, b, c, d, e).

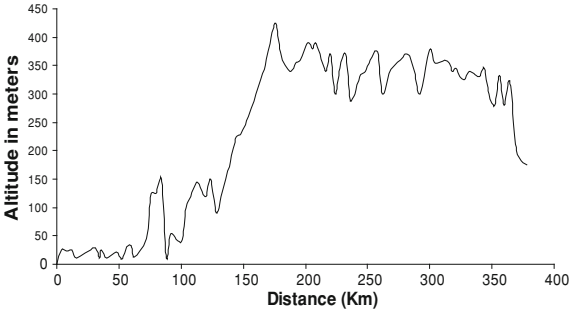


Fig. 13.1 Topography of a petroleum product transporting pipeline

The specification for diesel oil allows a maximum of 0.05% water (Bento et al. 2004). This is 0.5 ml/L—quite sufficient for the initial growth of microorganisms. Although Hill and Hill (1993) stated that 0.1% water is needed for the substantial microbial growth, a fine film of water on the tank surfaces or a few microliters water in the fuel is enough to allow microorganisms to begin growth, and cell metabolism, once begun, results in the production of more water. Thus, the cycle continues further.

Oxygen is normally present in sufficient quantities in distillate fuels (Hill 1987), and is continually replenished when tanks are refilled. However, even if the fuel becomes anaerobic, it is not protected from microbial attack, since facultative organisms, such as *Bacillus*, and anaerobes, such as sulfur-reducing bacteria (SRB), continue to thrive. The limiting factor to growth is probably availability of minerals, particularly phosphorus, which is generally present at <1 ppm in the fuel (Hill and Hill 1993). Nitrogen and iron may also be important limiting nutrients (Turner et al. 1983). Many laboratory-based studies have shown that fungi could grow much more readily in a fuel system containing mineral salts solution as the aqueous phase than in water or even tank drainage water (Bento and Gaylarde 1998). However, apart from minerals entering in water or aerial contaminants, many of the additives now used in the fuel industry contain these vital mineral elements, removing one of the factors that limited growth in earlier times. In diesel fuel, microbial contamination may contribute to aging instability (Litman 1980), but in general the most important consequences are microbially induced corrosion of the storage tanks and pipe work and formation of microbial mats, which may block filters and pipelines, and increase wear in pumps. According to Irish and Richardson (1981), as little as 1 mg particulates/100 ml fuel can cause filtration problems. In addition to microorganisms, these may include dirt, dust, sand, components of other filters, such as paper or cotton, pump wear particles, corrosion debris and material removed from tank or pipe linings, such as fiberglass (Table 13.1). Corrosion from within tanks and pipelines can be intense when microbial contamination is present.

Table 13.1 Consequences of microbial growth in fuel systems

Problems	Principal types of microorganisms
Blockage of pipes, valves, filters and incorrect readings from fuel probes	Fungi; polymer-producing bacteria
Increased water content	All
Sludge formation	All
Surfactant production, causing oil/water emulsification, entry of cells into the oil phase and coalescer malfunction	Fungi and aerobic bacteria
Corrosion of storage tanks and lines	Fungi and anaerobic bacteria
Production of suspended solids in the fuel	All
Breakdown of hydrocarbons	Fungi and aerobic bacteria
Shortened filter life	All
Fouling of injectors	Aerobic bacteria and fungi
Increased sulfur content of fuel	SRB
Shortened life of engine parts	Undetermined
Penetration of protective tank linings	Fungi
Heath problems	Endotoxin-producing bacteria, opportunistic pathogens, SRB

Sources Hill (1987), Hill and Hill (1993), Bento and Gaylarde (1996)

13.3 Role of Microbes in Petroleum Hydrocarbon Degradation

Twenty-one years after ZoBell's (1946) classic review, the super tanker Torrey Canyon sank in the English Channel. With this incident, attention of the scientific community was dramatically focused on problems of oil pollution. After this event, several studies were initiated to study the fate of oil pollution. Several studies were also initiated on the fate of petroleum in varied ecosystems. Biodegradation of petroleum products in natural ecosystem is no doubt a complex issue. The degradation of the hydrocarbon mixture depends on the nature of the oil, the nature of the microbial community, and a variety of environmental factors, which influence microbial activities (Gibson 1968; ZoBell 1973; Atlas 1981; Michaud et al. 2004). The ability to degrade petroleum hydrocarbons is not restricted to a few microbial genera (Whyte et al. 1998), but a diverse group of bacteria and fungi. ZoBell (1946), in his review, noted that more than 100 species representing 30 microbial genera had been shown capable of utilizing or degrading hydrocarbons. In a review, Bartha and Atlas (1977) also listed 22 genera of bacteria, one algal genus and 14 genera of fungi, which had been demonstrated to contain various members, utilize petroleum hydrocarbons. All of these microorganisms had been isolated from aquatic environments. The most important (based on frequency of isolation) genera of hydrocarbon utilizers in aquatic environments were *Pseudomonas* sp., *Achromobacter* sp., *Micrococcus* sp., *Nocardia* sp., *Candida* sp., *Vibrio* sp., *Acinetobacter* sp., *Brevibacterium* sp., *Rhodotorula* sp., *Sporobolomyces* sp., *Corynebacterium* sp., and *Flavobacterium* sp. (Bartha and

Atlas 1977). Walker and Colwell (1975) compared the abilities of bacteria and fungi in the degradation of hydrocarbons. The following genera were included in their study: *Candida* sp., *Sporobolomyces* sp., *Hansenula* sp., *Rhodotorula* sp., *Cladosporium* sp., *Penicillium* sp., *Aspergillius* sp., *Pseudomonas* sp., *Vibrio* sp., *Nocardia* sp. and *Rhobium* sp. Walker and Colwell (1976) isolated *Vibrio* sp., *Pseudomonas* sp. and *Acinatobacter* sp. from oil-contaminated sediments and *Pseudomonas* sp. and *Coryneform* sp. from oil free sediments. A large number of *Pseudomonas* sp. have been isolated that are capable of utilizing petroleum hydrocarbons. The genetics and enzymology of hydrocarbon degradation by *Pseudomonas* sp. have been extensively studied (Chakrabarty 1972; Chakrabarty et al. 1973; Dunn and Gunsalus 1973). The genetic information for hydrocarbon degradation in these organisms has been generally found to occur on plasmids. Numerical taxonomy has also been used to examine petroleum-degrading bacteria (Priest and Austin 1993). Recently, Rahman et al. (2002a) reported 130 bacterial cultures as crude oil degraders in Bombay high, India. Dela Fuente et al. (1991) studied the role of *Serratia marcescens* in oxidation of aromatic aldehydes into the corresponding acids in high yield under mild conditions. Verma et al. (2006) isolated *Bacillus* sp. SV9, *Acinetobacter* sp. SV17 and *Pseudomonas* sp. SV17 from the contaminated soil in Ankleshwar, India which were tested for their ability to degrade the complex mixture of petroleum hydrocarbons. This study revealed that after 5 days, the *Bacillus* strain was able to degrade oily sludge components of chain length of C_{12}–C_{30} and aromatics more effectively than the other two strains. Degradation of diesel oil by *Pseudomonas aeruginosa* strain WatG was verified in soil microcosms. This strain exhibited a high diesel oil (mainly n-alkanes) degradation ability in soil provided with rich nutrients and also it produced dirhamnolipid to facilitate the bioaugmentation ability (Wongsa et al. 2004). Degradation of polycyclic aromatic hydrocarbons by a newly discovered enteric bacterium *Leclercia adecarboxylata* was reported from oily sludge contaminated soil (Sarma et al. 2004). Bacterial consortium isolated from oil contaminated sites degraded alkanes in oil sludge and diesel contaminated soil (Rahman et al. 2002a, b, 2007, 2010). The introduction of 16S rRNA sequence analysis was heralded as the new era for bacterial systematics which could be used to rectify the mistakes in bacterial identification. Although numerical and chemotaxonomic methods were primarily employed to resolve these problems, 16S rRNA has been invaluable for the identification of evolutionary relationships not just in bacteria, but also in all living organisms. This technology was the first module examined for studying prokaryotic phylogenies, as a little was known about bacterial phylogeny earlier. One of the major successes was the reclassification of all life on this planet into three domains *Bacteria*, *Archae* and *Eucarya* (Woese et al. 1990). As a result of further work by Woese et al. (1990), the bacterial world is now divided into some 18 major lines of descent, referred to as phyla or divisions (Ludwig and Schleifer 1999). There are undoubtedly strong supports for 16S rRNA sequence analysis. Ludwig and Schleifer (1999) advocated the acceptance of 16S rRNA based phylogenetic trees as the "gold standard" which was supported by other workers

(Kimura 1980; Law et al. 1988; Weisburg et al. 1991; Larkin et al. 2007; Tamura et al. 2007).

The preliminary identification of bacteria by biochemical tests indicated that the isolates belonged to the genera *Bacillus* sp., *Pseudomonas* sp., *Kelebsiella* sp. and *Serratia* sp. The phenotypic profile of strains is shown in Tables 13.2 and 13.3. These bacteria were identified from the petroleum transporting pipeline.

Amplification of gene encoding for small subunit ribosomal RNA of DDB (diesel degrading bacteria) and NDB (naphtha degrading bacteria) was done using eubacterial 16S rDNA primers. The 16S rDNA amplicons derived from DDB and NDB was cloned in pTZ57R/T vectors. The recombinant plasmid (pACE2, harboring 16S rDNA insert) was partially sequenced. The sequence obtained was matched with the previously published sequences available in NCBI using BLAST. Sequence alignment of DDB and comparison revealed more than 99% similarity with *S. marcescens* ACE2, *Bacillus cereus* ACE4, *Bacillus subtilis* AR12, *B. cereus* AR14, *P. aeruginosa* AI1, *Klebsiella* sp. ACP, *Pseudomonas* sp. AP2, *Bacillus litoralis* AN1, *B. cereus* AN4 and *Bacillus* sp. AN5 and NDB comparison revealed more than 99% similarity with *S. marcescens* AR1, *Bacillus pumilus* AR2, *Bacillus carboniphilus* AR3, *Bacillus megaterium* AR4, and *B. cereus* AR5 has been deposited. The nucleotide sequences of 16S rDNA sequenced has been deposited in GenBank database under accession numbers DQ092416, AY912105, EF535590, EF535591, EF535592, EF535593, EF535594, EF535595, EF535596 and EF535597 (Rajasekar et al. 2010). Five naphtha degrading bacteria including representative strains of the two classified species *S. marcescens* AR1, *B. pumilus* AR2, *B. carboniphilus* AR3, *B. megaterium* AR4, and *B. cereus* AR5 were isolated and identified in a naphtha transporting pipeline. The 16S rDNA sequences are deposited in the gene bank with accession numbers DQ207558, DQ207559, DQ207560, DQ207561 and DQ207562.

The phylogenetic trees were constructed to find out the evolutionary interrelationship among various bacterial species present in the environment. The percentage distribution of individual species in diesel pipeline is presented in Fig. 13.2. 30% *B. cereus*, 10% of *S. marcescens*, 10% each species of *Bacillus substilis*, *Klebiella* sp., *B. litoralis*, *Bacillus* sp., *Pseudomonas* sp. and *P. aeruginosa.* Strains isolated from the corrosion product of naphtha transporting pipeline at South West India are presented in Fig. 13.3.

Sequence alignment and comparison revealed more than 99% similarity with *S. marcescens* AR1, *B. pumilus* AR2, *B. carboniphilus* AR3, *B. megaterium* AR4, *B. cereus* AR5, The nucleotides sequences data have been deposited in GenBank under the sequence numbers DQ207558, DQ207559, DQ207560, DQ207561 and DQ207562. The four isolates (AR2, AR3, AR4 and AR5) belong to the Bacillaceae family and one isolate AR1 to Enterbacteriacae. *Bacillus* sp. is the dominating genus in the pipeline, although all the five species distributed equally. Among the fifteen sequences in petroleum transporting pipeline, ten sequences were from Gram-positive bacteria. The dominant bacterial species in diesel and naphtha pipeline were identified as *B. cereus* and *S. marcescens.* All the bacterial strains exhibit catalase and oxidase positive. The naphtha and diesel degraders

Table 13.2 Biochemical characterization of isolates from diesel pipeline

Characteristics	ACE2	ACE4	ARI2	AR4	AI1	ACP	AP2	AN1	AN4	AN5
Cell morphology										
Gram stain	Negative	Positive	Positive	Positive	Positive	Negative	Positive	Positive	Positive	Positive
Shape	Rod	Rod	Rod	Rod	Rod	Small rod	Rod	Rod	Rod	Rod
Motility	+	+	+	+	+	+	+	+	+	+
Sporulation	−	+	+	+	+	−	+	+	+	+
Growth at										
20°C	+	+	+	+	+	+	+	+	+	+
30°C	+	+	+	+	+	+	+	+	+	+
40°C	+	+	+	+	+	+	+	+	+	+
Hydrocarbon utilization										
Diesel	+	+	+	+	+	+	+	+	+	+
Biochemical reaction										
Indole production test	−	+	−	−	−	−	+	+	−	+
Methyl red test	−	−	+	+	+	−	−	−	+	+
Voges Proskauer test	+	−	−	−	−	+	−	−	−	−
Citrate utilization test	+	−	+	+	+	+	−	+	+	−
Oxidase test	+	+	+	+	+	+	+	+	+	+
Catalase test	+	+	+	+	+	+	+	+	+	+
Production of acid from										
Glucose	+	+	+	+	+	+	+	+	+	+
Galactose	−	−	+	+	−	+	−	−	−	−
Fructose	+	+	+	+	+	+	+	+	+	+
Sucrose	−	+	−	−	−	−	+	−	−	−
Mannitol	−	−	−	−	−	+	−	−	+	−
Lactose	−	−	+	+	+	−	−	+	+	+
Cellobiose	−	−	+	+	+	−	−	+	+	+
Adonitol	−	−	−	−	−	+	−	−	−	−

(continued)

Table 13.2 (continued)

Characteristics	ACE2	ACE4	ARI2	AR4	AI1	ACP	AP2	AN1	AN4	AN5
Arabinose	−	+	−	−	+	−	+	−	−	+
Raffinose	−	−	−	−	−	+	−	−	+	−
Inositol	−	−	−	−	−	+	−	+	−	−
Hydrolysis of										
Starch	−	+	+	+	+	+	+	+	+	+
Cellulose	−	−	−	−	−	+	+	−	+	−
Casein	−	+	−	−	−	+	+	+	−	+
Gelatin	−	+	+	+	+	−	−	+	+	+
Urea	−	+	−	−	−	−	+	−	−	−
Tween 80	+	+	−	−	−	+	+	+	+	+

ACE2—*Serratia marcescens*, ACE4—*Bacillus cereus*, AR12—*Bacillus subtilis*, AR14—*Bacillus cereus*, AP2—*Pseudomonas stutzeri*, ACP—*Klebsiella oxytoca*, AI1—*Pseudomonas aeruginosa*, AN1—*Bacillus litoralis*, AN4—*Bacillus cereus* and AN5—*Bacillus* sp.

Table 13.3 Partial biochemical characterization of isolates from naphtha pipeline

Characteristics	AR1	AR2	AR3	AR4	AR5
Cell morphology					
Gram stain	Negative	Positive	Positive	Positive	Positive
Shape	Rod	Rod	Rod	Rod	Rod
Motility	+	+	+	+	+
Sporulation	−	+	+	+	+
Growth at					
20°C	+	+	+	+	+
30°C	+	+	+	+	+
40°C	+	+	+	+	+
Hydrocarbon utilization					
Diesel	+	+	+	+	+
Biochemical reaction					
Indole production test	−	+	−	−	−
Methyl red test	−	−	+	−	+
Voges Proskauer test	+	−	−	+	−
Citrate utilization test	+	−	+	−	−
Oxidase test	+	+	+	+	+
Catalase test	+	+	+	+	+
Production of acid from					
Glucose	+	+	+	−	+
Fructose	+	+	+	+	+
Sucrose	+	+	−	+	−
Mannitol	−	−	−	−	−
Lactose	−	−	+	+	−
Cellobiose	−	−	+	+	−
Adonitol	+	+	+	+	+
Arabinose	+	+	+	+	+
Raffinose	+	+	+	+	+
Inositol	−	−	−	−	−
Hydrolysis of					
Starch	−	+	+	+	−
Cellulose	−	−	−	+	+
Casein	−	+	−	+	+
Gelatin	−	+	+	+	+
Urea	−	+	−	+	+
Tween 80	+	+	−	+	+

AR1—*Serratia marcescens*, AR2—*Bacillus pumilus*, AR3—*Bacillus carboniphilus*, AR4—*Bacillus megaterium*, AR5—*Bacillus cereus*

were able to growth at 20, 30 and 40°C. Most of the Gram-positive species identified are clustered while Gram-negative species, such as *Pseudomonas* sp. and *Klebsiella* sp. are grouped away from the former species (Fig. 13.4).

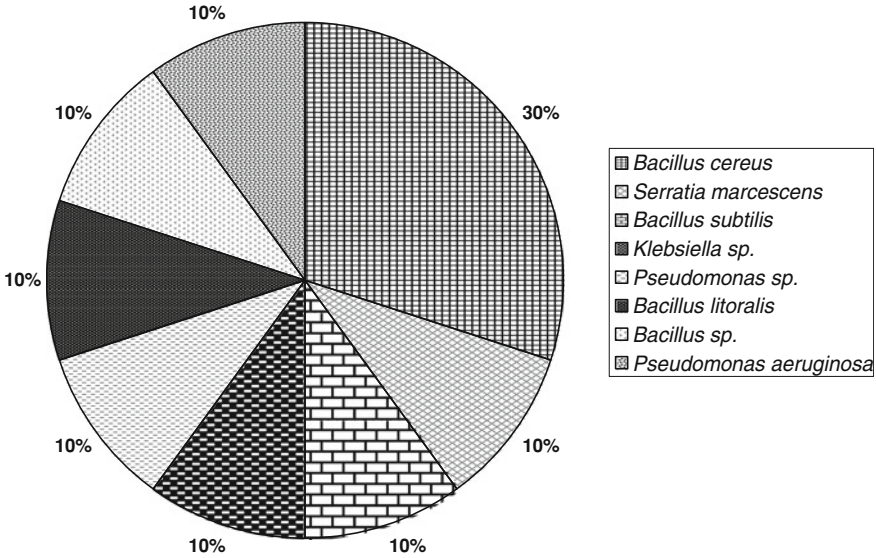


Fig. 13.2 Distribution of diesel degrading bacteria isolated from diesel pipeline

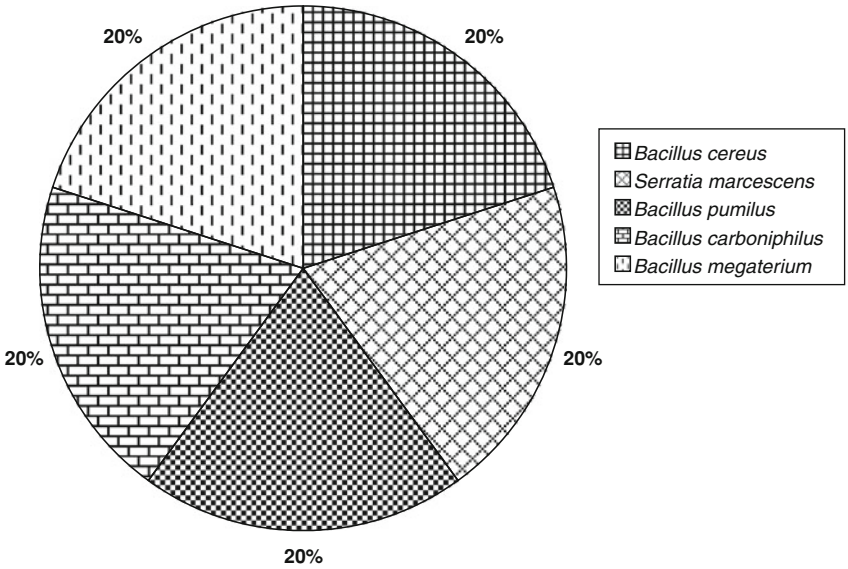


Fig. 13.3 Distribution of naphtha degrading bacteria isolated from naphtha pipeline

The similarity and species identified with the phylogenetic analysis are given in Table 13.3. In the *Bacillus* genera tree, most of the isolates belonging to *Bacillus* genus exhibited a high nucleotide sequence similarity with *B. cereus* species

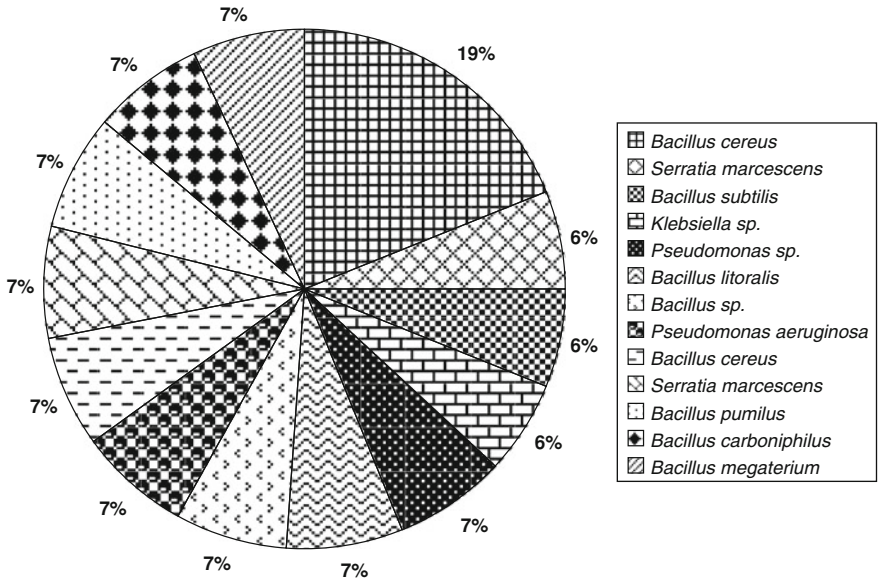


Fig. 13.4 Distribution of diesel/naphtha degrading bacteria isolated from diesel and naphtha pipeline

(99.5%). Similarly, in the tree corresponding to the *Bacillus* genus, most isolates were related to *B. subtilis*, *B. pumilus*, *B. carboniphilus*, *B. litoralis* and *B. megaterium* with high similarity values (98%). In the Classes Enterobacteriales, two isolates of the genus *Serratia* exhibited high sequence similarity with *S. marcescens* (99%) and one isolate of *Klebsiella* genus (*Klebsiella oxytoca*). Two isolates of genera *Pseudomonas* had high similarities with *P. aeruginosa* and *Pseudomonas stutzeri* (98%) in the Pseudomonadales tree. Phylogenetic relationship was analyzed between the 16S rDNA sequences of the bacterial phyla Firmicutes (Fig. 13.5) and Proteobacteria in the three phylogenetic trees (Figs. 13.6, 13.7). The phylogenetic analysis of the isolates showed the dominance of *Bacillus* sp., while *B. cereus* could be seen in dominant species among other species of genus *Bacillus*. *Serratia* sp., *Pseudomonas* sp. and *Klebsiella* sp. were present among phylum Gamma proteobacteria (Table 13.4).

The ability of enteric bacterium, *S. marcescens* to degrade hydrocarbon appears to be an unexpected finding since this feature has always been associated with typical soil bacteria. Besides, enteric bacterium is mainly regarded as an inhabitant of animal guts (Holt et al. 1994). Present understanding of role of hydrocarbon degradation on corrosion by enteric bacterium is still in its infancy. This study provides a new insight into the influence of degradation of diesel fuel on corrosion by enteric bacteria. Such studies can help in adopting efficient and predictable bioremediation strategies. However, only a few reports

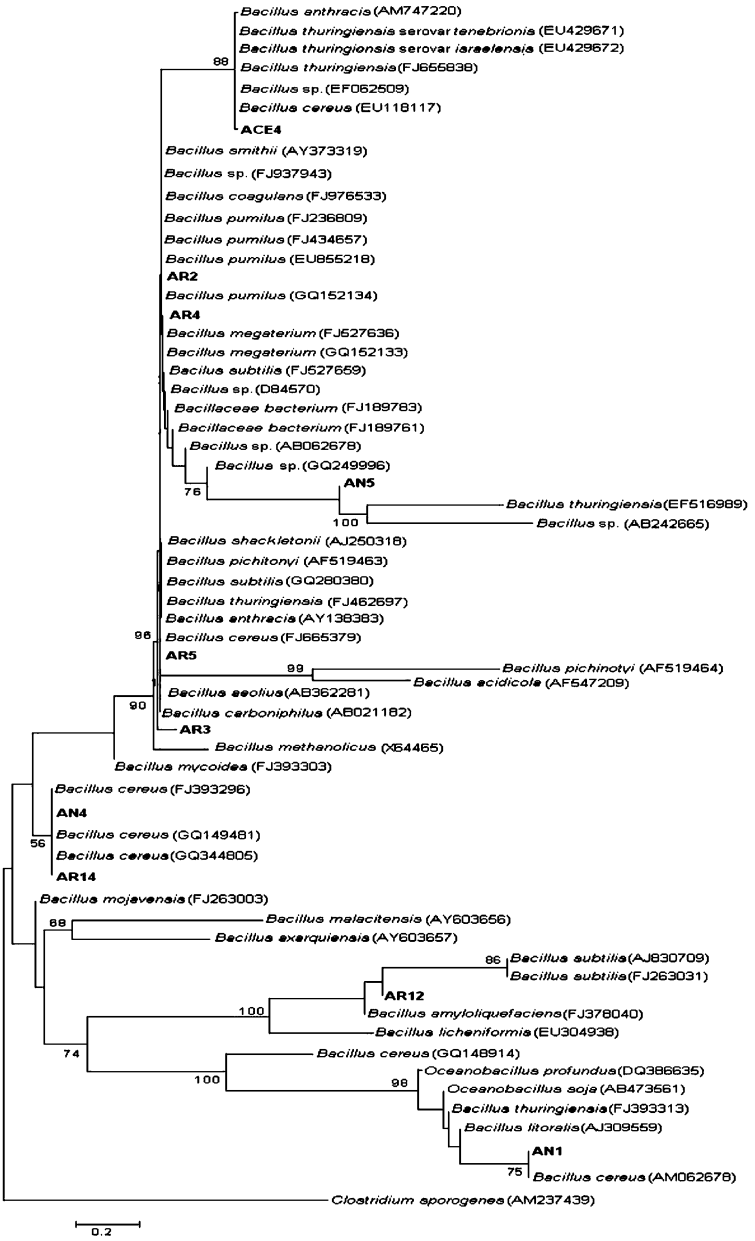


Fig. 13.5 Neighbor-joining tree based on 16S rRNA gene sequences, showing phylogenetic relationships between sequences of the bacterial phylum Firmicutes (*Bacillus* related species). *Clostridium sporogenes* was used as a bacterial outgroup. Numbers at nodes indicate bootstrap values >50% from 1000 replicates. GenBank accession numbers are given in parentheses. The scale bar indicate sequence divergence. ACE4—*Bacillus cereus*, AR2—*Bacillus pumilus*, AR4—*Bacillus megaterium*, AN5—*Bacillus* sp., AR5—*Bacillus cereus*, AR3—*Bacillus carboniphilus*, AN4—*Bacillus megaterium*, AR14—*Bacillus cereus*, AR12—*Bacillus subtilis*, AN1—*Bacillus litoralis*

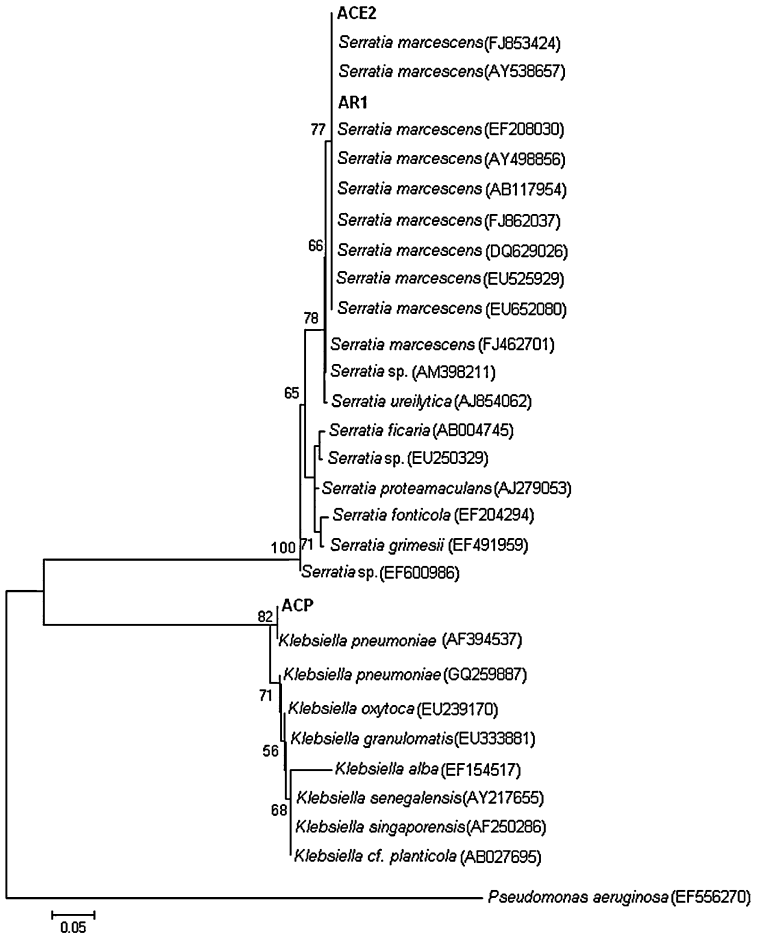


Fig. 13.6 Neighbor-joining tree based on 16S rRNA gene sequences, showing phylogenetic relationships between sequences of the Order Enterobacteriales (phylum Proteobacteria). *Pseudomonas aeruginosa* was used as the out group sequence. Numbers at nodes indicate bootstrap values >50% from 1000 replicates. GenBank accession numbers are given in parentheses. The scale bar indicate sequence divergence. AR1—*Serratia marcescens,* ACE2—*Serratia marcescens*, ACP—*Klebsiella oxytoca*

address utilization of aromatic compounds by *Enterobacteria*, particularly those of the genera *Klebsiella*, *Escherichia* and *Hafnia* (Grant 1967; Ijah 1998; Diaz et al. 2001). Although there are several reports of bioremediation of high-molecular-weight polyaromatic hydrocarbons (PAHs), research findings pertaining to biodegradation of these substances by enteric bacteria has been relatively rare (Diaz et al. 2001; Kanaly and Harayama 2000). Sarma et al. (2004) discovered a new enteric bacterium *L. adecarboxylata* PS 4040 in oil sludge-contaminated soil and reported the degradation of PAH at the Digboi Oil Refinery in Northeastern India.

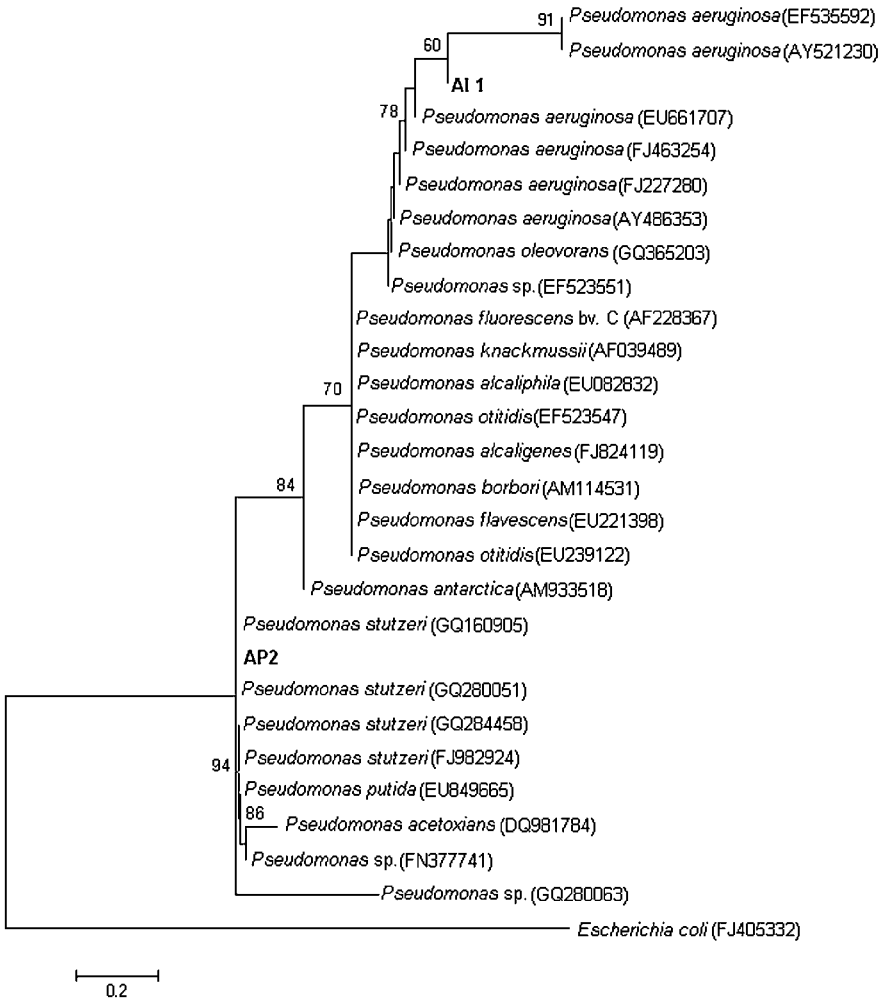


Fig. 13.7 Neighbor-joining tree based on 16S rRNA gene sequences, showing phylogenetic relationships between sequences of the Order Pseudomonadales (phylum Proteobacteria). *Escherichia coli* was used as the out group sequence. Numbers at nodes indicate bootstrap values >50% from 1000 replicates. GenBank accession numbers are given in parentheses. The scale bar indicate sequence divergence. AI1—*Pseudomonas aeruginosa*, AP2—*Pseudomonas stutzeri*

Recently, Wongsa et al. (2004) identified *S. marcescens* which has the ability to degrade the broad spectrum of hydrocarbons present in crude oil-contaminated soil. Muthukumar et al. (2003a, b) reported that *Brucella* sp. and *Gallionella* sp. could degrade diesel in a transporting pipeline in Northwest India, while Rajasekar et al. (2005) detected bacterial genera *Pseudomonas* sp., *Bacillus* sp., *Gallionella* sp., *Siderocapsa* sp., *Thiobacillus* sp., *Thiospira* sp., *Sulfolobus* sp. and *Vibrio* sp. in a naphtha pipeline, and reported on

Table 13.4 Cultivable bacterial isolates associated with biodegradation in petroleum product pipeline

S. no.	Strain	GenBank accession number	Best match
Diesel degrading bacteria			
1.	ACE2	DQ092416	*Serratia marcescens*
2.	ACE4	AY912105	*Bacillus cereus*
3.	AR12	EF535590	*Bacillus subtilis*
4.	AR14	EF535591	*Bacillus cereus*
5.	AI1	EF535592	*Pseudomonas aeruginosa*
6.	ACP	EF535593	*Klebsiella* sp.
7.	AP2	EF535594	*Pseudomonas* sp.
8.	AN1	EF535595	*Bacillus litoralis*
9.	AN4	EF535596	*Bacillus cereus*
10.	AN5	EF535597	*Bacillus* sp.
Naphtha degrading bacteria			
11.	AR1	DQ207558	*Serratia marcescens*
12.	AR2	DQ207559	*Bacillus pumilus*
13.	AR3	DQ207560	*Bacillus carboniphilus*
14.	AR4	DQ207561	*Bacillus megaterium*
15.	AR5	DQ207562	*Bacillus cereus*

interactions between heterotrophs and chemolithotrophs in naphtha-transporting pipelines Southwest India. Hence, an investigation on the identification of bacteria in oil transporting pipelines is worthwhile for petroleum degradation control.

13.4 Biodegradation of Petroleum Hydrocarbons in Petroleum Transporting/Storage Tanks

Biodegradation of diesel hydrocarbon by *B. cereus* ACE4 and *S. marcescens* ACE2 was confirmed by GC–MS, NMR and FT-IR analyses in petroleum transporting pipeline, India (Rajasekar et al. 2007b, e). From GC–MS analysis, it was observed that the control system consisted of *n*-alkanes (C_{10}–C_{20}), branched alkanes, naphthalene derivatives, substituted naphthalenes and iso preniod alkanes (pristane, phytane) (Fig. 13.8a). The inoculated *S. marcescens* ACE2 degraded almost all the *n*-alkanes (C_{10}–C_{20}) and many of the branched alkanes found in diesel (Fig. 13.8b). Inoculation of *B. cereus* ACE4 degraded more n-alkanes (C_{10}–C_{20}) (Fig. 13.8c) and many of the branched alkanes found in diesel when compared to ACE2 (Fig. 13.8b). The biodegradation efficiency of diesel hydrocarbon by the strains ACE2 and ACE4 is presented in Table 13.5. Bacteria ACE2 and ACE4 showed 69 and 77% biodegradation efficiency, respectively. The GC–MS analysis suggested that ACE2 and ACE4 degrade all the hydrocarbon present in diesel (C_{10} and C_{22}). Moderately degraded branched alkanes and naphthalene derivatives could be found in the degraded products, which is a

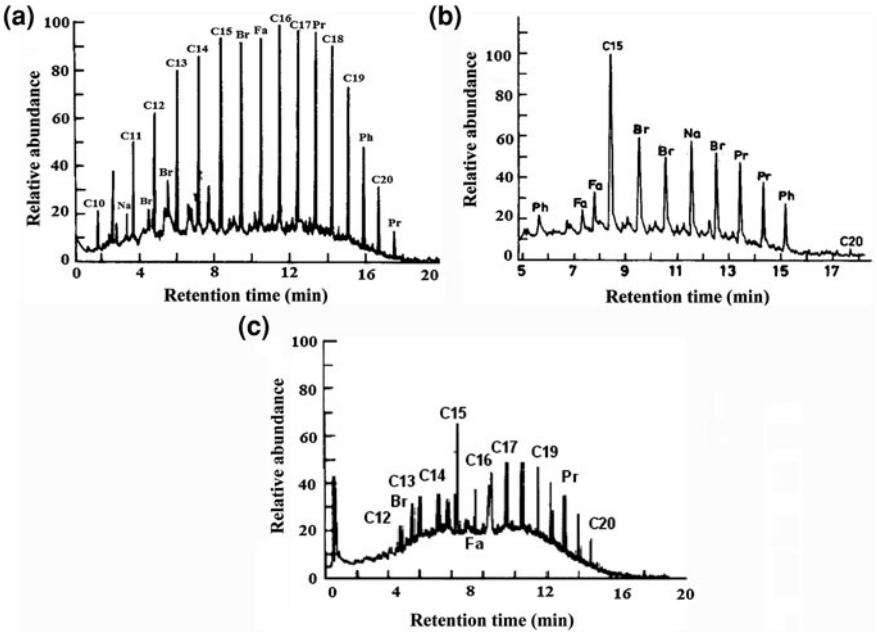


Fig. 13.8 **a** GC–MS profiles of abiotic control (uninoculated) C10 to C21, *n*-alkanes (numbers designate the number of C atoms); Br, branched alkanes; Na, substituted naphthalenes; Pr, pristane; Ph, phytane. The alkane, naphthalene, phytane, and pristane peaks were identified by comparison of their retention times and mass spectra with authentic standards. **b** GC–MS of diesel inoculated with *S. marcescens* ACE2. **c** GC–MS of diesel inoculated with *B. cereus* ACE4

common feature of many other alkane-degrading microorganisms (Van Beilen et al. 1994; Whyte et al. 1998; Wongsa et al. 2004).

In Fig. 13.9a, the FTIR spectrum of the control system (pure diesel) shows bands at 2904, 2923 and 2853 cm^{-1} (C–H aliphatic stretch); 1607 cm^{-1} (C=C stretch in aromatic nuclei); 1458 and 1375 cm^{-1} (CH def for methyl group), 808 and 726 cm^{-1} (disubstituted benzene). The FTIR spectrum for diesel in presence of *S. marcescens* (Fig. 13.9b) shows characteristic bands at 3331 cm^{-1} (OH peak); 2905 and 2853 cm^{-1} (C–H aliphatic stretch); 1647 cm^{-1} (C=C stretch); 1458 and 1375 cm^{-1} (CH aliphatic stretch for methyl groups). A new peak at 1647 cm^{-1} indicates carbonyl group (C=C) stretch and OH peak at 3331 cm^{-1}. Peaks due to aromatic nuclei at 847, 809, 872, 722, and 743 cm^{-1} (mono and di-substituted benzene) disappeared because of degradation. The FTIR spectra of experimental system (inoculated with ACE4) shows the characteristic bands (Fig. 13.9c) at 2853 and 2904 cm^{-1} (C–H aliphatic stretch); free OH peaks at 3315, 1632 cm^{-1} (C=C stretch); 1461 and 1376 cm^{-1} (CH aliphatic stretch for methyl groups). A new peak at 1632 cm^{-1} appeared which indicates a carbonyl group (C=O) stretch (Fig. 13.9b, c). The absence of aromatic nuclei peaks at 847, 809, 873, 722 and 743 cm^{-1} (Mono and di substituted benzene) indicates the utilization of aromatic

Table 13.5 Percentage of biodegradation efficiency of diesel degradation by *S. marcescens* ACE2/*B. cereus* ACE4

Retention time (time)	Compounds	Relative abundance (RA)		Biodegradation efficiency BE (%)	Strain ACE4	
		Control	Strain ACE2		Relative abundance (RA)	Biodegradation efficiency BE (%)
2	C 10	21	0	100	0	100
3	C 11	38	0	100	0	100
5.1	C 12	61	20	67.21	0	100
6	C 13	80	36	55	0	100
7.1	C 14	83	38	54	0	100
8.2	C 15	97	64	34	100	100
11.8	C 16	100	45	55	0	100
12.6	C 17	99	46	53	0	100
14.5	C 18	95	0	100	0	100
15.1	C 19	78	38	51	0	100
16.5	C 20	38	24	27	0	100
3.7	Napthalene derivatives Compound—I	18	0	100	0	100
4	Napthalene derivatives Compound—II	50	48	4	0	100
5.8	Branched alkanes Compound—I	39	0	100	37	5
9.5	Branched alkanes Compound—II	100	39	61	45	65
16	Phytane Compound—I	45	0	100	35	22
13.1	Pristine Compound—I	98	43	56	40	59
18.1	Pristine Compound—II	14	0	100	12	14
11.5	Farnithane Compound—I	98	38	61	35	64
7.6	Farnithane Compound—I	30	0	100	28	7
BE (Total percentage)				69		77

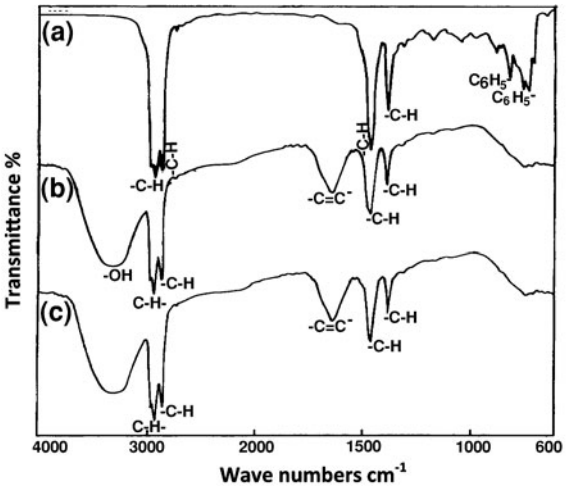


Fig. 13.9 FTIR spectrum of diesel and diesel inoculated with ACE and ACE4. **a** FTIR spectrum of diesel, **b** FTIR spectrum of diesel inoculated with ACE2, **c** FTIR spectrum of diesel inoculated with ACE4

hydrocarbon. The intensity of C–H aliphatic stretches at 2954, 2923 and 2854 cm^{-1} decreased which is indicative of the aliphatic hydrocarbon degradation (Fig. 13.9b, c).

The NMR spectrum (Fig. 13.10a) of the control system shows some major peaks at 0–3 chemical shifts (δ), suggesting the presence of aliphatic protons. The other peaks at 6–7 chemical shifts indicate the presence of benzene ring. In the presence of *S. marcescens* ACE2 and *B. cereus* ACE4 (Fig. 13.10b, c), the new peaks obtained at 4–5 δ indicate the presence of (–O–CH_2–) after the degradation by bacterial strains. Aliphatic proton (0–3 chemical shifts (δ) peaks and benzene peaks [6–7 chemical shifts (δ)] disappeared because of biodegradation. It reveals inclusion of oxygen with carbon atoms and it can be explained that aliphatic hydrogen (–CH_2–CH_2–) was consumed by degrading bacteria and that the carbon–hydrogen bond was cleaved into carbon–oxygen bond (–O–CH_2–). From this, it is evident that aliphatic protons (CH_2–CH_2) are completely degraded by bacterial species, and converted as (–O–CH_2) group. This confirms that this strain is involved in the utilization of both aliphatic and aromatic hydrocarbons present in the diesel. The ACE2 and ACE4 degrade both aliphatic and aromatic hydrocarbon present in the diesel. FTIR reveals the absence of aromatic nuclei peaks at 847, 809, 872, 722 and 743 cm^{-1} (Mono and di substituted benzene) during degradation process (Fig. 13.9b, c). GC–MS results reveal that bacteria ACE2 and ACE4 utilized almost all the n-alkanes (–CH_2–CH_2–) and moderately attacked the branched alkanes including phytane, pristane and naphthalene derivatives which is a common feature of many other hydrocarbon degraders (Whyte et al. 1998; Van Beilen et al. 1994). It was reported that benzene is converted to catechol (an orthohydroxy phenol) and fission occurs between the carbon atoms being two hydroxyl groups to form cis–cis-muconic acid which is then converted into 3-oxoadipic acid via the action of a catechol 1, 2-oxygenase (Traxler and Falnnery 1968). During degradation, n-alkanes were converted into (–O–CH_2–) which was

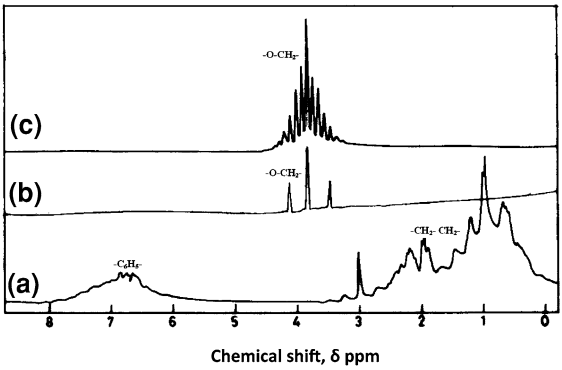


Fig. 13.10 NMR spectrum of diesel and diesel inoculated with ACE and ACE4. **a** NMR spectrum of diesel, **b** NMR spectrum of diesel inoculated with ACE2, **c** NMR spectrum of diesel inoculated with ACE4

identified in NMR studies (Fig. 13.10b, c). The conversion was due to hydrogen consumption and the very rapid addition of oxygen in the place of hydrogen by bacterial metabolic activity (Muthukumar et al. 2003b; Rajasekar et al. 2005). Overall, the bacterium is capable of utilizing a broad range of alkanes and aliphatic components present in diesel.

Total hydrocarbon in the presence and absence of bacteria is presented in Fig. 13.11a. The total hydrocarbon at the initial stage was about 30 mg/ml in absence of bacteria. After degradation by ACE2, the total hydrocarbon was 10 mg/ml, while after adding ACE4, the total hydrocarbon was 8.7 mg/ml. This may be due to the presence of higher activity of arylhydrocarbon hydroxylase (AHH) in ACE4 and further it indicates that the activity of ACE4 on degradation of diesel is more vigorous than ACE2. The activities of aryl hydrocarbon hydroxylase (AHH), during biodegradation of diesel are presented in Fig. 13.11b.

During biodegradation, in the beginning, the activity of AHH in ACE2 was measured as 11.38 p mole/min. After 20 days of bacterial inoculation in diesel, the quantity was totally decreased to below the detection limit (<0.001 p mole/min). Similarly, in ACE4 the activity of AHH is 15.91 p moles/min and the quantity was decreased after 20 days diesel incubation (<0.001 p moles/min). It indicates that at the initial stage, the inoculated bacteria may produce large quantity or detectable level of AHH enzyme. The *Bacillus* species mediated conversion of PAH with 3–5 rings (acenaphthene, fluoranthene, pyrene, benzo(e)pyrene) was demonstrated earlier by Feitkenhauer et al. (2003). Aitken et al. (1998) also observed mineralization of benzo(a)pyrene (PAH) by different type of bacterial strains. Since diesel is the major organic nutrient source for bacteria, it consumes the carbon from diesel with the help of hydrocarbon metabolizing enzymes. Thus, it shows a large quantity of monooxygenase enzymes, such as aryl hydroxylase for breaking the diesel for their metabolic activity at the initial stage. After degradation, the availability of simple molecule in diesel or metabolites of diesel hydrocarbons may inhibit aryl hydrocarbon hydroxylase activity and act as substrate inhibitor (Gerbal-Chaloin et al. 2006; Shimada and Guengerich 2006). In addition, with this enzyme assay, we measured the content of total hydrocarbons in the diesel at

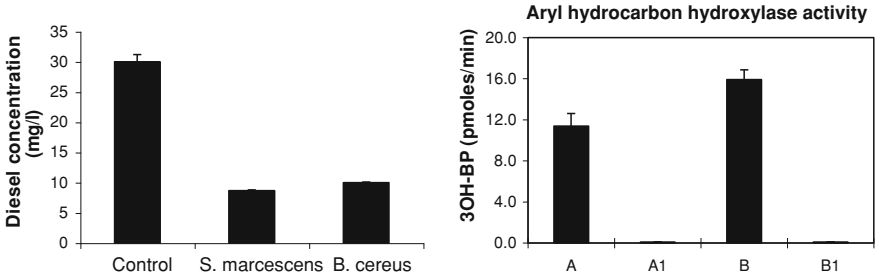


Fig. 13.11 **a** Total petroleum hydrocarbon in presence/absence of bacteria *S. marcescens* ACE2 and *B. cereus* ACE4. **b** Aryl hydrocarbon hydroxylase activity of bacteria ACE2 and ACE4, Enzyme Unit = 3OH-B(a)P pmoles/min. (*A*) Initial period of biodegradation (ACE2), (*B*) initial period of biodegradation (ACE4), (*A1*) after 20 days of biodegradation (ACE2), (*B1*) after 20 days of biodegradation (ACE2)

beginning stage and after 20 days of incubation (with two different bacterial cultures) period. It has been clearly observed that during the biodegradation experiment the content of the total hydrocarbon was completely decreased or metabolized by AHH enzymes on 20th day (Fig. 13.11b).

13.5 Factors Influencing in Petroleum Hydrocarbon Degradation

13.5.1 Nitrogen and phosphorus

There is an ambiguity regarding the limitation of petroleum biodegradation by available concentrations of nitrogen and phosphorus. Lepetit and Barthelemy (1968) reported that the concentration of available nitrogen and phosphorus in water are severe limiting factors for microbial hydrocarbon degradation. However, Kinney (1968) is of the opinion that nitrogen and phosphorus are not the limiting factors, since microorganisms require nitrogen and phosphorus for their incorporation into biomass and hence the availability of these nutrients is critical as that of hydrocarbon. Colwell et al. (1978) concluded that major oil was degraded slowly in the marine environment probably because of low nitrogen and phosphorus availability. The cultures studies have indicated that the available concentrations of nitrogen and phosphorus severely limit the extent of hydrocarbon degradation in the most major oil spills. Rates of nutrient replenishment generally are inadequate to support rapid biodegradation of large quantities of oil. The addition of nitrogen and phosphorus containing fertilizers can be used to stimulate microbial degradation of hydrocarbon.

13.5.2 Oxygen

Like nutrients, there has been controversy over whether oxygen is absolutely required for hydrocarbon degradation. In this regard, Grishchenkov et al. (2000), Zinjarde and Pant (2002) support the view that anaerobic degradation by microorganisms proceeds at negligible rates in nature. However, the existence of microorganisms, which are capable of carrying out anaerobic hydrocarbon metabolism, has not been excluded (Atlas 1981). During storage of oil products in underground, oxygen is usually limited in supply and it is of special concern, if hydrocarbon degradation is possible under anaerobic conditions. The initial attack on hydrocarbon requires oxygen, but for the subsequent steps, anaerobic processes may degrade partially oxygenated intermediates further. Under oxygen limited conditions, accumulation of degradation products occurs in the form of fatty acids. It has been seen that, even in a relatively static system the sulphate-reducing microbes in the fuel themselves can produce membranes, biosurfactants and gaseous by-products which, increase the surface area available for microbial activity. In fact, there have been several reports of isolated microorganisms, which are capable of alkane dehydrogenation under anaerobic conditions (Atlas 1981). These organisms have enzymatic mechanisms, which add oxygen at C=C bond, forming a secondary alcohol supporting anaerobic growth. Senez and Azouly (1961) reported that *Pseudomonas* sp. consumed oxygen when grown on heptane even though it had an n-heptane dehydrogenase enzyme. The importance of oxygen for hydrocarbon degradation is well indicated by the fact that the major degradative pathways for both saturated and aromatic hydrocarbons, involve oxygenases and molecular oxygen.

Degradation of hydrocarbons has been earlier reviewed by Atlas (1981). Sulfate reducing bacteria can use a wide range of fatty acids including acetate, lactate, propionate, butyrate and fatty acids up to C_{18} and some phenyl substituted acids (Savage et al. 2010). The generally accepted view is that anaerobic degradation of hydrocarbons is a very slow process as compared to aerobic degradation. Over very long periods, however, anaerobic pathway of degradation might be significant. Reports on the anaerobic degradation in natural ecosystems have suggested that nitrate or sulphate could serve as electron acceptors in place of oxygen (Atlas 1981).

13.5.3 Temperature and Pressure

Schwarz et al. (1974) examined the growth and utilization of hydrocarbons at ambient and in situ pressure for deep-sea bacteria. The rate of hydrocarbon utilization at high pressure and ambient temperatures were found to be significantly lower than the rates found under conditions of ambient temperatures and atmospheric pressure. For example, 94% hexadecane was utilized within 8 weeks at

1 bar while at 500 bars, it took 40 weeks for same level of degradation. It appears that oil, which enters deep-ocean environments, will be degraded very slowly and hence persists for long period of time. Biodegradation can occur over a wide range of temperatures from below 0 to 70°C (Atlas 1981, 1984). Most hydrocarbon degrading microorganisms prefer a near neutral pH, while a few of them can tolerate extreme acidity and alkalinity.

13.6 Consequences of Oil Degradation in Fuel Systems

13.6.1 Physical Debris

The most obvious and recognizable consequence of microbial activity is the formation of visible solids. These are the combinations of living and dead cellular materials, inorganic by-products and extraneous debris and biologically generated membranous materials. Although historical problems are associated with *Cladosporium resinae*, many yeasts and bacteria can also contribute significantly to the physical load. This material may block fuel lines, injectors, pipes, filters and oil/water separators as well as contribute to potential corrosion problems.

13.6.2 Fuel Turbidity/Cloudiness

For a fuel to be of marketable quality, it must be clear and bright (C + B). Microbial growth can often cause severe turbidity and cloudiness. This arises in one of two ways. Any mixing of interfacial growth can often produce particles of such a density that they remain in components. The hydrocarbon range of gasoline, C_5–C_9 is less likely to be degraded, but kerosene with a hydrocarbon range of C_{10}–C_{18} is more susceptible to degradation (Hill 1984). The preferential removal of C_{15}–C_{35} n-alkanes relative to branched and cyclic alkanes is recognized as standard feature in biodegradation of crude oils (Cannan 1984). Degradation normally proceeds by a mono terminal attack wherein usually a primary alcohol is formed followed by aldehyde and a mono carboxylic acid.

13.6.3 Chemical Alteration of Fuels

Often linked to increased corrosivity are microbially produced by-products that can often change the actual chemical properties of fuels in storage tanks and transporting pipelines. Gaseous by-products from microbial metabolism, such as H_2S, SO_2 and CO_2, are often generated in such quantities that they can dissolve in

the fuel, increasing the silver strip corrosivity index. The H_2S generation can also be a severe health hazard to the operating staff. Other metabolites, such as biosurfactants, can increase the emulsification of fuels.

13.7 Control of Microbial Contamination

As in most cases of microbial deterioration of materials, the best control treatment is prevention. Cleanliness and frequent drainage of water should ensure that problems are minimal. However, these standards are difficult to maintain in practice, as the result, storage systems have to be emptied for thorough cleaning (removal of biofilms) and biocides used. Corrosion in the tanks may be avoided by internal coatings which will be effective for some years. Although this is an expensive option, but it is increasingly employed. Cathodic protection (impressed current or the use of sacrificial anodes) is also used to combat corrosion, sometimes in addition to resistant coatings. Although expensive, this option may be less costly than the government-imposed fines which are levied in the USA against companies polluting the environment with leaked fuels. Such provisions, however, do not reduce the other consequences of contamination. Without adequate drainage, microbial slimes and sludges are likely to form in fuel systems and hence recourse to biocides may be necessary.

13.7.1 Control of Biodegradation of Fuels

13.7.1.1 Physical Control

Apart from maintaining fuels at temperatures unsuitable for microbiological growth which is often very difficult, the more obvious method of control is to eliminate water by good house keeping techniques, but this is often very difficult to achieve for the following reasons:

- Many fuels contain a degree of dissolved or chemically bound water which can later condense out during temperature changes and form free microbiologically available water.
- Atmospheric moisture may often enter the storage systems through breather vents, floating tank tops, ineffective seals etc.
- Other systems may actually purge delivery lines with various sources of water, for example cleaning of pig on muck collecting tanks.
- Complete water removal may be very difficult due to tank design. Since temperature control and water elimination are often impractical or costly to implement, the alternative is to look for some means of chemical control for the growth of fungi, yeasts and bacteria, i.e., a microbiocide or microbiostat.

13.7.1.2 Chemical Control

One of the main considerations in choosing microbiocide is whether the aim is to treat the water phase, fuel phase or both. There are advantages and disadvantages of treating either the water or fuel phase. But for long-term protection, a biocide that can treat the whole system appears to be an ideal solution.

Generally, the bacterial action in oil pipeline undergoes in two ways i.e. (1) consumption or degradation of oil, (2) conversion or storage or deposition of metals. Since these two processes are interlinked and dependent on the physiological activity of microbes, the corrosion action will be high in oil pipelines. Hence, MIC is an important aspect in oil industry and it should be considered as a major factor by the maintenance engineers.

13.7.1.3 Biocides

Selection of Biocides for Use in Fuel Systems

The criteria governing the selection of an effective biocide formulation can be summarized as follows:

- No adverse effects on engines, pumps etc.,
- Efficacy against a broad spectrum of microorganisms,
- Ability to penetrate microbial slime,
- Chemical and physical compatibility with the fuel and other additives (e.g., corrosion inhibitors),
- Suitable partition co-efficient (water–oil),
- Safety and ease of use and storage,
- Biodegradability, and
- Cost-effectiveness.

In aircraft fuel systems, routine maintenance, rigorous monitoring and treatment with allowed biocides when necessary (EGME in military aircraft, organoboramine, or isothiazolone (which is allowed by some manufacturers) keep risks of serious microbial growth to a minimum (Rajasekar and Ting 2011). Other fuels are generally not so well protected, but a wider variety of biocides is available. These include formulations to partition into the fuel phase and other water-soluble compounds. It is generally recommended that the biocides dissolve in the oil and then partition into the aqueous phase in sufficient quantity to protect the whole system and not merely the storage tank. For treatment of tanks only, water-bottom biocides may be used and this will be a cheaper option, since the volume to be treated is smaller. Some of the available biocides are shown in Table 13.6. The required properties are obtained by biocide formulation, not inherent to the chemical compound itself as mentioned below:

Table 13.6 Examples of oil–water—soluble biocides

Oil soluble	Water soluble
Isothiazolone formulations	Morpholines
Organoborinanes	Oxazolidines
Pyridinethione	Halides
Hexahydrotriazines	Aldehydes
Imidazolcarbamate	Phenolics

Source Shennan (1988)

- Oil-dispersible products partitioning completely into the water phase for treatment of water bottoms,
- Oil-soluble products which partition into the aqueous phase for use in clean fuels where little water is expected,
- Oil-soluble blends, separating into two components, one active in the water and the other in the oil, for protecting the interface and the water bottom, and
- Water-soluble products for addition to tank bottoms.

Biocides are the most underused, misunderstood, and misapplied chemical products in the petroleum industry for many reasons. Biocides are used to combat a problem that is subtle and difficult to detect. In general, biocides are needed to control the activity of the bacteria in a system. However, biocides alone cannot solve microbiological problem. Reliable means of monitoring are available to the supplier or the end user, and the benefits of biocide use take a long time to become evident. Finally, once bacteria are well established, it is nearly impossible to control without drastic measures. Hence, identification of microbes and also the role of inhibitors on microbial growth are to be studied in details.

Examples of Chemicals Used as Fuel System Biocides (Fuel+/or Water Soluble)

- Methylene bisthiocyanate
- Hexahydro-1,3,5-tris (2-hydroxyethyl)-S-triazine
- Mixture of 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazoline-3-one
- Ethylene or diethylene glycol monomethyl ether
- Mixture of 4-(2-nitrobutyl) morpholine and 4.4′-(2-ethyl-2-nitromethylene-dimorpholine
- Mixture of 2′s-oxy-bis(4,4,6-trimethyl-1,3,2-dioxaborinane) and 2,2-(1-methyl-trimethylene di-oxy) bis-(4-methyl-1,3,2-dioxaborinane)
- Methyl-1-(butyl carbomoyl)-2-benzimidazole carbamate-1-(2-hydroxyethyl-)-2-alkyl-(-C-18)-2-imidazoline etc.

13.8 Monitoring Program

Initial and continuous care is better, because once film is microfouled, it can be eradicated only with the addition of a microbiocides at very strong doses or by scrapping, which usually involves much trained labour and high operational costs. Hence, it is necessary to formulate a continuous monitoring programme by weight loss assessments and by microbiological analyses, if untreated water is used. A simple monitoring programme is given below:

- Chemical analysis of the water/oil used.
- Sampling and identification of the microbial population in the water/oil.
- Identification of microbes that form sessile consortia on the metals used in the site.
- Detection of low velocity/stagnant points in the flow lines.
- The quantity and the nutrient contents of the water, the interface between inhibitor and biocide, the degradation of inhibitors have to be analysed for effective usage of biocides.
- Selection of specific microbiocides with respect to the exact populations encountered at the site.
- Determination of exact concentration of microbiocide at which it ensures highest percent efficiency over sessile bacteria.
- If needed, dispersant can also be supplemented with the microbiocide.
- Immediately after pigging the line, a higher concentration of the microbiocide can be added followed by periodical additions at lower concentration (shock treatment). The quantity added should not be below the prescribed level, since a microbiocide at insufficient concentration promotes microbial growth.
- Monitoring the magnitude of bacteria before and after every four hours of biocide addition has to be carried out.
- Periodical monitoring of MIC by weight loss method/electrochemical probe technique is to be followed.
- The persistency and the level of the inhibitor/biocide should be checked by taking periodical samples at various pumping stations.

13.9 Conclusion

Biodegradation of hydrocarbon and its effect on corrosion causes hundreds of millions of dollars of damage to the production, transport and storage of petroleum products. Basic research on the understanding of the diversity of microbial species involved in biodegradation of petroleum hydrocarbon will be useful for the development of a new approach for the detection, monitoring and control of biodegradation and bio-corrosion. The role of individual hydrocarbon degrading bacteria identified from the diesel and naphtha pipeline has been studied. Until

now, no work has been reported on the biodegradation of petroleum products transporting pipeline. The mechanistic aspect of biodegradation in petroleum product pipeline has also not been given much attention in the investigation. Only limited reports are available in the literature on the biodegradation of biocides/inhibitors by microbes and the control of biocorroison and biodegradation in oil pipeline. Therefore, an in-depth study has been undertaken to identify the bacteria by 16S rDNA gene sequencing and to investigate the role of hydrocarbon utilizing bacteria in petroleum products and also to elucidate the mechanism of biodegradation by the individual native bacterial species.

This chapter describes the bacterial strains from the corrosion products collected from diesel and naphtha product pipelines which were identified by 16S rDNA analysis. In summary, this study provided an insight on the role of *S. marcescens* ACE2 and *B. cereus* ACE4 in biodegradation of petroleum hydrocarbon in the tropical country pipeline. It can be concluded that *B. cereus* ACE4 and *S. marcescens* ACE2 are the most proficient diesel degraders among the culturable organisms isolated from corrosion products collected from diesel transporting pipeline. ACE2 is a major aromatic and aliphatic degrader that breaks the benzene ring and aliphatic (–O–CH_2–) chain allowing inclusion of oxygen and accelerating the corrosion by ferric oxide formation in hydrocarbon environment. This investigation clearly indicates the possibility of the breakdown of diesel by *B. cereus* ACE4 at the stagnant areas of the pipelines. The role of enzyme aryl hydroxylase on degradation has been studied for first time by the authors. The degradation quality of hydrocarbon has been estimated by fluorescent spectrophotometer. The quality of degradation has been also studied by FTIR. The present study explains that both the strains *B. cereus* ACE4 and *Serratia marcenes* ACE2 were capable of utilizing both aliphatic and aromatic hydrocarbon present in the diesel. On inoculation, the bacteria produce a large quantity of aryl hydrocarbon hydroxylase at initial stage. In the later stage, the quantity of the enzyme is low. Since diesel is a major organic nutrient source for bacteria, it cannot consume the carbon from diesel directly. Hence, it secretes a large quantity of aryl hydroxylase enzyme for breaking the diesel for their metabolic activity. After degradation, the availability of simple molecule in diesel does not encourage the secretion of aryl hydroxylase. On the basis of total degradation of hydrocarbon, it was concluded that the activity of ACE4 on degradation of diesel is more vigorous than ACE2. Degraded carbon (diesel) acts as a good nutrient for bacteria, which increases the proliferation of bacteria on the steel and determine the nature of degradation and corrosion. Eventhough these bacteria may be useful in the bioremediation of a diesel polluted habitat, their presence in diesel pipeline and transportation facilities would lead to reduction in the quality of diesel, and in turn, incur huge economic loss. This chapter provides a summary on the knowledge of bacterial biofilm communities associated to steel pipelines and recognizes the corrosive potential of bacteria of the Enterobacteriaceae family (*K. oxytoca* and *S. marcescens*).

References

Aitken MD, Stringfellow WT, Nagel RD, Kazunga C, Chen SH (1998) Characteristics of phenanthrene-degrading bacteria isolated from soils contaminated with polycyclic aromatic hydrocarbons. Can J Microbiol 44:743–752

Atlas RM (1981) Microbial degradation of petroleum hydrocarbons, an environmental perspective. Microbiol Rev 45:180–209

Atlas RM (1984) Petroleum microbiology. Macmillan, New York

Bartha R, Atlas RM (1977) The microbiology of aquatic oil spills. Adv Appl Microbiol 22:225–266

Batts BD, Fathomi AZ (1991) A literature review on fuel stability studies with particular emphasis on diesel oil. Energy Fuels 5:2–21

Bento FM, Gaylarde CC (1996) Microbial contamination of stored diesel oil in Brazil. Rev Microbiol 27:192–196

Bento FM, Gaylarde CC (1998) Biofilms formed on coated or uncoated metal surfaces into diesel/water systems. In: Symposium aquatic microbiology, Manchester, proceedings of SAM, London, IMS, p. 64

Bento FM, Englert GE, Gaylarde CC, Muller IL (2004) Influence of aqueous phase on electrochemical biocorrosion tests in diesel/water systems. Mater Corros 55(8):577–585

Cannan J (1984) Biodegradation of crude oil in reservoirs. In: Brooks J, Welbe DH (eds) Advance in petroleum geochemistry. Academic Press, London, p 299

Chakrabarty AM (1972) Genetic basis of the biodegradation of salicylate in *Pseudomonas*. J Bacteriol 112:815–823

Chakrabarty AM, Chou G, Gunsalus IC (1973) Genetic regulation of octane dissimilation plasmid in *Pseudomonas*. Proc Natl Acad Sci USA 70:1137–1140

Colwell RR, Mills AL, Walker JD, Garcia-Tello P, Campos V (1978) Microbial ecology studies of the Metulla spill in the straits of Magellan. J Fish Res Board Can 35:573–580

Dela Fuente G, Perestelo F, Rodriguez Perez A, Falcon MA (1991) Oxidation of aromatic aldehydes by *Serratia marcescens*. Appl Environ Microbiol 57:1275–1276

Diaz E, Fernandez A, Prieto MA, Garcia JL (2001) Bioremediation of aromatic compounds by *Escherichia coli*. Microbiol Mol Biol Rev 65:523–569

Dunn NW, Gunsalus IC (1973) Transmisable plasmid coding early enzymes of naphthalene, oxidation in *Pseudomonas putida*. J Bacteriol 14:974–979

Fass R, Ben-Asher J, Shavit AB (1986) Effects of microbial contamination in storage tanks on the long-term stability of jet fuel. In: LL Stavinoah (ed) Proceedings of the 2nd international conference on long term stabilities of liquid fuels, SRI, San Antonio, TX, p 253

Feitkenhauer H, Muller R, Markl H (2003) Degradation of polycyclic aromatic hydrocarbons and long chain alkanes at 60–70 degrees C by *Thermus* and *Bacillus* spp. Biodegradation 14:367–372

Gaylarde CC, Bento FM, Kelley J (1999) Microbial contamination of stored hydrocarbon fuels and its control: a mini-review. Revista de Microbiologia 30:1–10

Gerbal-Chaloin S, Pichard-Garcia L, Fabre JM, Sa-Cunha A, Poellingerl L, Maurel P, Daujat-Chavanieu M (2006) Role of CYP3A4 in the regulation of the aryl hydrocarbon receptor by omeprazole sulphide. Cell Signal 18:740–750

Gibson DT (1968) Microbial degradation of aromatic compounds. Science 161:1093–1097

Grant DJW (1967) Kinetic aspects of the growth of *Klebsiella aerogenes* with some benzenoid carbon sources. J Gen Microbiol 46:213–224

Grishchenkov VG, Townsend RT, McDonald TJ, Autenrieth RL, Bonner S, Boronin AM (2000) Degradation of petroleum hydrocarbon by facultative anaerobic bacteria under aerobic and anaerobic conditions. Process Biochem 35:889–896

Groysman A (2005) Corrosion of aboveground fuel storage tanks. Mater Perform 44:44–48

Groysman A (2010) In corrosion for everybody. Springer, New York, p 368

Hill EC (1984) Petroleum microbiology. In: Atlas RM (ed) Biodegradation of petroleum products. Macmillan, New York, p 579
Hill EC (1987) Fuels. In: Hill EC, Shennan J, Watkinson R (eds) Microbial problems in the offshore oil industry. Wiley, New York, pp 219–230
Hill EC, Hill GC (1993) Microbiological problems in distillate fuels. Trans Inst Marine Eng 104:119–130
Holt JG, Kreig NR, Sneath PHA, Stanely JT (1994) In: Williams ST (ed) Bergey's manual of determinative bacteriology. Williams and Wilkins Publishers, Maryland
Ijah JJ (1998) Studies on relative capability of bacterial and yeast isolates from tropical soil degrading crude oil. Waste Manag 18:293–299
Irish GE, Richardson RL (1981) ASTM STP 751:76
Kanaly RA, Harayama S (2000) Bio-degradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria. J Bacteriol 182:2059–2067
Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120
Kinney D (1968) Biodegradation of hydrocarbons in the marine environment. Appl Environ Microbiol 27:125–129
Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23:2947–2948
Law RJ, Fileman TW, Portmann JE (1988) Aquatic environmental protection: analytical methods. MaFF Direct Fish Res Lowestoft 2:25
Lepetit J, Barthelemy MH (1968) Le probleme de l'epuration des zones littorales par les microorganisms. Ann Inst Pasteur (Paris) 114:149
Litman ES (1980) Microbiological contamination of fuels during storage. In: Stavinoah LL, Henry EI (eds) Distillate fuel stability and cleanliness, ASTM 04-751000-12, pp 136–144
Ludwig W, Schleifer K-H (1999) Phylogeny of bacteria beyond the 16S rRNA standard. ASM News 65:752–757
Maruthamuthu S, Mohanan S, Rajasekar A, Muthukumar N, Ponmarippan S, Subramanian P, Palaniswamy N (2005) Role of corrosion inhibitors on bacterial corrosion in petroleum product pipeline. Indian J Chem Technol 12:567–575
Michaud L, Giudice AL, Saitta M, De Domenico M, Bruni V (2004) The biodegradation efficiency on diesel oil by two psychrotrophic Antarctic marine bacteria during a two-month-long experiment. Marine Res Bull 49:405–409
Muthukumar N, Mohanan S, Maruthamuthu S, Subramanian P, Palaniswamy N, Raghavan M (2003a) Role of *Brucella* sp. and *Gallionella* sp. in oil degradation and corrosion. Electrochem Comm 5:422–427
Muthukumar N, Rajasekar A, Ponmarriappan S, Mohanan S, Maruthamuthu S, Muralidharan S, Subramanian P, Palaniswamy N, Raghavan M (2003b) Microbiologically influenced corrosion in petroleum product pipelines: a review. Indian J Exp Biol 41:1012–1022
Obuekwe CO, Westlake DWS (1987) Occurrence of bacteria in Pembina oil pipeline system and their role in corrosion process. Appl Microbiol Biotechnol 26:389–393
Priest F, Austin B (1993) Modern bacterial taxonomy, 2nd edn. Chapman and Hall, London Chapter 1
Rahman KSM, Thahira-Rahman J, Lakshmanaperumalsamy P, Banat IM (2002a) Towards efficient crude oil degradation by a mixed bacterial consortium. Biores Technol 85:257–261
Rahman PKSM, Rahman TJ, Lakshmanaperumalsamy P, Banat IM (2002b) Occurrence of crude oil degrading bacteria in gasoline and diesel station soil. J Basic Microbiol 42:284–291
Rahman PKSM, Rahman TJ, Banat IM, Lord RA, Street G (2007) Bioremediation of petroleum sludge using bacterial consortium with biosurfactant. In: Singh SN, Tripathi RD (eds) Environmental bioremediation technologies. Springer, Berlin, pp 391–407
Rahman PKSM, Pasirayi G, Auger V, Ali Z (2010) Production of rhamnolipid biosurfactants by *Pseudomonas aeruginosa* DS10-129 in a microfluidic bioreactor. Biotech Appl Biochem 55:45–52

Rajasekar A, Ting YP (2010) Microbial corrosion of aluminum 2024 aeronautical alloy by hydrocarbon degrading bacteria *Bacillus cereus* ACE4 and *Serratia marcescens* ACE2. Ind Eng Chem Res 49:6054–6061

Rajasekar A, Ting YP (2011) Inhibition of biocorrosion of aluminum 2024 aeronautical alloy by conductive ladder polymer poly(o-phenylenediamine). Ind Eng Chem Res 50:2040–2046

Rajasekar A, Maruthamuthu S, Muthukumar N, Mohanan S, Subramanian P, Palaniswamy N (2005) Bacterial degradation of naphtha and its influence on corrosion. Corros Sci 47:257–271

Rajasekar A, Ganesh Babu T, Karutha Pandian S, Maruthamuthu S, Palaniswamy N, Rajendran A (2007a) Biodegradation and corrosion behaviour of *Bacillus cereus* ACE4 in diesel transporting pipeline. Corros Sci 49:2694–2710

Rajasekar A, Ganesh Babu T, Karutha Pandian S, Maruthamuthu S, Palaniswamy N, Rajendran A (2007b) Role of *Serratia marcescens* ACE2 on diesel degradation and its influence on corrosion. J Ind Microbiol Biotechnol 34(9):589–598

Rajasekar A, Ganesh Babu T, Maruthamuthu S, Karutha Pandian S, Mohanan S, Palaniswamy N (2007c) Biodegradation and corrosion behavior of *Serratia marcescens* ACE2 isolated from Indian-diesel transporting pipeline. World J Microbiol Biotechnol 23:1065–1074

Rajasekar A, Maruthamuthu S, Palaniswamy N, Rajendran A (2007d) Biodegradation of corrosion inhibitors and their influence on petroleum product pipeline. Microbiol Res 162:355–368

Rajasekar A, Ponmariappan S, Maruthamuthu S, Palaniswamy N (2007e) Bacterial degradation and corrosion of naphtha in transporting pipeline. Curr Microbiol 55:374–381

Rajasekar A, Maruthamuthu S, Ting YP (2008) Electrochemical behavior of *Serratia marcescens* ACE2 on carbon steel API5-LX-60 in organic-aqueous phase. Ind Eng Chem Res 47:6925–6932

Rajasekar A, Anandkumar B, Maruthamuthu S, Ting YP, Rahman PKSM (2010) Characterization of corrosive bacterial consortia isolated from petroleum product transporting pipelines. Appl Microbiol Biotechnol 85:1175–1188

Sarma PM, Bhattacharya D, Krishnan S, Lal B (2004) Degradation of polycyclic aromatic hydrocarbons by a newly discovered enteric bacterium, *Leclercia adecarboxylata*. Appl Environ Microbiol 70:3163–3166

Savage KN, Krumholz LR, Gieg LM, Parisi VA, SuflitaJM, Allen J, Philp RP, Elshahed MS (2010)Biodegradation of low-molecular-weight alkanes undermesophilic, sulfate-reducing conditions: metabolicintermediates and community patterns. FEMS MicrobiolEcol 72:485–495.

Schwarz JR, Walker JD, Colwell RR (1974) Growth of deep-sea bacteria on hydrocarbons at ambient and in situ pressure. Dev Ind Microbiol 15:239–249

Senez JC, Azoulay E (1961) Dehydrogenation of paraffinic hydrocarbon by resting cells and free extract of *Pseudomonas aeruginosa*. Biochem Biophys Acta 471:307

Shennan JL (1988) Control of microbial contamination of fuels in storage. In: Houghton DR, Smith RN, Eggins HOW (eds) Bio-deterioration. Elsevier, Barking, pp 248–254

Shimada T, Guengerich FP (2006) Inhibition of human cytochrome P450 1A1-, 1A2- and 1B1-mediated activation of procarcinogens to genotoxic metabolites by polycyclic aromatic hydrocarbons. Chem Res Toxicol 19:288–294

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Traxler RW, Flannery WL (1968) Mechanism of hydrocarbon degradation. P44-53. In: Walters HA, Elphick J (eds) Biodeterioration of materials, microbiological and allied aspects. Elsevier Publishing Co. Ltd, Amsterdam

Turner APF, Eaton RA, Jones EBG (1983) Nutritional aspects of ship fuel system contamination by *Cladosporium resinae*. In: Oxley TA, Barry S (eds) Biodeterioration. Wiley, London, pp 507–516

Van Beilen JB, Wubbolts MG, Witholt WE (1994) Genetic of alkane oxidation by *Pseudomonas oleovorans*. Biodegradation 5:161–174

Verma S, Bhargava R, Pruthi V (2006) Oily sludge degradation by bacteria from Ankleshwar, India. Intl Biodeter Biodegrad 57:207–213

Walker JD, Colwell RR (1975) Some effects of petroleum on estuarine and marine microorganisms. Can J Microbiol 21:305–313

Walker JD, Colwell RR (1976) Measuring the potential activity of hydrocarbon-degrading bacteria. Appl Environ Microbiol 31:189–197

Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA for phylogenetic study. J Bacteriol 173:697–703

Whyte LG, Hawari J, Zhou E, Bourbonniere L, Inniss WE, Greer CW (1998) Biodegradation of variable-chainlength alkanes at low temperatures by a psychrotrophic *Rhodococcus* sp. Appl Environ Microbiol 64:2578–2584

Woese CR, Kandler O, Wheelis ML (1990) Towards a natural system of organisms: proposal for the domains Arachea, Bacteria and Eucarya. Proc Natl Acad Sci USA 87:4576–4579

Wongsa P, Tanaka M, Ueno A, Hasanuzzaman M, Yumoto I, Okuyama H (2004) Isolation and characterization of novel strains of *Pseudomonas aeruginosa* and *Serratia marcescens* possessing high efficiency to degrade gasoline, kerosene, diesel oil, and lubricating oil. Curr Microbiol 49:415–422

Zinjarde S, Pant A (2002) Hydrocarbon degraders from tropical environments. Mar Pollut Bull 44:118–121

ZoBell CE (1946) Action of microorganisms on hydrocarbon. Bacteriol Rev 10:1–49

ZoBell CE (1973) Microbial degradation of oil: present status, problems and perspectives. In: Ahearn DG, Meyers SP (eds) The microbial degradation of oil pollutants. Georgia State University, Atlanta

Chapter 14
Polyurethane Biodegradation

Gary T. Howard

14.1 Introduction

Polyurethanes represent a class of polymers that have found a widespread use in the medical, automotive and industrial fields. Polyurethanes can be found in products such as furniture, coatings, adhesives, constructional materials, fibers, paddings, paints, elastomers and synthetic skins. Polyurethane is abbreviated as PUR in compliance with official German and International standards. However, the abbreviation PU is more commonly used in English texts.

Advantages of polyurethanes are that they increase the tensile strength and melting points making them more durable (Bayer 1947). Their resistance to degradation by water, oils, and solvents makes them excellent materials for the replacement of plastics (Saunders and Frisch 1964). As coatings, they exhibit excellent adhesion to many substances, abrasion resistance, electrical properties and weather resistance for industrial purposes (Saunders and Frisch 1964; Urbanski et al. 1977; Fried 1995). Depending on the chemical structures of the polyisocyanates and polyols, PU can adopt various forms ranging from flexible to rigid and from low density to solid elastomer.

The chemical composition of PU precludes them from being classified as pure plastics and hence called as a mixed polymer. The urethane group, which is the basis of this class of mixed polymer, represents a small part of the macromolecule and some PU products do not contain a urethane group. Despite the lack of this base unit, all PU are based on the composition of polyisocyanates. The polyisocyanate polyaddition is distinct from polymerization and polycondensation for the production of synthetic polymers and this feature explains their versatility.

G. T. Howard (✉)
Department of Biological Sciences, Southeastern Louisiana University,
Hammond, LA 70402, USA
e-mail: ghoward@selu.edu

S. N. Singh (ed.), *Microbial Degradation of Xenobiotics*,
Environmental Science and Engineering, DOI: 10.1007/978-3-642-23789-8_14,

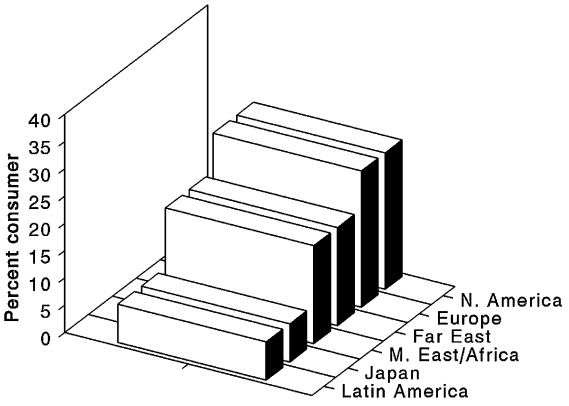


Fig. 14.1 The main global consumers for polyurethane are North America (25%), Europe (25%), the Far East (18%), Japan (7%), Latin America (7%), and the remaining split between the Middle East and Africa (Uhlig 1999)

Isocyanate precursors react with hydroxyl groups of polyols, compounds with two or more hydroxyl groups, to form PU. When the polyol is a polyester resin, the product is polyester PU. By varying the isocyanate and polyol composition, chemists can synthesize an enormous diversity of PU materials, including flexible polyester PU that forms the binding agent in most aircraft topcoat paints.

The global plastic consumption in 1997 totalled about 145 million tons with polyurethanes comprising a 5% share resulting in PU being fifth in global plastic consumption (Uhlig 1999). Over three-fourths of the global consumption of PU is in the form of foams. In the United States alone, the production of PU increased from 45,000 tons in 1960 to 2,722,000 tons in 2004. The main global consumers of polyurethane are summarized in Fig. 14.1.

14.2 Physical and Chemical Properties

Polyurethanes were first produced and investigated by Dr. Otto Bayer in 1937. Polyurethane is a polymer in which the repeating unit contains a urethane moiety. Urethanes are derivatives of carbamic acids which exist only in the form of their esters (Dombrow 1957). This structure can be represented by the following, generalized amide-ester of carbonic acid:

$$\begin{array}{c} \mathrm{O} \\ \| \\ \mathrm{R{-}O{-}C{-}NH2} \end{array}$$

Variations in the R group and substitutions of the amide hydrogen produce multiple urethanes. Although PU may contain urethane groups, other moieties such as urea, ester, ether or an aromatic may also be included (Saunders and Frisch 1964). The addition of these functional groups may result in fewer urethane moieties in the polymer than functional groups.

The urethane linkage results most readily through the reaction of an isocyanate with an alcohol (Dombrow 1957; Kaplan et al. 1968). The hydrogen atom of the

Table 14.1 Raw materials for synthesis of polyurethane

Polyisocyanate		2,4-Tolylene diisocyanate
		4,4′-Diphenylmethane diisocyanate
		1,3-Xylylene diisocyanate
		Hexamethylene diisocyanate
		1,5-Naphthalene diisocyanate
Polyol		
	Polyester-type	
		Poly(butylene adipate)
		Poly(ethylene butylene adipate)
		Poly(ethylene adipate)
		Polycaprolactone
		Poly(propylene adipate)
		Poly(ethylene propylene adipate)
	Polyether-type	
		Poly(oxytetramethylene) glycol
		Poly(oxypropylene) glycol
		Poly(oxypropylene)-poly(oxyethylene) glycol
Chain extension/ crosslinking agent		
		1,4-Butanediol
		Ethylene glycol
		1,3-Butanediol
		2,2-Dimethyl-1,3-propanediol
		Trimethylopropane
		Glycerol
		1,2,6-Hexanetriol

hydroxyl group is transferred to the nitrogen atom of the isocyanate (Bayer 1947). The major advantage of PU is that the chain is not composed exclusively of carbon atoms but rather of heteroatoms, oxygen, carbon and nitrogen (Bayer 1947). The simplest formula for PU is linear and represented by:

$$(\text{–R–O–}\overset{\overset{\text{O}}{\|}}{\text{C}}\text{–NH–R2–NH–}\overset{\overset{\text{O}}{\|}}{\text{C}}\text{–O–})_n$$

R represents a hydrocarbon containing the alcohol group, R2 is a hydrocarbon chain and n is the number of repetitions. Diisocyanates are employed in PU production reactions because they will react with any compound containing active hydrogen (Dombrow 1957).

For industrial applications, a polyhydroxyl compound can be used. Similarly, polyfunctional nitrogen compounds can be used at the amide linkages. By changing polyhydroxyl and polyfunctional nitrogen compounds, different PU can be synthesized (Dombrow 1957). Polyester or polyether resins containing hydroxyl groups are used to produce polyester- or polyether-PU, respectively (Urbanski et al. 1977). Examples of the raw materials used in the synthesis of PU are summarized in Table 14.1.

Variations in the number of substitutions and the spacing between and within branch chains produce PU ranging from linear to branched and flexible to rigid. Linear PU is used for the manufacture of fibers and molding (Urbanski et al. 1977). Flexible PU is used in the production of binding agents and coatings (Saunders and Frisch 1964). Flexible and rigid foamed plastics, which make up the majority of PU produced, can be found in various forms in the industry (Fried 1995). Using low molecular mass pre-polymers, various block copolymers can be produced. The terminal hydroxyl group allows alternating blocks, called segments, to be inserted into the PU chain. Variation in these segments results in varying degrees of tensile strength and elasticity. Blocks providing rigid crystalline phase and containing the chain extender are referred to as hard segments (Fried 1995). Those yielding an amorphous rubbery phase and containing the polyester/polyether are called soft segments. Commercially, these block polymers are known as segmented PU (Young and Lovell 1994).

14.3 Polyurethane Degradation

Research has been initiated to elucidate whether additives to the chemical structure of PU could decrease biodegradation. Kanavel et al. (1966) observed that sulfur-cured polyester and polyether PU had some fungal inertness. However, they noted that even with fungicides added to the sulfur- and peroxide-cured PU, fungal growth still occurred on the polyester PU and most fungicides had adverse effects on the formulations. Kanavel et al. (1966) also recognized the need for physical testing of the PU after extended exposure to the activity of fungi.

Santerre et al. (1994) studied the amount of degradation products released by varying the physical makeup of the polyester PU, as coatings on glass tubes or as films. This implied that while urethane and urea groups are susceptible to hydrolysis, they are not always accessible to the enzyme and degradation may never proceed past the polymer surface. Although the polyether PU showed no significant degradation, they consistently showed higher radiolabel products release from soft-segment-labeled, enzyme-incubated samples than controls. The author has attributed these results to the shielding of ester sites by secondary structures and hydrogen bonding within the hard segment.

Santerre and Labow (1997) tested the effect of hard segment size on the stability of PU against cleavage. Analysis was performed with polyether PU and their susceptibility to cholesterol esterase. Three polyether PU were synthesized with varying molar ratios of [^{14}C]-diisocyanate to chain extender and constant polyether makeup. A 10-fold increase in enzyme concentration of cholesterol esterase previously used (Santerre et al. 1994) was used to approach plateau values for polyether PU hydrolysis. Upon treatment with cholesterol esterase, Santerre and Labow (1997) observed that radiolabel release was significantly dependent on the amount of hard segment contained within the polymer. In the polymer with the lowest concentration of hard segment, higher numbers of carbonyl groups are

exposed to the surface. With increased hard segment size, a greater number of carbonyl groups are integrated into secondary hard segment structures through hydrogen bonding. The investigators also concluded that an increase in hard segment size does lead to restrictions in polymer chain mobility.

In the medical field, PU show resistance to macromolecular oxidation, hydrolysis and calcification (Marchant 1992). Polyurethane elastomers are being used in place of other elastomers due to higher elasticity and toughness, and resistance to tear, oxidation and humidity (Dombrow 1957; Saunders and Frisch 1964; Ulrich 1983). In addition, polyether derivatives are inexpensive to produce as prepolymers, which can lower the overall cost of polymer production.

Huang and Roby (1986) tested the biodegradability of polyamide-urethanes for medical purposes. They synthesized PU with long repeating units and alternating amide and urethane groups from 2-aminoethanol. The resulting partial crystalline fibers were observed to undergo hydrolysis by subtilisin less readily than polyamideesters with degradation proceeding in a selective manner. The amorphous regions on the PU were being degraded prior to the crystalline regions. These fibers showed promise as absorbable sutures and implants where in vivo degradation is needed. The investigators also noted that PU with long repeating units and hydrophilic groups would less likely to pack into high crystalline regions as normal PU, and these polymers were more accessible to biodegradation.

Tang et al. (1997) added surface-modifying macromolecules (SMM) containing fluorinated end groups to the base PU to reduce the material's susceptibility to hydrolysis by lysosomal enzymes. Synthesized polyester urea-urethanes were radiolabled with [^{14}C] and coated onto small hollow tubes. Biodegradation experiments were carried out using methods previously established by Santerre et al. (1994). Results indicated that degradation was inhibited by the SMM surface. Different SMM formulations provided varying degrees of enzyme resistance. It was noted that some SMM formulations were incompatible with the PU and led to increased biodeterioration. The mechanism of inhibition was not deduced and will be the subject of further study.

In an attempt to increase biocompatibility and reduce bacterial adhesion on PU surfaces, Baumgartner et al. (1997) synthesized phosphonated PU. They used glycerophosphorylcholine (GPC) as the chain extender, which incorporated phosphorylcholine head groups into the PU backbone. This gave the PU surface some characteristics of a red blood cell surface. Physical tests on the PU showed a small decrease in tensile strength and transition temperature with increasing GPC concentration. Water absorption by the PU was increased with increased GPC content. To test bacterial adhesion to the PU, Baumgartner et al. (1997) used a radial flow chamber. They passed a culture of *Staphylococcus aureus* across phosphonated and unphosphonated PU at a rate of 8 ml min^{-1}. The phosphonated PU showed a decrease in bacterial adhesion with increased GPC content.

Lack of degradability and increasing depletion of landfill sites as well as growing water and land problems have led to concern about plastics (Kawai 1995). As more and more raw materials (e.g. crude oil) become in short supply for the synthesis of plastics, recycling of waste plastics is becoming important

(Schnabel 1981). Degradability problems promoted researchers to investigate modification or productions that led to either chemically degradable or biodegradable PU.

Huang et al. (1981) derived polyester PU from polycaprolactonediols in an effort to produce biodegradable PU for use in the medical field. Several different PU were made containing polyester subunits of various lengths. The polymers were subjected to degradation by the enzyme axion and two species of fungi. The enzyme and fungi degraded each PU. In addition, it was also noted that there was an increase in the biodegradability of the polyester PU with increase in the chain length of the polyesters.

In a later study, Phua et al. (1987) observed that two proteolytic enzymes, papain and urease degraded a medical polyester PU. The PU they tested was Biomer®, segmented, cross-linked polyester PU. Although cross linking was previously described as a way of inhibiting degradation (Kaplan et al. 1968), papain (molecular weight 20.7 kDa) had little difficulty in diffusing into the film and causing breaks in the structural integrity. Urease activity, because of its size (molecular weight 473 kDa), was limited to the PU surface and therefore degradation was not significant. Phua et al. (1987) also proposed that papain degraded the polymer by hydrolyzing the urethane and urea linkages producing free amine and hydroxyl groups. The effect of papain on polyether PU was assessed by Marchant et al. (1987). Comparison of papain activity to aqueous hydrolysis resulted in both releasing degradation products. Ether linkages were non-enzymatic ally hydrolyzed by water while degradation of the urethane groups was dependent on the presence of the proteolytic enzyme.

Labrow et al. (1996) treated polyester PUs and polyether PU with human neutrophil elastase and porcine pancreatic elastase. The polyester PUs was readily degraded by porcine pancreatic elastase at a rate ten times higher than by human neutrophil elastase. The rate of polyester PU degradation by porcine pancreatic elastase was also ten times higher than its activity against the polyether PUs. Human neutrophil elastase had no significant activity against the polyether PUs. These results indicate a distinct similarity to the degradation of PUs by cholesterol esterase (Santerre et al. 1993, 1994; Santerre and Labrow 1997). Inhibition of porcine pancreatic elastase was achieved with the elastase specific inhibitor NMSAAPVCMK.

14.4 Fungal Biodegradation

After years of production of PUs, manufacturers found them susceptible to degradation. Variations in the degradation patterns of different samples of PUs were attributed to the many properties of Pus, such as molecular orientation, crystallinity, cross-linking, and chemical groups presented in the molecular chains which determine the accessibility to degrading-enzyme systems (Pathirana and Seal 1983). The regularity in synthetic polymers allows the polymer chains to pack easily, resulting in the formation of crystalline regions. This limits accessibility of

the polymer chains to degradation, whereas amorphous regions on the PU can degrade more readily. Huang and Roby (1986) observed PU degradation proceeded in a selective manner, with the amorphous regions being degraded prior to the crystalline regions. Also, it was observed that PUs with long repeating units and hydrolytic groups would be less likely to pack into high crystalline regions as normal polyurethanes, and these polymers were more accessible to biodegradation. Several investigators have suggested microbial attack on PUs could be through enzymatic action of hydrolases such as ureases, proteases and esterases (Evans and Levisohn 1968; Hole 1972; Flilip 1978; Griffin 1980).

Several reports have appeared in the literature on the susceptibility of PUs to fungal attack (Darby and Kaplan 1968; Kaplan et al. 1968; Ossefort and Testroet 1966). These studies revealed that polyester-type PUs are more susceptible to fungal attack than other forms. In addition, polyether PUs were noted to be moderately resistant. Boubendir (1993) isolated enzymes with esterase and urethane hydrolase activities from the fungi *Chaetomium globosum* and *Aspergillus terreus*. These organisms did not grow solely on PUs and the enzymes had to be induced. Induction of the enzymes was accomplished by addition of liquid polyester PU to the growth media. Activity of the enzymes was determined by assays based on ethyl carbamate (urethane) as artificial substrate.

In a more recent study, Cosgrove et al. (2007) reported on involvement of soil fungal communities in the biodegradation of polyester polyurethane. Fungal communities on the surface of the PU were compared to the native soil communities using culture-based and molecular techniques. Putative PU-degrading fungi were common in both soils, as <45% of the fungal colonies cleared the colloidal PU dispersion Impranil on solid medium. Denaturing gradient gel electrophoresis revealed that fungal communities associated with the PU coupons were less diverse than in the soil, and only a few species in the PU communities were detectable in the soil indicating that only a small sub-set of the soil fungal communities colonized the PU. Soil type influenced the composition of the PU fungal communities. *Geomyces pannorum* and a *Phoma* sp. were the dominant species recovered by culturing from the PU buried in the acidic and neutral soils, respectively. Both fungi degraded Impranil and represented >80% of cultivable colonies. However, PU was highly susceptible to degradation in both soils, losing up to 95% of its tensile strength. Therefore, different fungi are associated with PU degradation in different soils, but the physical process is independent of soil type. As a follow up study, Cosgrove et al. (2010) investigated soil microcosms that were biostimulated with the PU dispersion agent "Impranil" and/or yeast extract or were bioaugmented with PU-degrading fungi, and the degradation of subsequently buried PU was determined. Their results indicated that biostimulation with yeast extract alone or in conjunction with Impranil increased PU degradation to 62% compared to the degradation in untreated control soil and was associated with 45% increase in putative PU degraders colonizing PU. Specific fungi were enriched in soil following biostimulation; however, few of these fungi colonized the surface of buried PU. Fungi used for soil bioaugmentation were cultivated on the surface of sterile wheat to form a mycelium-rich inoculum. Wheat,

when added alone to soil, increased PU degradation by 28%, suggesting that wheat biomass had a biostimulating effect. Addition of wheat colonized with *Nectria haematococca*, *Penicillium viridicatum*, *Penicillium ochrochloron*, or an unidentified *Mucormycotina* sp. increased PU degradation further by 30–70%, suggesting that biostimulation and bioaugmentation were operating in concert to enhance PU degradation. A few of the inoculated fungi were detected by DGGE in the soil or on the surface of the PU after four weeks of inoculation. Bioaugmentation did, however, increase the numbers of indigenous PU-degrading fungi and caused an inoculum-dependent change in the composition of the native fungal populations, which may explain the increased degradation. These results demonstrate that both biostimulation and bioaugmentation may be viable tools for the remediation of environments contaminated with polyurethane waste.

In another study, four species of fungi, *Curvularia senegalensis, Fusarium solani, Aureobasidium pullulans,* and *Cladosporium* sp. were isolated based on their ability to utilize a colloidal polyester PU (Impranil DLN^{TM}) as the sole carbon and energy source (Crabbe et al. 1994). *Curvularia senegalensis* was observed to have a higher PU-degrading activity and therefore, subsequent analysis of this fungal isolate was carried out. An extracellular polyurethanase (PUase) displaying esterase activity was purified from this organism. The protein has a molecular mass of 28 kDa, is heat stable at 100°C for 10 min and inhibited by phenylmethylsulphonylfluoride (PMSF).

Wales and Sagar (1988) proposed a mechanism for the degradation of polyester PUs by extracellular esterases. Polyurethane degradation is the result of synergistic activity between endopolyurethanases and exopolyurethanases. Endoenzymes hydrolyze the PU molecule at random locations throughout the polymer chain leading to loss of tensile strength. Exoenzymes remove successive monomer units from the chain ends showing little loss of tensile strength.

14.5 Bacterial Biodegradation

In a large-scale test of bacterial activity against PUs, Kay et al. (1991) investigated the ability of 16 bacterial isolates to degrade polyester PU. Seven of the isolates tested degraded PU when the media was supplemented with yeast extract. Two isolates, *Corynebacterium* sp. and *Pseudomonas aeruginosa*, could degrade PU in the presence of basal media. However, none of the isolates grew on PU alone. Physical tests of the degraded polyester PU revealed different but significant decreases in tensile strength and elongation for each isolate. In a further study, Kay et al. (1993) tested the chemical and physical changes in degraded polyester PU. Polyurethanes taken from *Corynebacterium* sp. cultures had significant reductions in both tensile strength and elongation after three days of incubation. Infra-red spectrophotometer analysis revealed the ester segment of the polymer to be the main site of attack. The investigators noted that supplementing the media with

glucose inhibited esterase production. However, addition of PU did not increase esterase activity.

Halim et al. (1996) tested the growth of several species of bacteria on PU military aircraft paint. The investigators isolated *Acinetobacter calcoaceticus*, *Pseudomonas cepacia* and *Arthrobacter globiformis*. In addition, the U.S. Navy supplied two strains of *A. calcoaceticus*, *Pseudomonas aeruginosa* and *Pseudomonas putida*. All species were capable of utilizing the polyurethane paint as a sole carbon and energy source with the exception of *P. cepacia*. Using fluorescein diacetate as an esterase substrate, the remaining species showed esterase activity in the absence of PU. This data indicated that the PUases were constitutively expressed.

14.5.1 Polyurethane Degradation by Bacillus

Blake and Howard (1998) reported bacterial degradation of a polyester PU (Impranil DLN) by a species of *Bacillus*. The pattern of degradation involved the binding of cells to the polymer with subsequent floc formation, and the degradation of substrate. The growth of the *Bacillus* sp. on a solid medium resulted in the visual disappearance of the polyurethane. The complexity of the bacteria-polyurethane interaction was more apparent when grown on a polyurethane liquid medium. Incubation of the *Bacillus* sp. in media supplemented with polyurethane resulted in the appearance of a chalky precipitate that appeared to be resistant to further degradation.

Electrophoretic mobility, electrical impedance, and dynamic light diffraction measurements were performed on the *Bacillus*-polyurethane system (Fig. 14.2). *Bacillus* cells had a relatively weak net negative charge corresponding to a zeta potential of −6 mV. Colloidal polyurethane had a strongly negative charge with a zeta potential of −42 mV. Complex formation between the PU and cells results in a zeta potential of −20 mV.

Electrical impedance data showed that on average the *Bacillus* cell had a volume of around 3.9 mm^3 corresponding to a spherical equivalent diameter of just over 2 mm. The majority of the polyurethane particles were sufficiently small to be below the detection limit, 0.6 mm, for electrical impedance. The relative volumes as a function of size for polyurethane and *Bacillus* were determined by static light diffraction methods. The results from the static light diffraction methods verified the electrical impedance results.

The above methods were then used to examine the formation of a complex between *Bacillus* and polyurethane. The electrophoretic mobility data showed that the peaks that were associated with the free polyurethane and the free *Bacillus* were replaced by a single peak that possessed the size and charge properties anticipated for a complex of the large *Bacillus* with the strongly negatively charge polyurethane (Fig. 14.2). This evidence was corroborated with electrical impedance measurements that showed there was an increase in the total volume of the

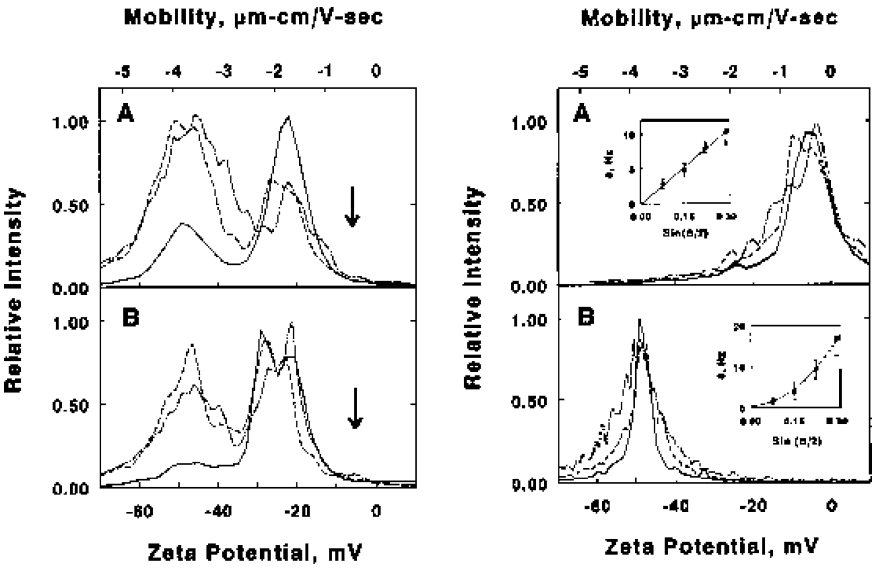


Fig. 14.2 (*Left*) Characterization of *Bacillus* and polyurethane by methods commonly employed in particle analysis *A* and *B*, electrokinetic measurements of *Bacillus* and polyurethane, respectively, in defined YES medium. Mobility (*top*) and zeta potential (*bottom*) spectra were calculated from frequency spectra determined by Doppler electrophoretic light scattering measurements performed in a DELSA 339. The conductivity was 1.46 and 1.58 mS/cm in experiments *A* and *B*. The angles of the photodiode light scattering detectors relative to the transmitter (corrected for the refractive index of water) were 34.7° (*dash-dot line*), 26.0° (*dashed line*) and 17.4° (*solid line*). Each inset is a plot of δ the half-width at half-height of the frequency spectrum, as a function of the sine of the bisected scattering angle. Each datum and error bar in the insets represents the mean and standard deviation of at least three determinations. The line drawn through the data points in the inset of *A* was determined by linear regression analysis. The line drawn through the data points in the inset of *B* was plotted according to the quadratic expression $\delta = \alpha \sin2(\theta/2)$, where the value of a was determined by a linear regression analysis of δ versus $\sin2(\theta/2)$ (plot not shown). (*Right*) One hundred ml of a 3.0 g/l suspension of polyurethane in defined YES medium was inoculated with 1.0 ml of an overnight culture of *Bacillus* grown on LB media. Electrokinetic measurements were performed on samples withdrawn from the mixture of *Bacillus* and polyurethane at 15 (*A*) and 30 (*B*) min. The arrows in each panel indicate the average position of the peaks of the mobility spectra observed with *Bacillus* in the absence of polyurethane

Bacillus cells as a function of time as they were mixed with an excess of polyurethane. Evidence that the increase in cell size occurred at the expense of the polyurethane came from light diffraction measurements. Further evidence that the *Bacillus* cell forms a complex with polyurethane was obtained through microscopic observations. These observations showed that the majority of the cells in the presence of polyurethane were coated with small particles of various dimensions (Fig. 14.3).

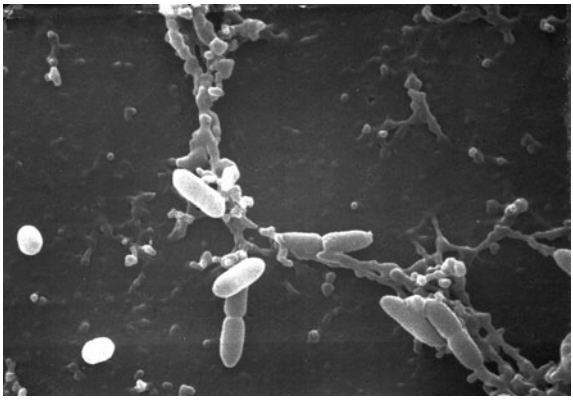


Fig. 14.3 Scanning Electron Micrograph of complex formed between *Bacillus* cells and polyurethane after 4 h exposure

This evidence indicates that two populations exist in polyurethane cultures: one that is coated with polyurethane and one that is not. At lower concentrations of polyurethane, it may be that the two populations of bacteria are dependent on different sources of nutrition. The first population is coated with polyurethane and the polyurethane is metabolized into small, soluble metabolites, which are released into the medium. The second population, which is not covered in polyurethane, uses the small, soluble metabolites produced by the first population to grow. At higher concentrations of polyurethane, all the cells present in the media may be coated with polyurethane. The more cells coated with polyurethane, the more polyurethane that is degraded and the more metabolites available for growth. This would result in polyurethane-coated cells, which are not free in solution and therefore, not detectable.

A follow up study (Rowe and Howard 2002) revealed that when grown on 1% Impranil DLN^{TM}, a lag phase growth was noted for the first 5 h which was followed by logarithmic growth for 8 h, reaching a cell density of $2.60 \times 10^8 \pm 1.17 \times 10^7$. The Monod plot for all concentrations of polyurethane tested did not follow simple Monod kinetics. At higher concentration (9.0 to 3.0 mg ml^{-1}) of Impranil DLN^{TM} Monod kinetics were not observed. The μ values dramatically decreased at a concentration of 3.0 mg ml^{-1} from 1.5 mg ml^{-1} to 0.466 doublings h^{-1} from 0.721 doublings h^{-1}. The μ continued to drop at higher concentrations from 0.466 doublings h^{-1} at 3.0 mg ml^{-1} to 0.369 doublings h^{-1} at 9.0 mg ml^{-1}. This dramatic decrease in μ may be explained by observations in a previous study by Blake and Howard (1998) that polyurethane was observed to accumulate on the cell surface of a *Bacillus* sp.

14.5.2 Polyurethane Degradation by Pseudomonas

Three Pseuomonads have been isolated for their ability to utilize a polyester PU as the sole carbon and energy source. Interestingly, three species of bacteria produce

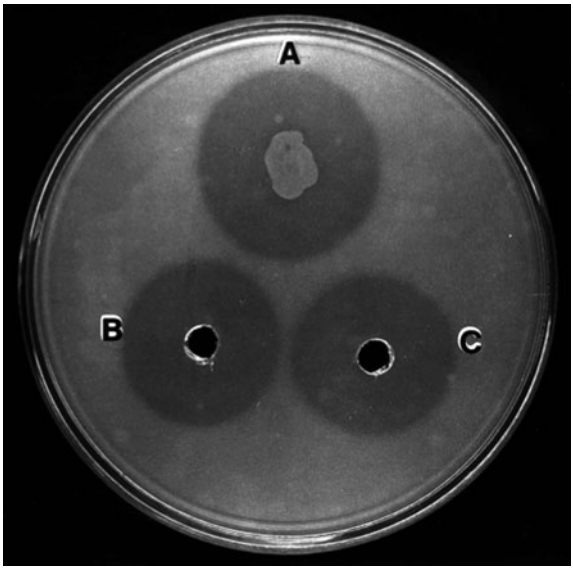


Fig. 14.4 Hydrolysis of Impranil DLN polyurethane produces clear zones in agar. *A*. Colony of *Pseudomonas fluorescens* bacteria. *B–C*. Extracellular proteins from *P. fluorescens* grown in LB or Impranil media, respectively

different PUase activities that are inhibited by serine hydrolase inhibitors. These data suggest that either esterase and/or protease activities are involved in the degradation of Impranil (Fig. 14.4).

Growth of *Comamonas acidovorans* on colloidal polyester-polyurethane resulted in the growth parameters for K_s and μ_{max} of 0.3 mg ml^{-1} and 0.7 doublings h^{-1}, respectively (Allen et al. 1999). A 42 kDa PUase enzyme displaying esterase/protease activity has been purified and characterized (Allen et al. 1999). Nakajima-Kambe et al. (1995, 1997) reported a strain of *C. acidovorans* that could utilize solid polyester PU as the sole carbon and nitrogen source. These authors indicated the role of an extracellular membrane bound esterase activity in PU degradation. Purification of the membrane bound esterase revealed a thermally labile protein having a 62 kDa molecular mass (Akutsu et al. 1998). *C. acidovorans* strain TB-35 was isolated from the soil samples for its ability to degrade polyester PU (Nakajima-Kambe et al. 1995). Solid cubes of polyester PU were synthesized with various polyester segments. The cubes were completely degraded after 7 days incubation when they were supplied as the sole carbon source and degraded 48% when they were the sole carbon and nitrogen source. Analysis of the breakdown products of the PU revealed that the main metabolites were derived from the polyester segment of the polymer. Gas chromatographic analysis revealed the metabolites produced were diethylene glycol, trimethylolpropane, and dimethyladipic acid. In agreement with these findings, Gautam et al. (2007) examined the biodegradation of polyester-polyurethane foam by *P. chlororaphis* ATCC 55729. Concentrations of ammonia and diethylene glycol increased over time with an increase of bacterial growth and a decrease in PU mass. A possible biodegradative pathway of PU is shown schematically (Fig. 14.5). Further analysis of strain

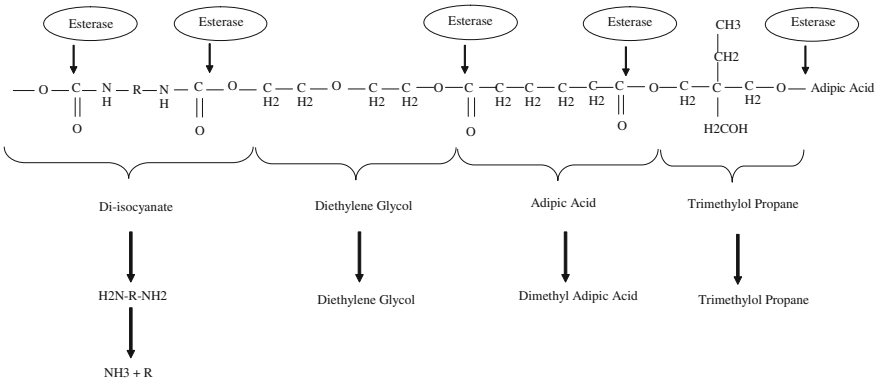


Fig. 14.5 Theoretical degradative pathway of polyester-polyurethane by esterase activity of *Pseudomonas*

TB-35 revealed that the degradation products from the polyester PU were produced by an esterase activity (Nakajima-Kambe et al. 1997). Strain TB-35 possesses two esterase enzymes, a soluble, extracellular and one membrane-bound. The membrane-bound enzyme was found to catalyze the majority of the polyester PU degradation. The membrane-bound PUase enzyme was purified and characterized (Akutsu et al. 1998). The protein has a molecular mass of 62 kDa, heat stable up to 65°C and is inhibited by PMSF. The structural gene, *pudA*, for the PU esterase was cloned in *Escherichia coli*. Upon nucleotide sequencing of the open reading frame (ORF), the predicted amino acid sequence contained a Gly-X -Ser-X-Gly motif characteristic of serine hydrolases. The highest degree of homology was detected with the *Torpedo californica* acetylcholinesterase (T ACh E), possessing the Ser-His-Glu catalytic triad, with the glutamate residue replacing the usual aspartate residue. Similarity in the number and positions of cysteine and salt bonds was very apparent between PudA and T AchE, as were also identities of sequences and their positions in the α-helix and β-strand regions between the two. In the neighborhood of the glutamate residue of the Ser^{199}-His^{433}-Glu^{324} catalytic domain of PudA, there were three hydrophobic domains, one of which constituted the surface-binding domain, which occurred in the C-terminus of most bacterial poly(hydroxyalkanoate)(PHA) depolymerases.

Growth of *Pseudomonas fluorescens* on PU resulted in values of 0.9 mg ml^{-1} and 1.6 doublings h^{-1} for K_s and μ_{max}, respectively (Howard and Blake 1999). Two PUase enzymes have been purified and characterized from this bacterial isolate, a 29 kDa protease (Howard and Blake 1999) and a 48 kDa esterase (Vega et al. 1999). In addition, to the enzymology of the Puases, the gene encoding a 48 kDa protein has been cloned and expressed in *E. coli* (Vega et al. 1999). The gene encoding PulA has been sequenced (Genbank, Accession AF144089). The deduced amino acid sequence has 461 amino acid residues and a molecular mass of 49 kDa. The PulA amino acid sequence showed high identity with Group I lipases (58–75%).

Growth of *Pseudomonas chlororaphis* on polyurethane resulted in values of 0.9 mg ml^{-1} and 1.3 doublings h^{-1} for K_s and μ_{max}, respectively (Ruiz et al. 1999a). Two PUase enzymes have been purified and characterized, a 65 kDa esterase/protease and a 31 kDa esterase (Ruiz et al. 1999b. A third PUase enzyme, 60 kDa esterase, has been partially purified and characterized (Ruiz et al. 1999a). Two genes encoding PUase activity from *P. chlororaphis* have been cloned in *E. coli* (Stern and Howard 2000; Howard et al. 2001). Both genes can be expressed in *E. coli*. However, the PueA enzyme is secreted in the recombinant *E. coli* and displays a beta-zone of clearing on polyurethane agar plates while PueB is not secreted in the recombinant *E. coli* and displays an alpha-zone of clearing on polyurethane agar plates. In addition, PueB has been noted to display esterase activity towards ρ-nitrophenylacetate, ρ-nitrophenylpropionate, ρ-nitrophenylbutyrate, ρ-nitrophenylcaproate, and ρ-nitrophenylcaprylate while PueA has been reported to display esterase activity only towards ρ-nitrophenylacetate and ρ-nitrophenylpropionate.

Upon cloning PueA (Stern and Howard 2000) and PueB (Howard et al. 2001) from *P. chlororaphis* in *Escherichia coli*, the recombinant proteins were noted to have a high homology to Group I lipases. This family of lipases and other serine hydrolases, are characterized by an active serine residue that forms a catalytic triad in which an aspartate or glutamate and a histidine also participate (Jaeger et al. 1994; Persson et al. 1989; Winkler et al. 1990). Sequence analysis of the two-polyurethanase genes revealed that both encoded proteins contain serine hydrolase-like active site residues (G-H-S-L-G) and a C-terminal nonapeptide tandem called repeat in toxin (RTX), (G-G-X-G-X-D-X-X-X) repeated three times. Group I lipases lack an N-terminal signal peptide but instead contain a C-terminal secretion signal. The secretion of these enzymes occurs in one step through a three-component, ATP-binding cassette (ABC) transporter, Type I secretion system (Arpigny and Jaeger 1999). Proteins secreted by Type I systems typically exhibit two features: (1) an extreme C-terminal hydrophobic secretion signal located within the last 60 amino acids that is not cleaved as part of the secretion process and (2) roll structure stabilized by glycine-rich RTX motifs. The RTX repeats form a Ca^{2+} roll. These ions co-ordinated between adjacent coils of the motifs are thought to be important for proper presentation of the secretion signal to the secretion machinery, but their exact role is controversial.

Comparison between the amino acid and nucleotide sequences of these two genes revealed that they share 42 and 59% identity, respectfully (Table 14.2). Parsimony analysis of the predicted amino acid sequences for PueA, PueB, PudA, and PulA polyurethanase enzymes and similar lipase enzymes was also performed (Fig. 14.6). Interestingly the PUase enzymes do not form a single cluster, but appear to be distributed among multiple lineages (Howard et al. 2001). These analyses suggest that the PUase enzymes so far studied have evolved from lipases, and are not derived from a single source.

Howard et al. (2007) identified a gene cluster resembling a binding-protein-dependent ABC transport system in *Pseudomonas chlororaphis* in connection with PueA and PueB (Fig. 14.7). The identified ABC transport system

Table 14.2 Identity comparison of PueB and other serine hydrolases

Protein	Length (aa/nt)	%Identity (aa/nt)[a]	Strain	Accession number
PueB	567/1704	100/100	*Pseudomonas chlororaphis*	EF175556
PueA	617/1801	42/59	*Pseudomonas chlororaphis*	EF175556
PulA	451/1353	24/41	*Pseudomonas fluorescens*	AF144089
PudA	548/1644	11/31	*Comamonas acidovorans*	AB009606
TliA	476/1428	26/40	*Pseudomonas fluorescens* B52	AF083061
LipA	613/1789	36/53	*Serratia marcescens* SM6	BAA02519
Lipase	617/1801	39/55	*Pseudomonas* sp. MIS38	BAA84997
LipApf33	476/1428	27/41	*Pseudomonas fluorescens* 33	BAA36468
Lipase	449/1338	25/39	*Pseudomonas fluorescens* SIK W1	JQ1227

[a] Amino acid and nucleotide identities were determined with Bioedit version 4.8.8 program

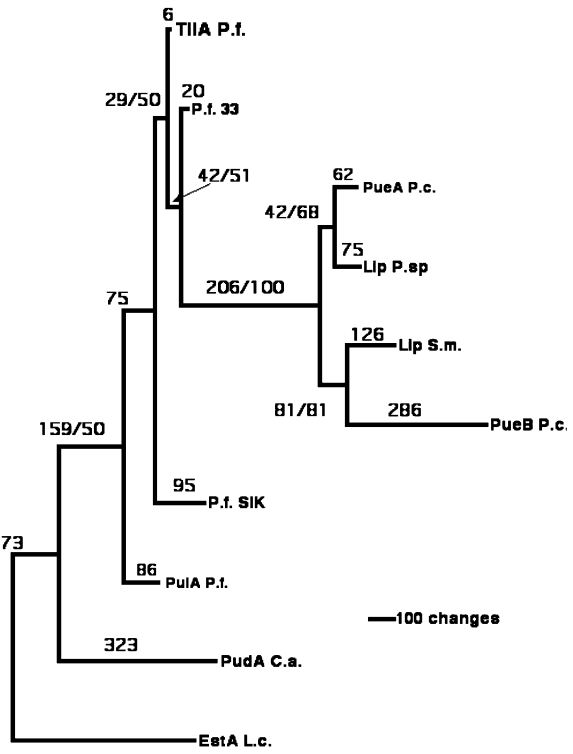


Fig. 14.6 Single most parsimonious tree inferred from the phylogenetic analysis of polyurethanases and lipases. The numbers above the branches depict total character support/ bootstrap support for each branch and node. Branch lengths reflect number of changes estimated along each branch

consists of three components: an ATPase-binding protein (ABC), an integral membrane protein (MFP), and an outer membrane protein (OMP). The ABC pathway has been shown to mediate translocation of an alkaline protease in *Pseudomonas aeruginosa* (Doung et al. 1994). Also, the ABC pathway has been shown to be involved in secretion of a lipase from *Serratia marcescens*

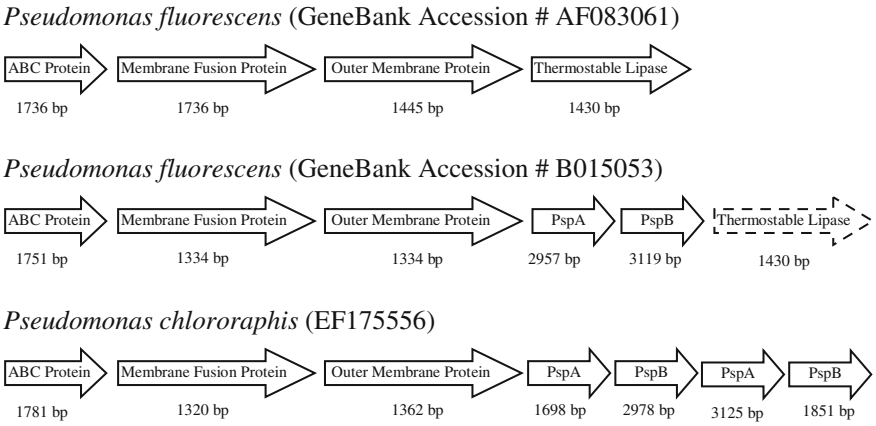


Fig. 14.7 Comparison of the gene clusters from two strains of *Pseudomonas fluorescens* and the PUase gene cluster from *Pseudomonas chlororaphis*. The ABC Reporter Protein, Membrane Fusion Protein and Outer Membrane Protein are involved in Type I translocation of the extracellular protein. The PspA and PspB proteins are serine protease homologues

(Akatsuka et al. 1995), which is located separately from the lipase gene on the chromosome and secretes protease, lipase and S-layer proteins (Kawai et al. 1998). A gene cluster (accession number AF083061) was identified for an ABC transporter specific for a lipase in *Pseudomonas fluorescens* SIK W1 (Ahn et al. 1999) and a similar gene cluster (accession number AB015053) was identified in *Pseudomonas fluorescens* 33 for a lipase gene and two serine proteases (Kawai et al. 1999). Interestingly, when the two ABC exporter gene clusters of *Pseudomonas fluorescens* are compared to the ABC exporter gene cluster of the one found in *Pseudomonas chlororaphis*, a unique gene arrangement is observed (Fig. 14.7). It appears that the novel gene arrangement observed is a combination of the two *P. fluorescens* gene clusters, and may have resulted through either a rearrangement or an insert ional event between the two ABC gene clusters observed in *P. fluorescens*.

Further investigation of the gene cluster involved growth studies to compare the effects of a PueA deficient strain and a PueB deficient strain with the wild type strain in polyurethane utilization (Table 14.3). *Pseudomonas chlororaphis* wild type and its PueA derivatives when grown on 1% Impranil DLN^{TM} YES medium exhibited a lag phase growth for the first 3 h and then was followed by logarithmic growth for 6 h. The wild type reached a cell density of $2.31 \times 10^8 \pm 0.87$. The PueA mutant, *P. chlororaphis pue*A::Kan^r, had an 80% decrease in cell number ($4.66 \times 10^7 \pm 0.13$), whereas both the complements, *P. chlororaphis pue*A::Kan^r pPueA-1 and *P. chlororaphis* pPueA-1 had an increase in cell densities, $2.86 \times 10^8 \pm 0.09$ (25% increase) and $3.85 \times 10^8 \pm 0.98$ (65% increase), respectively. The results obtained from the cell densities of each strain were reflected in the growth kinetic studies. Values for K_s and μ_{max} for polyurethane utilization were elucidated by varying the Impranil concentration from 0.18 to

Table 14.3 Growth kinetic analysis of *P. chlororaphis* and its derivatives using polyurethane as the sole carbon source

Strain	μ_{max}	Doubling time (min.)	K_s (mg ml^{-1})	Cell density (cells ml^{-1})
P. chlororaphis (wild type)	1.32	31.5	0.800	$2.31 \times 10^8 \pm 0.87$
P. chlororaphis pueA::Kan^r	1.09	38.2	0.917	$4.66 \times 10^7 \pm 0.13$
P. chlororaphis pueA::Kan^r (pPueA-1)	1.41	29.5	0.710	$2.86 \times 10^8 \pm 0.09$
P. chlororaphis (pPueA-1)	1.54	27.0	0.649	$3.85 \times 10^8 \pm 0.98$
P. chlororaphis pueB::Kan^r	1.19	34.9	0.893	$2.35 \times 10^8 \pm 0.148$
P. chlororaphis pueB::Kan^r (pPueB-1)	1.37	30.4	0.735	$3.59 \times 10^8 \pm 0.187$
P. chlororaphis (pPueB-1)	1.41	29.5	0.781	$3.99 \times 10^8 \pm 0.813$

The concentrations of Impranil DLN^{TM} used were: 9.0, 6.0, 3.0, 1.5, 0.75, 0.54, 0.375, and 0.18 mg ml^{-1} . Each concentration was prepared in triplicate

9.0 mg ml^{-1}. *P. chlororaphis* wild type exhibited a μ_{max} of 1.32 whereas, the PueA insert ional mutant, *P. chlororaphis pue*A::Kan^r, exhibited a μ_{max} of 1.09. It would be hypothesized that a deletion of the *pue*A gene would result in a decrease in growth rate. However, a large decrease in growth obtained from the insert ional mutant may indicate that PueA plays a major role as compared to PueB in polyurethane degradation by *P. chlororaphis*. When multiple copies of *pue*A gene were introduced into either the wild type, *P. chlororaphis* pPueA-1, a μ_{max} value of 1.54, or the mutant, *P. chlororaphis pue*B::Kan^r, pPueA-1, a μ_{max} value of 1.41, was obtained. An increase in the growth rate seems plausible since more PueA produced from the added plasmid would reflect more polyurethane degraded, resulting in an increase in the amount of nutrients available to the cells.

The PueB mutant, *P. chlororaphis pue*B::Kan^r, had a 18% decrease in cell number ($2.35 \times 10^8 \pm 0.148$) whereas, both the complement, *P. chlororaphis pue*B::Kan^r pPueB-1 and *P. chlororaphis* pPueB-1 had an increase in cell densities, $3.59 \times 10^8 \pm 0.187$ and $3.99 \times 10^8 \pm 0.813$, respectively. The results obtained from the cell densities of each strain were reflected in the growth kinetic studies. Values for K_s and μ_{max} for polyurethane utilization were elucidated by varying the Impranil concentration from 0.18 to 9.0 mg ml^{-1}. *P. chlororaphis* wild type exhibited a μ_{max} of 1.31. When multiple copies of the *pue*B gene were introduced into the wild type, *P. chlororaphis* pPueB-1, a μ_{max} value of 1.41 was obtained which was similar to the complement, *P. chlororaphis pue*B::Kan^r pPueB-1, μ_{max} value of 1.37. An increase in growth rate seems plausible since more PueB produced would reflect more polyurethane degraded resulting in an increase in the amount of nutrients available to the cells. However, these values are small and may indicate that PueB plays a minor role as compared to PueA in polyurethane degradation by *P. chlororaphis*. The insertion mutant, *P. chlororaphis pue*B::Kan^r, displayed a μ_{max} value of 1.19. Again, it would be hypothesized that the deletion of the *pue*B gene would result in a decrease in growth rate.

However, this small variation compared to the wild type suggests that degradation of polyurethane by *P. chlororaphis* may be more dependent on PueA.

14.5.3 Binding of Polyurethane by Polyurethanase Enzymes

Enzyme molecules can easily come in contact with water-soluble substrates thus allowing the enzymatic reaction to proceed rapidly. However, the enzyme molecules are thought to have an extremely inefficient contract with insoluble substrates (e.g. PU). In order to overcome this obstacle, enzymes that degrade insoluble substrates posses some characteristic that allows them to adhere onto the surface of the insoluble substrate (Van Tilbeurgh et al. 1986; Fukui et al. 1988; Hansen 1992).

The observations made by Akutsu et al. (1998) for the polyurethanase PudA indicate that this enzyme degrades PU in a two-step reaction: hydrophobic adsorption onto the PU surface followed by the hydrolysis of the ester bonds of PU. The PU esterase was considered to have a hydrophobic-PU-surface binding domain (SBD) and a catalytic domain. The SBD was shown to be essential for PU degradation. The structure observed in PudA has also been reported in PHA depolymerase, which degrades PHA. PHA is insoluble polyester synthesized as a food reserve in bacteria. In PHA depolymerase enzymes, the hydrophobic SBD has been determined by amino acid sequence analysis and its various physicochemical and biological properties (Fukui et al. 1988; Shinomiya et al. 1997). Another class of enzymes that contain a SBD is cellulases. Several cellulase enzymes have been observed to contain three main structural elements: the hydrolytic domain, a flexible hinge region, and a C-terminus tail region involved in substrate binding (Knowles et al. 1987; Bayer et al. 1985; Langsford et al. 1987).

So far, only two types of PUase enzymes have been isolated and characterized: a cell associated, membrane bound PU-esterase (Akutsu et al. 1998) and soluble, extracellular PU-esterases (Ruiz et al. 1999b; Allen et al. 1999; Vega et al. 1999). The two types of PUases seem to have separate roles in PU degradation. The membrane bound PU-esterase would allow cell-mediated contact with the insoluble PU substrate while, the cell-free extracellular PU-esterases would bind to the surface of the PU substrate and subsequent hydrolysis. Both enzyme actions would be advantageous for the PU-degrading bacteria. The adherence of the bacteria cell to the PU substrate via the PUase would allow for the hydrolysis of the substrate to soluble metabolites which would then be metabolised by the cell. This mechanism of PU degradation would decrease competition between the PU-degrading cell with other cells and also allow for more adequate access to the metabolites. The soluble, extracellular PU-esterase would, in turn, hydrolyze the polymer into smaller units allowing for metabolism of soluble products and easier access for enzymes to the partially degraded polymer.

Studies addressing binding of PUase to soluble PU have been also performed. The equilibrium binding of Impranil DLN (polyester-polyurethane) to purified PueA from *Pseudomonas chlororaphis* was studied by kinetic exclusion assays

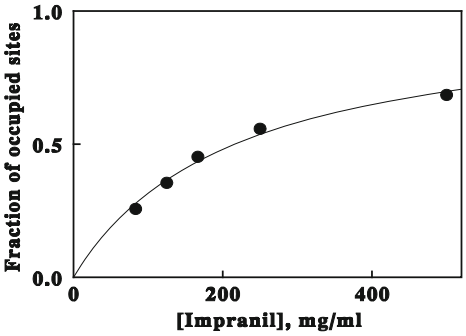


Fig. 14.8 Equilibrium binding of Impranil DLN to PueA. The concentration of occupied polyurethane binding sites present in different reaction mixtures of PueA and soluble Impranil DLN were determined by kinetic exclusion assays on a flow fluorimeter as described in the text. Each determination was expressed as a fraction of the total PueA in solution and plotted versus the concentration of free soluble polyurethane. Each datum represents the average of at least two determinations. The parameters for the curve drawn through the data were determined by nonlinear regression analysis using a one-site homogeneous binding model

conducted on a KinExA flow fluorimeter. Briefly, the KinExA comprises an immunoassay instrument that exploits an immobilized form of the polyurethane substrate to separate and quantify the fraction of unoccupied binding sites that remain in solution reaction mixtures of PueA and soluble polyurethane. In this case, the immobilized polyurethane was Bayhydrol 110 adsorption coated onto polystyrene beads, while the soluble polyurethane was Impranil DLN. The results of these binding studies are summarized in Fig. 14.6. Kinetic exclusion assays conducted with 6.6 μg ml^{-1} PueA in the absence of soluble polyurethane produced fluorescence signals of greater than 2.2 V with mvolt noise. In the presence of increasing concentrations of soluble Impranil DLN, the fluorescence signal attributed to PueA with unoccupied binding sites decreased to an extrapolated constant value at an infinitely high concentration of the soluble polyurethane that represented nonspecific binding to the beads. The fraction of soluble PueA that contained unoccupied polyurethane binding sites was calculated as the ratio of the difference between the fluorescence signal observed in the absence of Impranil DLN minus that observed in its presence, divided by the difference in fluorescence signals between zero and an infinitely highly high concentration of the soluble polyurethane.

The binding data in Fig. 14.8 were fit to a one-site homogeneous binding model with an apparent equilibrium dissociation constant of 220 ± 30 mg ml^{-1} Impranil DLN. Since both the soluble Impranil DLN and the immobilized Bayhydrol 110 are hydrolysable substrates for the active PueA enzyme, care was taken to perform individual measurements in such a manner as to minimize the time of exposure of the polyurethane substrates to the active PueA. Thus the PueA-Impranil DLN mixtures were assayed within two minutes of mixing, while the PueA captured on the immobilized Bayhydrol was exposed to the fluorescent labeling reagents and

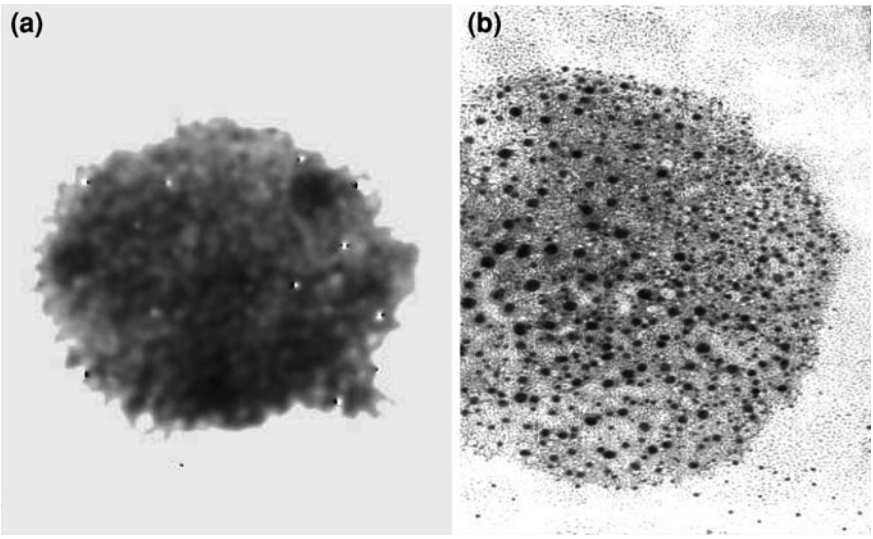


Fig. 14.9 Electron micrographs of embedded Bayhydrol 110^{TM} polyurethane particles. **a** Electron micrograph of polyurethane particles taken at a magnification of ×15,000. **b** Electron micrographs of Immunogold-labeled PueA (1:5,000,000,000 dilution of 0.83 mg ml^{-1} PueA) bound to embedded polyurethane particle at ×15,000 magnification

wash buffer within 4 min of the initial exposure of the hydrolase to the immobilized substrate. Control experiments demonstrated that much longer exposure times (at least 3-fold longer) were required before a time-dependent deterioration in individual fluorescence signals could be detected.

Electron micrographs were used in conjunction with the analysis of binding via the KinExA 3000, Kinetic Exclusion Assay unit. Grids were analyzed at high magnification and electron micrographs were produced from sections incubated in 1:5,000,000 PU and 1:5,000,000,000 PueA (Fig. 14.9). The TEM analysis of PueA, showed PueA to have a high affinity for the polyurethane substrate. Binding was found to be so extensive, that only the most dilute concentrations of PueA could be used to allow for visualization of areas with individual immunogold labeling.

14.6 Conclusions

The regularity in synthetic polymers allows polymer chains to pack easily, resulting in the formation of crystalline regions. Crystallinity limits accessibility of polymer chains to degradation, whereas amorphous regions within PU can degrade more readily. In addition, polyester-type PU is considered to be more susceptible to microbial attack than polyether-type PU. The hydrolysis of ester bonds in the polyester segments of PU has been shown to occur through esterase activity.

Little information has been available on the degradation of the isocyanate segment of PU however; the production of ammonia indicates that attack does occur.

A diverse group of microorganisms including fungi and bacteria capable of PU degradation can be isolated from soil. The majority of information available to date concerning the mechanisms that bacteria use in biodegradation of PU is from the Pseudomonad group. The esterase enzymes responsible for PU degradation were noted to have a high homology to Group I lipases. Upon nucleotide sequencing of these ORFs, the predicted amino acid sequence contained a Gly-X -Ser-X-Gly motif characteristic of serine hydrolases. Parsimony analysis of the predicted amino acid sequences for the PueA, PueB, PudA, and PulA polyurethanase enzymes and similar lipase enzymes have been performed. Interestingly, the PUase enzymes do not form a single cluster, but appear to be distributed among multiple lineages. These analyses suggest that PUase enzymes, so far studied, have evolved from lipases, and are not derived from a single source. Learning more about the pathways for degradation and the genes involved in PU degradation is essential in developing either recombinant derivatives or enriching for indigenous PU-degrading microorganisms for bioremediation.

References

Ahn JH, Pan JG, Rhee JS (1999) Identification of the tliDEF ABC transporter specific for lipase in *Pseudomonas fluorescens* SIK W1. J Bacteriol 181:1847–1852

Akatsuka H, Kawai E, Omori K, Shibatani T (1995) The three genes lipB, lipC, and lipD involved in the extracellular secretion of the *Serratia marcescens* lipase which lacks an N-terminal signal peptide. J Bacteriol 177:6381–6389

Akutsu Y, Nakajima-Kambe T, Nomura N, Nakahara T (1998) Purification and properties of a polyester polyurethane-degrading enzyme from *Comamonas acidovorans* TB-35. Appl Environ Microbiol 64:62–67

Allen A, Hilliard N, Howard GT (1999) Purification and characterization of a soluble polyurethane degrading enzyme from *Comamonos acidovorans*. Int Biodeter Biodegrad 43:37–41

Arpigny JL, Jaeger KE (1999) Bacterial lipolytic enzymes: classification and properties. Biochem J 343:177–183

Baumgartner JN, Yang CZ, Cooper SL (1997) Physical property analysis and bacterial adhesion on a series of phosphonated polyurethanes. Biomaterials 18:831–837

Bayer O (1947) Polyurethanes. Mod Plast 24:149–152

Bayer EA, Setter E, Lamed R (1985) Organization and distribution of the cellulosome in *Clostridium thermocellum*. J Bacteriol 163:552–559

Blake RC, Howard GT (1998) Adhesion and growth of a *Bacillus* sp on a polyesterurethane. Int Biodeter Biodegrad 42:63–73

Boubendir A (1993) Purification and biochemical evaluation of polyurethane degrading enzymes of fungal origin. Diss Abstr Int 53:4632

Cosgrove L, McGeechan PL, Robson GD, Handley PS (2007) Fungal communities associated with degradation of polyester polyurethane in soil. Appl Environ Microbiol 73:5817–5824

Cosgrove L, McGeechan PL, Handley PS, Robson GD (2010) Effect of biostimulation and bioaugmentation on degradation of polyurethane buried in soil. Appl Environ Microbiol 76:810–819

Crabbe JR, Campbell JR, Thompson L, Walz SL, Schultz WW (1994) Biodegradation of a collodial ester-based polyurethane by soil fungi. Int Biodeter Biodegrad 33:103–113
Darby RT, Kaplan AM (1968) Fungal susceptibility of polyurethanes. Appl Microbiol 16:900–905
Dombrow BA (1957) Polyurethanes. Reinhold Publishing Corporation, New York
Doung F, Soscia C, Lazdunski A, Marjier M (1994) The *Pseudomonas fluorescens* lipase has a C-terminal secretion signal and is secreted by a three-component bacterial ABC-exporter system. Mol Microbiol 11:1117–1126
Evans DM, Levisohn I (1968) Biodeterioration of polyester-based polyurethane. Int Biodeter Bull 4:89–92
Flilip Z (1978) Decomposition of polyurethane in a garabage landfill leakage water and by soil microorganisms. Eur J Appl Microbiol Biotechnol 5:225–231
Fried JR (1995) Polymer Science and Technology. Prentice Hall PTR, Englewood Cliffs
Fukui T, Narikawa T, Miwa K, Shirakura Y, Saito T, Tomita K (1988) Effect of limited trypic modifications of a bacterial poly(3-hydroxybutyrate) depolymerase on its catalytic activity. Biochimica Biophysica ACTA 952:164–171
Gautam R, Bassi AS, Yanful EK, Cullen E (2007) Biodegradation of automotive waste polyester polyurethane foam using *Pseudomonas chlororaphis* ATCC55729. Int Biodeter Biodegrad 60:245–249
Griffin GJL (1980) Synthetic polymers and the living environment. Pure Appl Chem 52:389–407
Halim El-Sayed AHMM, Mahmoud WM, Davis EM, Coughlin RW (1996) Biodegradation of polyurethane coatings by hydrocarbon-degrading bacteria. Int Biodeter Biodegrad 37:69–79
Hansen CK (1992) Fibronectin type III-like sequences and a new domain type in prokaryotic depolymerases with insoluble substrates. FEBS Lett 305:91–96
Hole LG (1972) Artificial leathers. Rep Prog Appl Chem 57:181–206
Howard GT, Blake RC (1999) Growth of *Pseudomonas fluorescens* on a polyester-polyurethane and the purification and characterization of a polyurethanase-protease enzyme. Int Biodeter Biodegrad 42:213–220
Howard GT, Crother B, Vicknair J (2001) Cloning, nucleotide sequencing and characterization of a polyurethanase gene (*pueB*) from *Pseudomonas chlororaphis*. Int Biodeter Biodegrad 47:141–149
Howard GT, Mackie RI, Cann IKO, Ohene-Adjei S, Aboudehen KS, Duos BG, Childers GW (2007) Effect of insertional mutations in the pueA and pueB genes encoding two polyurethanases in *Pseudomonas chlororaphis* contained within a gene cluster. J Appl Microbiol 103:2074–2083
Huang SJ, Roby MS (1986) Biodegradable polymers poly(amide-urethanes). J Bioact Compat Polym 1:61–71
Huang SJ, Macri C, Roby M, Benedict C, Cameron JA (1981) Biodegradation of polyurethanes derived from polycaprolactonediols. In: Edwards KN (ed) Urethane chemistry and applications. American Chemical Society, Washington, DC, pp 471–487
Jaeger KE, Ransac S, Dijkstra BW, Colson C, Van Heuvel M, Misset O (1994) Bacterial lipases. FEMS Microbiol Rev 15:29–63
Kanavel GA, Koons PA, Lauer RE (1966) Fungus resistance of millable urethanes. Rubber World 154:80–86
Kaplan AM, Darby RT, Greenberger M, Rodgers MR (1968) Microbial deterioration of polyurethane systems. Dev Ind Microbiol 82:362–371
Kawai F (1995) Breakdown of plastics and polymers by microorganisms. Adv Biochem Eng/Biotech 52:151–194
Kawai E, Akatsuka H, Idei A, Shibatani T, Omori K (1998) *Serratia marcescens* S-layer protein is secreted extracellularly via an ATP-binding cassette exporter, the Lip system. Mol Microbiol 27:941–952
Kawai E, Idei A, Kumura H, Shimazaki K, Akaksuka H, Omori K (1999) The ABC-exporter genes involved in the lipase secretion are clustered with the genes for lipase, alkaline protease and serine protease homologous in *Pseudomonas fluorescens* no. 33. Biochim Biophys Acta 1446(3):377–382

Kay MJ, Morton LHG, Prince EL (1991) Bacterial degradation of polyester polyurethane. Int Biodeter Bull 27:205–222

Kay MJ, McCabe RW, Morton LHG (1993) Chemical and physical changes occurring in polester polyurethane during biodegradation. Int Biodeter Biodegrad 31:209–225

Knowles J, Lehtovaara P, Teeri T (1987) Cellulase familes and their genes. Trends Biotechnol 5:255–261

Labrow RS, Erfle DJ, Santerre JP (1996) Elastase-induced hydrolysis of synthetic solid substrates: poly(ester-urea-urethane) and poly(ether-urea-urethane). Biomaterials 17:2381–2388

Langsford ML, Gilkes NR, Sing S, Moser B, Miller RC Jr, Warren RAJ, Kilburn DG (1987) Glycosylation of bacterial cellulases prevents proteolytic cleavage between functional domains. FEBS Lett 225:163–167

Marchant RE (1992) Biodegradability of biomedical polymers. In: Hamid SH, Amin MB, Maadhah AG (eds) Handbook of polymer degradation. Marcel Dekker, Inc, New York, pp 617–631

Marchant RE, Zhao Q, Anderson JM, Hiltner A (1987) Degradation of a poly(ether urethane urea) elastomer: infra-red and XPS studies. Polymer 28:2032–2039

Nakajima-Kambe T, Onuma F, Kimpara N, Nakahara T (1995) Isolation and characterization of a bacterium which utilizes polyester polyurethane as a sole carbon and nitrogen source. FEMS Microbiol Lett 129:39–42

Nakajima-Kambe T, Onuma F, Akutsu Y, Nakahara T (1997) Determination of the polyester polyurethane breakdown products and distribution of the polyurethane degrading enzyme of *Comamonas acidovorans* steain TB-35. J Ferment Bioeng 83:456–460

Ossefort ZT, Testroet FB (1966) Hydrolytic stability of urethane elastomers. Rubber Chem Technol 39:1308–1327

Pathirana RA, Seal KJ (1983) *Gliocladium roseum* (Bainier), a potential biodeteriogen of polyester polyurethane elastomers. Biodeterioration 5:679–689

Persson B, Bentsson-Olivecrona G, Enerback S, Olivecrona T, Jornvall H (1989) Structure features of lipoprotein lipase: lipase family relationships, binding interactions, non-equivalence of lipase cofactors, vitellogenin similarities and functional subdivision of lipoprotein lipase. Eur J Biochem 179:39–45

Phua SK, Castillo E, Anderson JM, Hiltner A (1987) Biodegradation of a polyurethane in vitro. J Biomed Mater Res 21:231–246

Rowe L, Howard GT (2002) Growth of *Bacillus subtilis* on polyurethane and the purification and characterization of a polyurethanase-lipase enzyme. Int Biodeter Biodegrad 50:33–40

Ruiz C, Hilliard N, Howard GT (1999a) Growth of *Pseudomonas chlororaphis* on a polyester-polyurethane and the purification and characterization of a polyurethanse-esterase enzyme. Int Biodeter Biodegrad 43:7–12

Ruiz C, Main T, Hilliard N, Howard GT (1999b) Purification and characterization of two polyurethanse enzymes from *Pseudomonas chlororaphis*. Int Biodeter Biodegrad 43:43–47

Santerre JP, Labrow RS (1997) The effect of hard segment size on the hydrolytic stability of polyether-urea-urethanes when exposed to cholesterol esterase. J Biomed Mater Res 36:223–232

Santerre JP, Labrow RS, Adams GA (1993) Enzyme-biomaterial interactions: effect of biosystem on degradation of polyurethanes. J Biomed Mater Res 27:97–109

Santerre JP, Labow RS, Duguat DG, Erfle D, Adams GA (1994) Biodegradation evaluation of polyether and polyester-urethanes with oxidative and hydrolytic enzymes. J Biomed Mater Res 28:1187–1199

Saunders JH, Frisch KC (1964) Polyurethanes: chemistry and technology, part II: technology. Interscience Publishers, New York

Schnabel W (1981) Polymer degradation: principles and potential applications. Macmillan Publishing Co. Inc, New York, pp 178–215

Shinomiya M, Iwata T, Kasuya K, Doi Y (1997) Cloning of the gene for poly(3-hydroxybutyric acid) depolymerase of *Comamonas testosteroni* and functional analysis of its substrate-binding domain. FEMS Microbiol Lett 154:89–94

Stern RS, Howard GT (2000) The polyester polyurethanase gene (*pueA*) from *Pseudomonas chlororaphis* encodes a lipase. FEMS Microbiol Lett 185:163–168

Tang YW, Santerre JP, Labrow RR, Taylor DG (1997) Application of macromolecular additives to reduce the hydrolytic degradation of polyurethanes by lysosomal enzymes. Biomaterials 18:37–45

Uhlig K (1999) Discovering polyurethanes. Hanser Publisher, Munich

Ulrich H (1983) Polyurethane. In: Modern plastics encyclopedia, vol 60. McGraw-Hill, New York, pp 76–84

Urbanski J, Czerwinski W, Janicka K, Majewska F, Zowall H (1977) Handbook of analysis of synthetic polymers and plastics. Ellis Horwood Limited, Chichester

Van Tilbeurgh H, Tomme P, Claeyssens M, Bhikhahai R, Pettersson G (1986) Limited proteolysis of the cellobiohydrolase I from *Trichoderma reesei*. FEBS Lett 204:223–227

Vega R, Main T, Howard GT (1999) Cloning and expression in *Escherichia coli* of a polyurethane-degrading enzyme from *Pseudomonas fluorescens*. Int Biodeter Biodegrad 43:49–55

Wales DS, Sagar BR (1988) Mechanistic aspects of polyurethane biodeterioration. In: Houghton DR, Smith RN, Eggins HOW (eds) Biodeterioration, 7th edn. Elsevier Applied Science, London, pp 351–358

Winkler FK, D'Arcy A, Hunzinger W (1990) Structure of human pancreatic lipase. Nature 343:7–13

Young RJ, Lovell PA (1994) Introduction to polymers', 2nd edn. Chapman & Hall, London

Chapter 15
Microbial Degradation of Recalcitrant PAHs-Microbial Diversity Involving Remediation Process

Shelly Sinha, Pritam Chattopadhyay and Sukanta K. Sen

15.1 Introduction

Domestic pollutants, largely due to population explosion and industrial inputs lead to the accumulation of various types of recalcitrant xenobiotic compounds (Hadibarata et al. 2009; Igwo-Ezikpe et al. 2010). As majority of them persist for longer period of time and are carcinogenic in nature, their disposal is a matter of global concern (Jain et al. 2005). Primarily, xenobiotic compounds are anonymous to living organisms and also have a tendency to get accumulated in the environment (Sinha et al. 2009). They encompass pesticides, fuels, solvents, alkanes, synthetic azo dyes, polyaromatic, nitroaromatic, chlorinated and polycyclic hydrocarbons. Amongst them, the presence of polycyclic aromatic hydrocarbons (PAHs) in the environment causes acute health hazard with their intrinsic chemical stability, high recalcitrance ability against different types of degradation and high toxicity to living organisms for their mutagenic or carcinogenic properties (Zhang et al. 2006). Apart from it, they are ubiquitous and prevail as persistent bioaccumulative toxins (PBT) (NiChadhain et al. 2006). For instance, phenanthrene, a lipophilic and relatively insoluble in water, is skin photosensitizer and mild allergenic to human (Hafez et al. 2008). It is also found as an inducer of the

S. Sinha
Rabindra Mahavidyalaya, Champadanga, Hooghly,
West Bengal, India

P. Chattopadhyay
Plant Biotechnology Laboratory, Department of Botany,
Visva-Bharati, Santiniketan, India

S. K. Sen (✉)
Microbiology Division, Department of Botany, Visva-Bharati,
Santiniketan, India
e-mail: sksenvb@rediffmail.com

S. N. Singh (ed.), *Microbial Degradation of Xenobiotics*,
Environmental Science and Engineering, DOI: 10.1007/978-3-642-23789-8_15,

sister chromatid exchange process (Popp et al. 1997) and a potent inhibitor of gap-junction intercellular communications (Bláha et al. 2002).

PAHs get absorbed to organic-rich soils and sediments, accumulate in fish and other aquatic organisms and enter into food chain through seafood consumption (Mrojik et al. 2003). These compounds require activation by electrophilic metabolites to exert their carcinogenic or mutagenic or teratogenic effects. Primarily, three principal pathways are proposed for metabolic activation of PAH compounds; one via the bay region of dihydrodiol epoxide by cytochrome P450 enzymes (CYPs), other via radical cation by one-electron oxidation and the third one via *ortho*-quinone pathway by dihydrodiol dehydrogenase (Xue and Warshawsky 2005). Besides these major pathways, it may also follow minor metabolic activation pathway that contains a primary benzylic alcoholic group or secondary hydroxyl group(s) (Liang et al. 2006).

The main processes for their removal are microbial transformation and/or degradation (Sinha et al. 2009). Biodegradation is one of the natural processes that helps to remove xenobiotic chemicals from the environment by microorganisms (Singh 2008). Soil microorganisms are able to degrade PAHs by any of the three metabolic pathways stated earlier. However, the physico-chemical characteristics of compounds and biological properties of soils significantly influence the degradation ability of naturally occurring microorganisms for field bioremediation. Detoxifying abilities (i.e. mineralization, transformation and/or immobilization of pollutants) of bacteria play a fundamental role in biogeochemical cycles for sustainable development of the biosphere (NiChadhain et al. 2006). Modern biological techniques are widely used to promote the efficiency of microbial PAH-degradation and to understand the biodegradation pathways more clearly. *Burkholderia* (Lloyd-Jones et al. 1999), *Pseudomonas* (Ma et al. 2006), *Sphingobium* (Liu et al. 2004), *Staphylococcus* (Mallick et al. 2007), *Bacillus* constitute the major bacterial genera for microbial transformation of PAH compounds.

15.2 Structure of PAHs

The polynuclear aromatic hydrocarbons are composed of two or more aromatic (benzene) rings which are fused together in a linear, angular or cluster arrangement when a pair of carbon atoms is shared between them (Dhote et al. 2010). PAHs toxicity is quite structurally dependent, with isomers (PAHs with the same formula and number of rings) varying from non-toxic to extremely toxic. Thus, highly carcinogenic PAHs may be small or large. The PAH compound, benzo[α]pyrene (BAP), was notable as the first discovered chemical carcinogen (Johnsen et al. 2005). The US Environmental Protection Agency (EPA), has classified seven PAHs as probable human carcinogens: benzo[α]anthracene, BAP, benzo[β]fluoranthene, benzo[κ]fluoranthene, chrysene, dibenzo(α,η)anthracene and indeno(1,2,3)pyrene. PAHs known for their carcinogenic, mutagenic and teratogenic properties, are benzo[α]anthracene and chrysene ($C_{18}H_{12}$), benzo[β]fluoranthene,

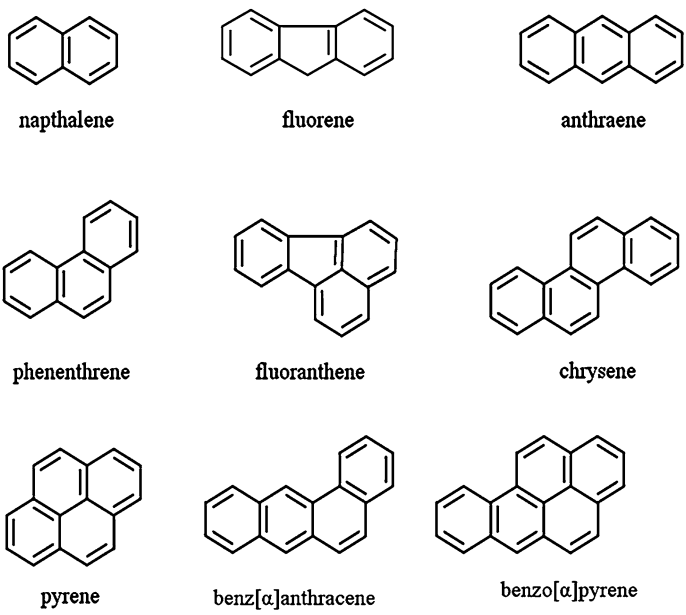


Fig. 15.1 Schematic diagrams of different PAHs

benzo[κ]fluoranthene ($C_{20}H_{12}$), benzo[α]pyrene ($C_{20}H_{12}$), coronene ($C_{24}H_{12}$), dibenz(α,η)anthracene ($C_{20}H_{14}$), and ovalene ($C_{32}H_{14}$) (Luch 2005; Xue and Warshawsky 2005). High prenatal exposure to PAH is associated with lower IQ of the progeny. Generally, PAH solubility and hydrophobicity are inversely proportional to an increase in number of fused benzene rings, whereas volatility decreases with an increasing number of fused rings (Kanaly et al. 2000b). The fused aromatic rings of different PAHs are well illustrated in Fig. 15.1.

15.3 Metabolism of PAH Compounds

PAHs with two to three fused aromatic rings are considered as low molecular weight (LMWPAHs) such as, naphthalene, anthracene and phenanthrene, whereas those with four and more fused rings are high molecular weight (HMWPAHs) that include chrysene, fluoranthene and pyrene, etc. (Igwo-Ezikpe et al. 2010). Isolation of highly efficient PAHs (containing four aromatic rings), degrading bacterium is the pioneering study that opened the possibility of PAH biodegradation (Heitkamp and Cerniglia 1988). Recent work was reported from the contaminated soils of Nigeria where bacterial isolates were used to biodegrade HMWPAHs like chrysene, pyrene and fluoranthrene (Igwo-Ezikpe et al. 2010). During metabolism, the chemical structure of PAHs is altered. Pathways of biotransformation, that operate in major groups of organisms, are of ancient origin where reactions mainly

operate to detoxify poisonous compounds, but in some cases, the metabolic intermediates are more toxic causing deleterious effects (Kanaly et al. 2000a).

Exposure to numerous PAHs signifies to a multiple compounds. However, difference occurs in combination of these particular compounds due to severity of different PAHs. Actually, understanding the dynamics of complex metabolism with reference to single metabolism of PAHs is necessary with respect to their possible effects on the toxicity expression measured accurately for impact assessment and to guide for possible remediation strategies. PAH necessitates activation of electrophilic metabolites to exert their deleterious effects. DNA damage caused by the reactive metabolites of PAH is concerned with the DNA covalent bond formation to form stable or depurinating adducts, resulting in apurinic sites and oxidative damage (Popp et al. 1997).

Studies are made to compare the metabolism of phenanthrene (PHE), flouranthene (FLA) and BAP in single, binary and ternary mixtures by monitoring the disappearance of the parent compound (Sinha et al. 2009). PAH metabolism with primary (single) PAH differed from metabolism in both binary and ternary mixtures. Enzyme competitiveness was evident in the metabolism of mixtures and differed significantly with the metabolism pattern of individual PAH. PAH structure also affected metabolism in mixtures and caused toxicity effects during complete metabolism. PAH concentration changed over time and found faster depletion with single PAH metabolism, followed by ternary mixture and finally binary metabolism. Such studies affirm the importance of substrate interactions for consideration in the risk assessment approaches of the danger posed by PAHs exposure.

15.4 Role of Microbes

Microbial diversity, the richness of species in environmental sites, provides a huge reservoir of resources that can be utilized for our benefit. However, the major bottleneck is our knowledge about the true diversity of bacterial life (Jain et al. 2005). Moreover, they have evolved the ability to utilize such highly reduced and recalcitrant compounds as potential source of carbon and energy (Phale et al. 2007). Therefore, mining out the array of microbial diversity would play a key role to develop effective and environment friendly ‘green’ technologies. Aerobic degradation of aromatics is initiated by oxidizing the ring, making them more susceptible to cleavage by ring-cleaving dioxygenases. Predominance of aromatic degradation genes in plasmids, transposons, and integrative genetic elements (and their shuffling through horizontal gene transfer) has lead to the evolution of novel aromatic degradative pathways. This enables the microorganisms to utilize a multitude of aromatics via common routes of degradation leading to metabolic diversity.

Several bacterial, fungal and algal strains are able to degrade a wide variety of PAHs. Most commonly reported bacterial species include *Acinetobacter*

calcoaceticus, *Alcaligens denitrificans*, *Mycobacterium* sp., *Pseudomonas putida*, *Pseudomonas fluorescens*, *Pseudomonas vesicularis*, *Pseudomonas cepacia*, *Rhodococcus* sp., *Corynebacterium renale*, *Moraxella* sp., *Bacillus cereus*, *Beijerinckia* sp., *Micrococcus* sp., *Pseudomonas paucimobilis* and *Sphingomonas* sp. (Chowdhury et al. 2008). Bacteria develop strategies for deriving energy virtually from every compound under oxic or anoxic conditions, using ultimate electron acceptors such as nitrate, sulfate, and ferric ions. Amongst PAH compounds, benzene ring is next to glucosyl residues and most extensively found unit of chemical structure in nature. Either aerobically or anaerobically, the bacterial enzymes are highly efficient to break resonance structure and to help in operating carbon cycle (Diaz 2004). Gene clusters, that code for the catabolism of aromatic compounds, are frequently found in transposons and plasmids which assist their horizontal gene transfer and enhance adaptation of specific bacterial genera to novel pollutants.

15.5 Biochemical Pathway

PAH degradation by microbial transformation mainly initiates with the action of intracellular dioxygenases after the toxic compound is taken up by the cells. Bacteria most often oxidize PAHs to *cis*-dihydrodiols by incorporation of both atoms of an oxygen molecule. The *cis*-dihydrodiols are further oxidized, first to the aromatic dihydroxy compounds (catechols) and then channeled through the *ortho*- or *meta*-cleavage pathways (Cerniglia 1984; Karthikeyan and Bhandari 2001).

The aerobic biotransformation of PAHs has been extensively studied (Cerniglia 1984; Jain et al. 2005). In response to immense turnover of the aromatic compounds in the carbon cycle, well organized channels of aerobic catabolism have been evolved separately during evolution (Chauhan et al. 2008). The aerobic strategy of degradation shows advantages over anaerobic ones. Commonly, the aerobic organisms overcome the problem of degradation with oxygenases that initially reduce elemental oxygen to activate it, permitting to insert into inert PAH compound. The oxygen of water is inserted into double bonds with hydratases and carbonic acid is added onto molecules with carboxylases. Compared to oxygen, most of the alternative electron acceptors have low standard reduction potential ($E^{\circ\prime}$) values, and convert into less standard Gibbs free energy change ($\Delta G^{\circ\prime}$) when coupled with the oxidation of any given substrate. Thus, it appears that aerobic culture techniques are relatively simple, as well as, efficient and is applicable for oxidative degradation (Adriaens and Vogel 1995).

The aerobic catabolic funnel mostly includes peripheral pathways involving oxygenation reactions carried out by monooxygenases or hydroxylating dioxygenases and generate dihydroxy aromatic compounds. These intermediate compounds were processed through either *ortho* or *meta* cleavage leading to central intermediates such as protocatechuates, catechols, gentisates, homoprotocatechuates, homogentisates, hydroquinones and hydroxyquinols which are further

transformed to tricarboxylic acid cycle intermediates (Mallick et al. 2007) and finally chanelled into the intermediates of Kreb's cycle. Aerobic bioremediation of HMWPAHs, including BAP, must utilize co-metabolic degradation that requires a carbon as an energy source and oxygen (Juhasz and Naidu 2000). The pathway of degradation described for *Sphingomonas yanoikuyae* JAR02 utilized salicylate as an inducer as well as a carbon and energy source. Previous studies also showed the ability of salicylate to stimulate HMWPAH mineralization, but no metabolites were identified (Chen and Aitken 1999).

In the absence of molecular oxygen, redox potential becomes a critical factor in determining the metabolic diversity of microbial population in soils, sediments, and aquifer systems (Karthikeyan and Bhandari 2001). PAHs, under anaerobic conditions, are mineralized if they serve as electron donating substrates for primary metabolism. Several PAHs themselves act as terminal electron acceptors (TEA), supporting growth of microorganisms by gaining energy from the oxidation of simple substrates (e.g. H_2). The degradation of xenobiotic compounds by anaerobes, such as *Pseudomonas*, *Desulfobacterium*, *Desulfovibrio*, *Methanococcus*, *Methanosarcina* and dehalogenating bacteria, become a subject of extensive research during last two decades (Gibson and Harwood 2002). In the absence of molecular O_2, alternative electron acceptors, such as NO^{3-}, Fe^{2+} and $SO_4{}^{3-}$ are used to oxidize aromatic compounds (Zhang et al. 2006; Chauhan et al. 2008). Benzoyl coenzyme-A (CoA) pathway is one of the centralized pathways in majority of denitrifying anaerobes where key enzyme is benzoate coenzyme. CoA ligases, oxidoreductases, and decarboxylases are suggested enzymes involved in anaerobic transformation of polycyclic aromatic compounds. Carboxylation, reductive dehydroxylation, reductive deamination, reductive dehalogenation, oxidation of carboxymethyl groups, methyl oxidation, *o*-demethylation, transhydroxylation, and decarboxylation are discussed as possible peripheral metabolic reactions that occur during the anaerobic transformation process. Under sulfate reducing conditions, carboxylation is reported as the initial step in PAH biotransformation (Meckenstock et al. 2000). Exception to that, in naphthalene biotransfomation, hydroxylation is the initial step (Bedessem et al. 1997).

Attempts were made to use toxic LMWPAHs which were found unsuitable for use as biostimulation strategy. The biotransformation of pyrene and BAP is well studied in different bacterial species, such as *Mycobacterium vanbaalenii* PYR-1, *M. flavescens* PYR-GCK, *Mycobacterium* RJGII-135, *Mycobacterium* KR2 and *Mycobacterium* AP1. *Mycobacterium* KMS was used to study the metabolism during pyrene transformation (Peng et al. 2008). In such transformation process, pyrene hydroxylation takes place at 1,2 positions, leading to the formation of 4-hydroxy-perinaphthenone which is the ultimate product only found in *M. vanbaalenii* PYR-1 cultures (Khan et al. 2001). Another pathway involves the accumulation of 6,6′-dihydroxy-2,2′-biphenyl-dicarboxylic acid in *Mycobacterium* AP1. Several key metabolites, including pyrene-4,5-dione, *cis*-4,5-pyrene-dihydrodiol, phenanthrene-4,5-dicarboxylic acid, and 4-phenanthroic acid are synthesized. Pyrene-4,5-dione, accumulates as a final product in some gram-negative

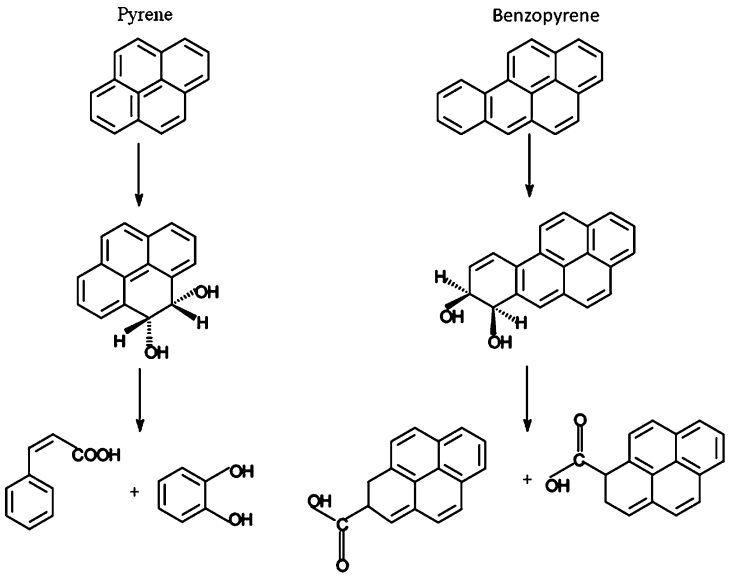


Fig. 15.2 Pathway of degradation of two significant PAH compounds

bacteria and is further utilized in biotransformation (Liang et al. 2006). The generalized pathway of biodegradation of pyrene and BAP is presented in Fig. 15.2. Pyrene-4,5-dione is formed following autooxidation of 4,5-dihydroxy-pyrene and is also a pyrene transformation metabolite of certain bacteria e.g. *S. yanoikuyae* R1 (Khan et al. 2001).

15.6 Expanding Catabolic Repertoire for PAHs Degradation

Degradation of PAH compounds is frequently intervened by a network of enzyme systems, having constraints in completion of the process. The manipulation of the catabolic genes from degradative enzymes is the possible way to solve the problem and boost up the process (Chauhan et al. 2008). Degradation primarily depends on the adapting response of the microbial communities which include both selective enrichment (amplification of genes) and genetic changes (gene transfer or mutation), simultaneously. Silent mobilization of sequences into the functional catabolic routes enhances advancement of substrate range by slow or spontaneous mutations, and lessens down the recalcitrance of several toxic PAHs (Hadibarata et al. 2009). In case of gram positive bacteria especially mycobacteria, genetical and biochemical data of HMWPAH degradation are relatively lower, as they possess extremely resistant cell surface compound, which results in lowering growth rate and triggers the activity of cell clumping. Also in gram positive

bacteria, *nid* and *pdo* genes encode HMWPAH dioxygenases whereas in gram negative bacteria *nah*, *pah* and *phn* genes encode LMWPAH dioxygenases (Liang et al. 2006).

Mobile genetic elements support horizontal gene transfer more accurately via conjugation or transformation that impart novel phenotypes or modify existing genes through mutational processes. Responsible catabolic plasmids are TOL, OCT, CAM, NAH, etc. (Mishra et al. 2001). Studies further revealed that horizontal transfer of catabolic genes occured mostly by means of plasmid-mediated conjugation in soil microcosms or in bioreactors by inoculation of a donor strain containing natural catabolic genes (Fetzer 2000; Peng et al. 2008).

15.7 Naphthalene Degradation

In addition to *Mycobacterium*, the biodegradative pathways are also reported from the genera *Corynebacterium*, *Aeromonas*, *Rhodococcus*, *Bacillus* and *Pseudomonas* (Mrojik et al. 2003). A self transferable plasmid with the highly versatile catabolic genes for naphthalene degradation was reported from *P. putida* G7 (Dunn and Gunsalus 1973). *P. stutzeri* AN10 is a naphthalene-degrading strain whose dissimilatory genes are chromosomally encoded. The entire naphthalene-degradating lower pathway genes of *P. stutzeri* AN10 are sequenced together with upper-pathway (Bosch et al. 1999). The *nahGTHINLOMKJ* genes encode enzymes for the metabolism of salicylate to pyruvate and acetyl-CoA, and *nahR* encodes the *nah*R regulatory protein. The catabolic modules are recruited through transposition events and recombination among *tnpA*-like genes. Subsequent rearrangements and deletions of non-essential DNA fragments are responsible in the formation of the actual catabolic pathway. Furthermore, the genes encoding the xylene or toluene-degradation enzymes of *P. putida* mt-2 (pWW0) were found to co-exist with the *nah* genes of the *P. stutzeri* AN10 ancestral genome (Bosch et al. 2000).

The complete 83,042 bp sequence of naphthalene degrading plasmid pDTG1 of *putida* NCIB 9816-4 was determined and the processes by which the *nah* and *sal* operons were compiled and distributed in nature were examined. Eighty-nine open reading frames were predicted using computer analyses, comprising 80.0% of the pDTG1 DNA sequence (Dennis and Zylstra 2004). The most distinctive feature of the plasmid was the upper and lower naphthalene degradation operons which occupied 9.5 and 13.4 kb regions, respectively, bordered by numerous defective mobile genetic fragments. It was further observed that the plasmid showed homologues of genes required for large plasmid replication, maintenance, and conjugation as well as synthesis of transposases, resolvases and integrases that manifest an evolution entailing the lateral transfer of DNA between bacterial species (Bosch et al. 2000). Also, there are genes that maintain a high degree of sequence similarity to other known degradation genes, as well as genes involved in chemotaxis.

15.8 Pyrene Degradation

Most abundant HMWPAH is none other than pyrene, present in environmental samples. The pathways for the biodegradation of pyrene are well documented for actinomycetes and fungal species (Kim et al. 2005b, 2008; Liang et al. 2006). However, in recent past, a few bacterial isolates, capable of metabolizing pyrene, were also reported (Sheng et al. 2009). Pyrene degradation studies in *Mycobacterium* PYR-1 showed involvement of ring oxidation and ring cleavage mechanisms. *M. vanbaalenii* PYR-1 was the first bacterium isolated by virtue of its ability to metabolize the HMWPAH pyrene.

Metabolic, genomic, and proteomic approaches were used to construct a complete and integrated pyrene degradation pathway for *M. vanbaalenii* PYR-1 (Liang et al. 2006). In compliance, genome sequence analyses to identify genes involved in the pyrene degradation pathway were worked out. Also to identify proteins involved in the degradation, proteome analysis of cells exposed to pyrene was undertaken using one-dimensional gel electrophoresis in combination with liquid chromatography–tandem mass spectrometry.

Database searching of the genome of *M. vanbaalenii* PYR-1 resulted in the identification of 1,028 proteins with a protein false discovery rate of <1%. Based on both genomic and proteomic data, it was possible to identify 27 enzymes involved in pyrene degradation pathway. The analyses indicate that the bacterium degrades pyrene to central intermediates through *o*-phthalate and the ketoadipate pathway (Kim et al. 2005b, 2007, 2008). Proteomic analysis also revealed that 18 enzymes in the pathway were upregulated more than twofold, as indicated by peptide counting. Three copies of the terminal subunits of ring-hydroxylating oxygenases (NidAB2, MvanDraft_0817/0818 and PhtAaAb), dihydrodiol dehydrogenase (MvanDraft_0815), and ring cleavage dioxygenase (MvanDraft_3242) were detected only in pyrene-grown cells. A comprehensive picture of pyrene metabolism in *M. vanbaalenii* PYR-1 was drawn and a useful framework for understanding cellular processes involved in PAH degradation was drafted (Kim et al. 2007). Recently, *Leclercia adecarboxylata* PS4040 is found as a potent degrader of pyrene. GC-Mass spectra confer that 1-hydroxypyrene, 2-henanthrenedicarboxylic acid, 2-carboxybenzaldehyde, *ortho*-phthalic acid and 1,2-benzene diol (catechol) are produced as possible metabolites (Sarma et al. 2010).

15.9 Anthracene Degradation

Anthracene is a persistent and toxic PAH used in dyes, wood preservatives, coal tar, insecticides and commonly found at sites of gas factory pollution. The toxicological data specific to anthracene is quite uncommon and somewhat conflicting. Anthracene biodegradation by Gram-negative and Gram-positive bacteria and numerous fungi has been observed (Cerniglia 1992). Plasmids pKAl, pKA2, and pKA3, approximately 100 kb size of each, were detected from these

isolates and characterized. These plasmids show homology between upper and lower NAH7 plasmid catabolic genes. These plasmids, including NAH7, are shown to mineralize phenanthrene, anthracene as well as naphthalene and are found to encode the genotype established conjugation experiments. *P. fluorescens* 5RL showed the complete lower pathway inactivation by transposon insertion in *nah*G (Karthikeyan and Bhandari 2001). This was the first direct evidence to indicate the involvement of NAH plasmid-encoded catabolic gene in degradation of polynuclear aromatic hydrocarbons other than naphthalene (Igwo-Ezikpe et al. 2010). The well-characterized plasmid-encoded naphthalene degradation pathway in *P. putida* PpG7 (NAH7) was used to investigate the role of the NAH plasmid-encoded pathway in mineralizing phenanthrene and plasmids of *Pseudomonas* sp. KA1, pKA2, and pKA3, approximately 100 kb in size, were characterized. In *P. fluorescens* 5RL, the complete inactivation of lower pathway by transposon insertion in *nah*G, accumulates a metabolite from phenanthrene and anthracene degradation. Association of catabolic plasmid in the degradation of anthracene by *Pseudomonas* strain E is identified through plasmid curing and agarose gel electrophoresis (Kumar et al. 2010).

Strains of *Sphingomonas* are reported to degrade a wide range of compounds such as substituted naphthalene, fluorene, substituted phenanthrene, pyrene, chlorinated diphenylether, chlorinated furan, chlorinated dibenzo-*p*-dioxin, carbazole, oestradiol, polyethylenglycols and an array of different herbicides and pesticides (Basta et al. 2005). The genes for catabolic pathways are often located in *Sphingomonas* strains, separately from each other, or, at least, are not organized in coordinately regulated operons. This was observed with the genes involved in the degradation of naphthalene, biphenyl and toluene by *S. yanoikuyae* B1 and *S. aromaticivorans* F199 (Cho and Kim 2001).

15.10 Phenanthrene Degradation

Phenanthrene, a PAH with three condensed rings fused in angular fashion, has a 'bay-region' and a 'K-region' and is often used as a model substrate for studies on the metabolism of carcinogenic PAHs (Igwo-Ezikpe et al. 2010). For last few decades, a number of studies on phenanthrene degradation by several Gram-negative and Gram-positive bacteria revealed metabolic diversity involved in phenanthrene degradation (Kim et al. 2005a). Universally, the metabolic pathway is initiated by the double hydroxylation of the bay-region of phenanthrene by a dioxygenase enzyme to form *cis*-3,4-phenanthrenedihydrodiol. The resultant dihydrodiol is then converted by the action of dihydrodiol dehydrogenase to 3,4-dihydroxyphenanthrene, which undergoes *meta*-cleavage, and in subsequent steps, the ring-cleavage product is converted to 1-hydroxy-2-naphthoic acid. 1-Hydroxy-2-naphthoic acid is further degraded by two distinct pathways reported so far (Evans et al. 1965). *Staphylococcus* PN/Y, capable of utilizing phenanthrene as a sole source of carbon and energy, was isolated from petroleum-contaminated soil. Phenanthrene degrading

plasmid was designated pPHN which had been confirmed by establishing a correlation between phenanthrene degradative ability and plasmid removal from strain PN/Y (Mallick et al. 2007).

15.11 Chrysene Degradation

It is a HMWPAH compound consisting of four fused benzene rings with solubility 0.006 mg/l and classified as priority pollutants by the US EPA (Smith et al. 1989). It is produced as gas during combustion of coal and petroleum products and is calcitrant. In pure solid form, chrysene is a colourless, crystalline solid which flouresces red–blue under UV light (Dhote et al. 2010). It is virtually insoluble in water, and only less soluble in alcohol, ether and glacial acetic acid (Hadibarata et al. 2009). Chrysene oxidation occurs by incorporation of an oxygen molecule in an aromatic ring and catalyzed by dioxygenase to a *cis*-dihydrodiol intermediate, which undergoes further metabolism via pyridine nucleotide dependent dehydrogenation reaction to produce catechols. These are substrates for ring cleavage enzymes which lead to the complete mineralization. The process indicates that bacteria use chrysene as the sole source of carbon and lead to complete mineralization (Hinchee et al. 1994; Igwo-Ezikpe et al. 2006).

Several factors determine its degradation rate and extent of metabolism, including molecular size, solubility, lipophilicity, volatility and concentration. It is moderately soluble in benzene at ambient temperature, while readily dissolves in boiling benzene and toluene. The environmental factors affecting degradation are temperature that affects kinetics of an enzyme, pH, oxygen availability for aerobic microorganisms, salinity, light intensity for photolysis, sedimentation ability for adsorption and the presence of growth-stimulating additives, such as co-metabolites. *Rhodococcus* sp. Strain UW1 (Walter et al. 1991) and *S. yanoikuyae* (Boyd et al. 1999) oxidize chrysene, while *P. fluorescens* utilizes chrysene and benz[α]anthracene as the sole carbon sources (Caldini et al. 1995). *Acinetobacter anitratus*, *A. mallei*, *Alcaligenes faecalis*, and *Micrococcus varians* are other bacterial species which are very efficient to degrade this HMWPAH compound (Igwo-Ezikpe et al. 2006).

15.12 Molecular Approach

Typically PAH degrading ability of bacteria anchors in plasmids that code for useful catabolic gene expression to empower them for certain strategies with proper management. So, ultimately degradation technology is spanning the spectrum from environmental monitoring to bioremediation (Singh 2008). This also encompasses molecular approach in a broad spectrum to characterize bacterial nucleic acids from environmental samples (Kumar et al. 2010).

Gene amplification, subsequent analysis of bacterial rRNA genes by sequencing, preparation of metagenomic libraries, RFLP, dot-blot, southern blot, denaturing gradient gel electrophoresis (DGGE), microarrays are numerous techniques applied for elucidating the essential genes required for degradation.

Ligation-mediated polymerase chain reaction (LMPCR) is a method for the detection of DNA adducts at individual nucleotide positions and is used to map the adducts of PAHs (Pfeifer et al. 1998). Metagenomics and its vast approach have attracted scientists for the detection of the desired catabolic genes. Primarily, it is a culture dependent genomic analysis, either function driven approach or sequence driven approach, of total microbial communities, which present admittance to recover unknown sequences (Schloss and Handelsman 2003). Nevertheless, the technique is relevant, still with numerous drawbacks, like less recovery of desired clone. However, the metagenomic libraries are particularly promising for locating denitrifying genes (Chauhan et al. 2008).

To investigate different degrading genes in relation to bacterial ecology, fingerprinting techniques are also used which are tagged to a PCR reaction to amplify selected sequences. For example, the amplified segment of *nah*Ac genes from a miscellaneous bacterial population may be of related size when amplified with a particular set of *nah*Ac specific degenerative primers; nevertheless contain similarity with certain variation within the PCR-amplified products (Schneegurt-Mark and Kulpa-Charler 1998; Kim et al. 2008). To evaluate the restriction fragments of PCR-amplified products, matrix-assisted laser desorption ionization time-of-flight mass spectrophotometry (MALDI-TOF-MS) was employed (Taranenko et al. 2002).

Other pioneering step is the use of modern molecular biological technology of PCR–RFLP, where the 16S rDNA is digested by different enzymes. Such techniques provide the basis for differentiating strains of bacteria through PCR–RFLP profiles. 16S rDNA sequences of these strains are aligned with the BLAST program on the NCBI website to draw the phylogenetic tree (Chang et al. 2005). Apart from that, enrichment cultures containing naphthalene, phenanthrene, fluoranthene or pyrene as a sole carbon and energy source can be well monitored by DGGE to detect changes in the bacterial-community profile during enrichment (Hilyard et al. 2008). The biotransformation of pyrene by *Mycobacterium* KMS was confirmed through the aid of proteomics with identification of almost all the enzymes required for the initial steps of degradation of this pericondensed PAH compound (Liang et al. 2006).

15.13 Conclusion

Rapid progress, in the last few years, has facilitated the understanding of process of biodegradation of numerous xenobiotic compounds by microbes. Use of culture independent molecular techniques support to understand the microbial community dynamics and structure has assisted in elucidating the particulars of PAH degradation. Synthetic strategies are expensive and are less feasible at a large scale,

whereas exploiting diverse microbial flora could solve the problem through various metabolic pathways. Use of different oxidative enzymes as biocatalysts for complacent environment expresses a promising potential because of less energy requirements as well as specificity. Further characterization is required to explore diverse bacterial flora, their novel catabolic genes, the substrate-specificity, kinetics and the stability of the encoded enzyme and efficient metabolic pathways which would elucidate novel environment friendly technologies for the removal of carcinogenic PAH compounds.

Acknowledgments Authors are thankful to Department of Botany (DST-FIST and UGC-DRS sponsored), Visva-Bharati, for necessary support.

References

Adriaens P, Vogel TM (1995) Biological treatment of chlorinated organics. In: Young LY, Cerniglia CE (eds) Microbial transformation and degradation of toxic organic chemicals. Wiley-Liss, New York, pp 435–486

Basta T, Buerger S, Stolz A (2005) Structural and replicative diversity of large plasmids from sphingomonads that degrade polycyclic aromatic compounds and xenobiotics. Microbiology 151:2025–2037

Bedessem ME, Swoboda-Colberg NG, Colberg PJS (1997) Naphthalene mineralization coupled to sulfate reduction in aquifer-derived enrichments. FEMS Microbiol Lett 152:213–218

Bláha L, Kapplová P, Vondráček J, Upham B, Machala M (2002) Inhibition of gap-junctional intercellular communication by environmentally occurring polycyclic aromatic hydrocarbons. Toxicol Sci 65:43–51

Bosch R, García-Valdés E, Moore ERB (1999) Genetic characterization and evolutionary implications of a chromosomally encoded naphthalene-degradation upper pathway from *Pseudomonas stutzeri* AN10. Gene 236:149–157

Bosch R, García-Valdés E, Moore ERB (2000) Complete nucleotide sequence and evolutionary significance of a chromosomally encoded naphthalene-degradation lower pathway from *Pseudomonas stutzeri* AN10. Gene 245:65–74

Boyd DR, Sharma ND, Hempenstal F, Kennedy MA, Malone JF, Allen CCR, Rensnick SM, Gibson DT (1999) Bis-cis-dihydrodiols: a new class of metabolites from biphenyl dioxygenase-catalyzed sequential asymmetric cis-dihydroxylation of polycyclic arenas and heteroarenes. J Org Chem 64:4005–4011

Caldini G, Cenci G, Manenti R, Morozzi G (1995) The ability of an environmental isolate of *Pseudomonas fluorescens* to utilize chrysene and other four-ring polynuclear aromatic hydrocarbons. J Appl Microbiol Biotechnol 44:225–229

Cerniglia CE (1984) Microbial metabolism of polycyclic aromatic hydrocarbons. Adv Appl Microbiol 30:31–71

Cerniglia CE (1992) Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation 3:351–368

Chang W, Um Y, Hoffman B, Holoman TCR (2005) Molecular characterization of polycyclic aromatic hydrocarbon (PAH) degrading methanogenic communities. Biotechnol Prog 21:682–688

Chauhan A, Faziur R, Oakeshott JG, Jain RK (2008) Bacterial metabolism of polycyclic aromatic hydrocarbons: strategies for bioremediation. J Ind Microbiol 48:95–113

Chen SH, Aitken MD (1999) Salicylate stimulates the degradation of high molecular weight polycyclic aromatic hydrocarbons by *Pseudomonas saccharophila* P15. Environ Sci Technol 33:435–439

Cho JC, Kim SJ (2001) Detection of mega plasmid from polycyclic aromatic hydrocarbon-degrading *Sphingomonas* sp. strain KS14. J Mol Microbiol Biotechnol 3:503–506

Chowdhury A, Pradhan S, Saha M, Sanyal N (2008) Impact of pesticides on soil microbiological parameters and possible bioremediation strategies. J Ind Microbiol 48:114–127

Dennis JJ, Zylstra GJ (2004) Complete sequence and genetic organization of pDTG1, the 83 kilobase naphthalene degradation plasmid from *Pseudomonas putida* strain NCIB 9816-4. J Mol Biol 341:753–768

Dhote M, Juwarkar A, Kumar A, Kanade GS, Chakrabarti T (2010) Biodegradation of chrysene by the bacterial strains isolated from oily sludge. World J Microbiol Biotechnol 26:329–335

Diaz E (2004) Bacterial degradation of aromatic pollutants: a paradigm of metabolic versatility. Ind Microbiol 7:173–180

Dunn NW, Gunsalus IC (1973) Transmissible plasmid coding early enzymes of naphthalene oxidation in *Pseudomonas putida*. J Bacteriol 114:974–979

Evans WC, Fernley HN, Griffiths E (1965) Oxidative metabolism of phenanthrene and anthracene by soil pseudonads. Biochem J 95:819–831

Fetzer JC (2000) The chemistry and analysis of the large polycyclic aromatic hydrocarbons. In: Fetzer JC (ed) Polycyclic aromatic compounds. Wiley, New York, pp 27–143

Gibson J, Harwood CS (2002) Metabolic diversity in aromatic compound utilization by anaerobic microbes. Ann Rev Microbiol 56:345–369

Hadibarata T, Tachibana S, Itoh K (2009) Biodegradation of chrysene, an aromatic hydrocarbon by *Polyporus* sp. S133 in liquid medium. J Hazard Mater 164:911–917

Hafez EE, Rashad M, Abd-Elsalam HE, Hanafy AA (2008) The polyaromatic hydrocarbons as a serious environmental pollutants and the role of bioremediation to overcome this problem. In: Basu SK, Datta BS (eds) Environment, health and nutrition—global issues. APH Publishing Corporation, New Delhi

Heitkamp MA, Cerniglia CE (1988) Mineralization of polycyclic aromatic hydrocarbons by a bacterium isolated from sediment below an oil field. Appl Environ Microbiol 54:1612–1614

Hilyard EJ, Jones-Meehan JM, Spargo BJ, Hill RT (2008) Enrichment, isolation, and phylogenetic identification of polycyclic aromatic hydrocarbon-degrading bacteria from Elizabeth river sediments. Appl Environ Microbiol 74:1176–1182

Hinchee RE, Leeson A, Ong SK, Semprini L (1994) Bioremediation of chlorinated and polycyclic aromatic hydrocarbon compounds. Lewis Publishers, London

Igwo-Ezikpe MN, Gbenle OG, Ilori MO (2006) Growth study on chrysene degraders isolated from polycyclic aromatic hydrocarbon polluted soils in Nigeria. Afr J Biotechnol 5:823–828

Igwo-Ezikpe MN, Gbenle OG, Ilori MO, Okpuzor J, Osuntoki AA (2010) High molecular weight polycyclic aromatic hydrocarbons biodegradation by bacteria isolated from contaminated soils in Nigeria. Res J Environ Sci 4:127–137

Jain RK, Kapur M, Labana S, Lal B, Sarma PM, Bhattacharya D, Thakur IS (2005) Microbial diversity: application of micro-organisms for the biodegradation of xenobiotics. Curr Sci 89:101–112

Johnsen AR, Wickb JY, Harms H (2005) Principles of microbial PAH-degradation in soil. Environ Pollut 133:71–84

Juhasz AL, Naidu R (2000) Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: a review of the microbial degradation of benzo[α]pyrene. Int Biodeterior Biodegrad 45:57–88

Kanaly RA, Bartha R, Watanabe K, Harayama S (2000a) Rapid mineralization of benzo[α]pyrene by a microbial consortium growing on diesel fuel. Appl Environ Microbiol 66:4205–4211

Kanaly RA, Harayama S, Watanabe K (2000b) Biodegradation of high molecular-weight polycyclic aromatic hydrocarbons by bacteria. J Bacteriol 182:2059–2067

Karthikeyan R, Bhandari A (2001) Anaerobic biotransformation of aromatic and polycyclic aromatic hydrocarbons in soil microcosms: a review. J Hazard Subst Res 3:1–19

Khan AA, Wang RF, Cao WW, Doerge DR, Wennerstrom D, Cerniglia CE (2001) Molecular cloning, nucleotide sequence and expression of genes encoding a polycyclic ring dioxygenase from *Mycobacterium* sp. strain PYR-1. Appl Environ Microbiol 67:3577–3585

Kim JD, Shim SH, Lee CG (2005a) Degradation of phenanthrene by bacterial strains isolated from soil in oil refinery fields in Korea. J Microbiol Biotechnol 15:337–345

Kim YH, Freeman JP, Moody JD, Engesser KH, Cerniglia CE (2005b) Effects of pH on the degradation of phenanthrene and pyrene by *Mycobacterium vanbaalenii* PYR-1. Appl Microbiol Biotechnol 67:275–285

Kim SJ, Kweon O, Jones RC, Edmondson RD, Cerniglia CE (2007) Complete and integrated pyrene degradation pathway in *Mycobacterium vanbaalenii* PYR-1 based on system biology. J Bacteriol 189:464–472

Kim SJ, Kweon O, Jones RC, Edmondson RD, Cerniglia CE (2008) Genomic analysis of polycyclic aromatic hydrocarbon degradation in *Mycobacterium vanbaalenii* PYR-1. Biodegradation 19:259–881

Kumar G, Singla R, Kumar R (2010) Plasmid associated anthracene degradation by *Pseudomonas* sp isolated from filling station site. Nat Sci 8:89–94

Liang Y, Gardener D, Miller CD, Chen D, Anderson AJ, Weimer BC, Sims RC (2006) Study of biochemical pathways and enzymes involved in pyrene degradation by *Mycobacterium* sp. strain KMS. Appl Environ Microbiol 72:7821–7828

Liu SL, Luo YM, Cao ZH, Wu LH, Ding KQ, Christie P (2004) Degradation of benzo[α]pyrene in soil with arbuscular mycorrhizal alfalfa. Environ Geochem Health 26:285–293

Lloyd-Jones G, Laurie AD, Hunter DWF, Fraser R (1999) Analysis of catabolic genes for naphthalene and phenanthrene degradation in contaminated New Zealand soils. FEMS Microbiol Ecol 29:69–79

Luch A (2005) The carcinogenic effects of polycyclic aromatic hydrocarbons. Imperial College Press, London

Ma Y, Wang L, Shao Z (2006) *Pseudomonas*, the dominant polycyclic aromatic hydrocarbon-degrading bacteria isolated from Antarctic soils and the role of large plasmids in horizontal gene transfer. Environ Microbiol 8:455–465

Mallick S, Chatterjee S, Dutta TK (2007) A novel degradation pathway in the assimilation of phenanthrene by *Staphylococcus* sp. strain PN/Y via *meta-cleavage* of 2-hydroxy-1-naphthoic acid: formation of *trans*-2,3-dioxo-5-(2′-hydroxyphenyl)-pent-4-enoic acid. Microbiology 153:2104–2115

Meckenstock RU, Annweiler E, Michaelis W, Richnow HH, Schink B (2000) Anaerobic naphthalene degradation by a sulfate reducing enrichment culture. Appl Environ Microbiol 66:2743–2747

Mishra V, Lal R, Srinivasan C (2001) Enzymes and operons mediating xenobiotic degradation in bacteria. Crit Rev Microbiol 27:133–166

Mrojik A, Piotrowska-Seget Z, Labuzek S (2003) Bacterial degradation and bioremediation of polycyclic aromatic hydrocarbons. Pol J Environ Stud 12:15–25

NiChadhain SM, Norman RS, Pesce KV, Kukor JJ, Zylstra GJ (2006) Microbial dioxygenase gene population shifts during polycyclic aromatic hydrocarbon biodegradation. Appl Environ Microbiol 72:4078–4087

Peng RH, Xiong AS, Xue Y, Fu XY, Gao F, Zhao W, Tian YS, Yao QH (2008) Microbial biodegradation of polyaromatic hydrocarbons. FEMS Microbiol Rev 32:927–955

Pfeifer A, Mark G, Leung S, Dougherty M, Spillare E, Kasid U (1998) Effects of c-raf-1 and c-myc expression on radiation response in an in vitro model of human small-cell-lung carcinoma. Biochem Biophys Res Commun 252:481–486

Phale PS, Basu A, Majhi PD, Deveryshetty J, Vamsee-Krishna C, Shrivastava R (2007) Metabolic diversity in bacterial degradation of aromatic compounds OMICS. J Integr Biol 11:252–279

Popp W, Vahrenholz C, Schell C, Grimmer G, Dettbarn G, Kraus R, Brauksiepe A, Schmeling B, Gutzeit T, Von-Bülow T, Norpoth K (1997) DNA single strand breakage, DNA adducts, and sister chromatid exchange in lymphocytes and phenanthrene and pyrene metabolites in urine of coke oven workers. Occup Environ Med 54:176–183

Sarma PM, Duraja P, Deshpande S, Lal B (2010) Degradation of pyrene by an enteric bacterium, *Leclercia adecarboxylata* PS4040. Biodegradation 21:59–69

Schloss PD, Handelsman J (2003) Biotechnological prospects from metagenomics. Curr Opin Microbiol 14:303–310

Schneegurt-Mark A, Kulpa-Charler FJR (1998) The application of molecular techniques in environmental biotechnology for monitoring microbial systems. Biotechnol Appl Biochem 27:73–79

Sheng XF, He LY, Zhou L, Shen YY (2009) Characterization of *Microbacterium* sp. F10a and its role in polycyclic aromatic hydrocarbon removal in low-temperature soil. Can J Microbiol 55:529–535

Singh DK (2008) Biodegradation and bioremediation of pesticide in soil: concept, method and recent developments. Ind J Microbiol 48:35–40

Sinha S, Chattopadhyay P, Pan I, Chatterjee S, Chanda P, Bandyopadhyay D, Das K, Sen SK (2009) Microbial transformation of xenobiotics for environmental bioremediation. Afr J Biotechnol 8:6016–6027

Smith JR, Nakles DV, Sherman DF, Neuhauser EF, Loehr RC (1989) Environmental fate mechanisms influencing biological degradation of coal-tar derived poly-nuclear-aromatic-hydrocarbons in soil system. In: Proceeding of 3rd international conference on new frontiers for hazardous waste management, US Environmental Protection Agency, Washington DC, pp 397–405

Taranenko NI, Hurt R, Zhou J, Isola NR, Huang H, Lee SH, Chen CH (2002) Laser desorption mass spectrometry for microbial DNA analysis. J Microbiol Method 48:101–106

Walter U, Beyer M, Klein J, Rehm HJ (1991) Degradation of pyrene by *Rhodococcus* sp. UW1. Appl Microbiol Biotechnol 34:671–676

Xue W, Warshawsky D (2005) Metabolic activation of polycyclic and heterocyclic aromatic hydrocarbons and DNA damage: a review. Toxicol Appl Pharm 206:73–93

Zhang XX, Cheng SP, Zhu CJ, Sun SL (2006) Microbial PAH-degradation in soil: degradation pathways and contributing factors. Pedosphere 16:555–565

Chapter 16
Microbial Degradation of Plastics and Water-Soluble Polymers

Fusako Kawai

16.1 Introduction

Polymer chemistry began approximately 90 years ago, when Staudinger established the theoretical background from which commercial production of synthetic polymers arose. Synthetic polymers, especially solid ones known as plastics, have been at the forefront since World War II. Annual worldwide production of plastics amounts to more than 200 million tons. Synthetic polymers were originally designed to replace natural polymers, with the advantages of long life (no decay), better performance, plasticity of form, and low cost of production, dependent on cheap petroleum. However, public concern over the use of synthetic polymers has been increasing since the end of 1980s as plastic bags have been polluting the environment. Plastic bags can be found everywhere from the deep sea to the highest mountains and can cause serious environmental problems by threatening wildlife and destroying scenic areas.

Synthetic polymers include all kinds of polymerized compounds, including water-soluble, oily, and solid polymers. Plastics are solid synthetic polymers of various forms. They are not water-soluble or miscible. Since plastics are highly visible, their fate in the environment and their recyclability cause public concern. On the other hand, water-soluble synthetic polymers are often neglected because they are invisible, although their total production corresponds to that of plastics. Environmental problems first attracted worldwide public concern at the end of the 1980s, and studies of biodegradation and the production of biodegradable synthetic polymers were promoted in the 1990s to establish standards for biodegradation and biodegradable synthetic polymers. A number of previous studies have shown that

F. Kawai (✉)
Center for Nanomaterials and Devices, Kyoto Institute of Technology,
Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan
e-mail: fkawai@kit.ac.jp

S. N. Singh (ed.), *Microbial Degradation of Xenobiotics*,
Environmental Science and Engineering, DOI: 10.1007/978-3-642-23789-8_16,

biodegradability is determined by the chemical structures and physico-chemical properties of polymers and not by their origins, whether natural or artificial. The biodegradability of many xenobiotic polymers has been verified, as described below. More recently, a paradigm shift from oil-based production to bio-based production has caused concern and attracted increasing interest in this area, with greater focus on bio-based production of existing monomers presently produced from oil to form polyolefins, polyesters and polyamides. At the same time, more light has been shed on microbial polyesters [poly(hydroxyalkanoate) (PHA)] and chemobiopolyesters [poly(lactic acid) (PLA)]. In the near future, significant ranges of xenobiotic polymers might be replaced with microbial polyesters and chemobiopolymers, which include not only PLA, but also other polymers from bio-based monomers, using environmentally friendly technology.

The biodegradability of xenobiotic polymers is highly dependent on their chemical structures and physical properties. The chemical structures of the major polymers are shown in Fig. 16.1. Most carbon-backbone polymers, barring polyvinyl alcohol (PVA), are generally recalcitrant to biodegradation, but many hetero-backbone polymers, such as polyethers, polyesters, and polyamides are biodegradable. Based on existing information on the degradability of synthetic polymers, copolymers consisting of different types of polymer chains or monomers have been designed, some of which are industrially produced as biodegradable polymers.

16.2 Microbial Degradation of Xenobiotic Polymers

What does "biodegradability of polymers" mean? The first criterion for biodegradation is enzymatic processing. In particular, hydrolyzable polymers such as polyesters and polyamides are enzymatically degraded into monomers (depolymerization process) that can easily enter central metabolic processes unless they are xenobiotic compounds. Most monomers are naturally occurring compounds, such as organic acids, alcohols/glycols, and amide compounds. Proteases, lipases, and esterases originating from animals, plants, and microbes can hydrolyze xenobiotic polymers such as polyamides and polyesters, and PLA, although their original substrates are proteinaceous compounds, lipids, and esters. This is well explained by the fact that enzymes cannot discriminate between original substrates and substrates analogous to them. For example, proteinase K can degrade PLA because lactic acid is analogous to alanine. The second and most important criterion for biodegradation is microbial degradation of target polymers, which are often assimilated by microorganisms as sole carbon and energy sources.

The biodegradability of many polymers has been investigated. The results are summarized in Table 16.1 (Kawai 2010b). Previous studies have revealed that depolymerization proceeds by two processes, exogenous and endogenous. Hydrolyzable polymers are readily depolymerized endogenously by hydrolytic enzymes to yield momoner units that are metabolized by central metabolic

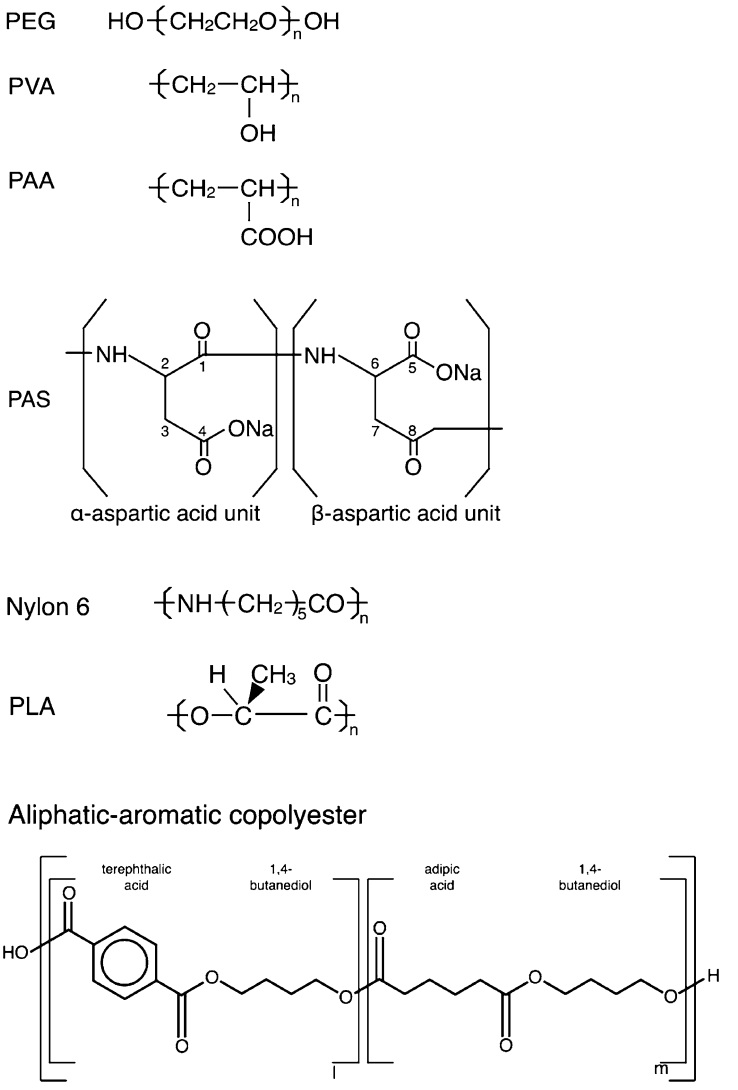


Fig. 16.1 Chemical structures of xenobiotic polymers discussed in this article

pathways. Generally, esterases/lipases contribute to the hydrolysis of synthetic polyesters. PHA- and PLA depolymerases are also categorized as polyester-degrading enzymes, but their enantioselectivities and substrate specificities indicate that there are three categories of polyester-degrading enzymes, general lipases, PHA depolymerases, and PLA-degrading enzymes (Tokiwa and Calabia 2004), although some cross these boundaries. PHA- and PLA-degrading microorganisms have different specific depolymerases for PHA and PLA, respectively.

Table 16.1 Biodegradability of xenobiotic polymers and oligomers by enzymes and microorganisms

Chemical structure	Biodegradable molecular size	Degradation mechanism	Relevant enzymes/ microorganism
A. Polyethers			
Polyethylene glycol (PEG)	MW: 20,000 (MW: 40,000)	Exogenous	Dehydrogenases; aerobic bacteria (anaerobic bacteria)
Polypropylene glycol (PPG)	MW: 3,000–4,000	Exogenous	Dehydrogenase: bacteria
Polytetramethylene glycol (PTMG)	Dimer–octamer	Exogenous	Dehydrogenase: bacteria
Polyputylene oxide (PBO)	MW: 2,000	Exogenous	Dehydrogenase; bacteria
B. Vinyl polymers			
PEwax	MW: 3,000	Exogenous	Bacteria and fungi
Polyvinyl alcohol (PVA)	MW: ca.100,000	Endogenous	Oxidase/dehydrogenase and hydrolase: bacteria and fungi
Polyacrylate (PAA)	MW: 4,500	Exogenous	Bacteria
Polyisoprene	MW: 1,000		Bacteria
Butadiene	Tetramer–decamer		Baceria
Styrene	Dimer		Bacteria
Acrylonitril	Trimer		Fungus
C. Polyamides			
Polyasparatate (PAS)	MW: ca. 20,000	Endogenous/exogenous	Hydrolases; bacteria
Nylon	Dimer–hexamer	Endogenous/exogenous	Hydrolases; bacteria
D. Polyesters			
Polycaprolactone (PCL)		Endogenous	Lipases and cutinases: bacteria and fungi
Polylactic acid (PLA)	Up to a few hundreds of thousands	Endogenous	Proteases and lipases (cutinases): actinomycetes and *Bacillus*

(continued)

Table 16.1 (continued)

Chemical structure	Biodegradable molecular size	Degradation mechanism	Relevant enzymes/ microorganism
Aliphatic		Endogenous	Lipases and cutianases: bacteria and fungi
Aromatic		Endogenous	Cutinases
Aliphatic-*co*- aromatic		Endogenous	Hydrolase; actinomycetes and *Bacillus*-related species
E. Polyurethane			
Ether type		?	?
Ester type		Endogenous	Lipases: bacteria and fungi

Thus the biodegradability of a given polymer is not uniform, and its evaluation requires substantial insight, even if the basic chemical structures and underlying mechanisms are the same.

On the other hand, some synthetic polymers are oxidized repeatedly and are cleaved exogenously by one terminal monomer unit that is assimilated as a carbon source into the central metabolic pathway. Typical examples of exogenous depolymerization are the oxidative degradation of polyethers, PAA, and probably polyolefins by a series of oxidative steps for polyethers, and β-oxidation-like processes for PAA and polyolefins. PVA is degraded by a combination of primary oxidation and hydrolysis of oxidized PVA, as described below. Except for PVA, high molecular weight carbon-backbone polymers are recalcitrant to biodegradation, although their oligomers, including polyethylene wax (PEwax), isoprene oligomers, styrene oligomers, and triacrylonitrile, are biodegradable (Kawai 1995). Hetero-backbone polymers are more susceptible to biodegradation than carbon-backbone ones. Examples of the former are polyethers, such as PEG, most polyesters, including PHA and PLA, and polyamides, such as PAS (polyaspartate).

16.3 Microbial Degradation of Synthetic Water-Soluble Polymers

The scale of the industrial production of synthetic water-soluble polymers is approximately the same as that of plastics. Examples of synthetic water-soluble polymers are polyethers, PVA, and PAA. Polyethers and PVA are often used in synthesizing copolymers to be used as biodegradable segments or to improve performance. Although these polymers are believed to be nontoxic to organisms, they have strong surface activity and produce large amounts of foam, and thus inhibit oxygen recovery in water. This poses a serious threat to water-borne organisms as well as to humans. Water-soluble polymers can be neither recycled nor incinerated after use, and eventually enter streams. Therefore, microbial degradation of these polymers is of great importance worldwide, since it is the only means to remove them from water systems.

16.3.1 Polyethers

Poly(alkylene glycol)s have a common structural formula: $HO[R–O]_nH$ [R=CH_2CH_2 for PEG, CH_3CHCH_2 for polypropylene glycol (PPG), a polymer of 1,2-propylene oxide, $(CH_2)_4$ for polytetramethylene glycol (PTMG), and $C_2H_5(CHCH_2)$ for polybutylene oxide (PBO), a polymer of 1,2-butylene oxide], where n represents the average range of units. The physical properties of PEGs vary from viscous liquids to waxy solids based on their molecular sizes, although

every PEG from oligomers up to polymers with a molecular weight (MW) of a few million is completely water-soluble. Commercially available PPG can be divided into two groups, the diol and triol types, based on the straight or branched chain structure of the polymer. The water solubility of PPGs is lost when the MW is increased to more than approximately 700 (triol type) and 1,000 (diol type) due to the inclusion of a methyl group in each monomer unit. Therefore, copolymers of PEG and PPG are used as detergents, where PEG is a hydrophilic constituent and PPG a hydrophobic one. Another copolymer is also used as a water-soluble flame-resisting pressure liquid, where ethylene oxide and propylene oxide are randomly copolymerized. PBO is an oily polymer due to its pendant ethyl groups. In general, PTMG is a waxy substance, from which water-soluble oligomers have been removed as impurities. PEG was the first member of the polyether group to be manufactured in large quantities and to be used as a commodity chemical in various industrial fields. The most common hydrophilic moieties in the nonionic surfactants are ethylene oxide polymers. The majority of PEGs produced are used in the production of nonionic surfactants, very important groups of industrial products with applications from domestic detergents to agrochemicals, food emulsifiers and other industrial preparations. These products ultimately constitute a significant burden on domestic and industrial wastewater systems. Therefore, their biodegradability characteristics have been observed over the past 50 years, which were reviewed by Kawai (1987, 2002, 2010b). Because of low toxicity and skin irritation, PEGs are widely used in the pharmaceutical industry in the preparation of ointments, suppositories, tablets, and solvents for injection, and also for the preparation of cosmetics, such as creams, lotions, powders, cakes, and lipstick. They are also used as intermediates in the production of resins, such as alkyd resin and polyurethane resin, and as components in the manufacture of lubricants, antifreeze agents, wetting agents, printing inks, adhesives, shoe polish, softening agents, sizing agents, and plasticizers. Furthermore, this material has been used in making resin gels to immobilize enzymes or microbial cells and in the chemical modification of enzymes. Although PEGs appear to be metabolically inert and nontoxic, they are sulfated in vitro by the rat and guinea pig liver (Roy et al. 1987), and repeated topical application of a PEG-based antimicrobial cream to open wounds in rabbits and burn patients has been found to cause a syndrome related to the metabolism of PEGs to various compounds, including mono- and diacids (Herald et al. 1989). Furthermore, the possibility, that PEG 400 and PEG oligomers are toxic, has also been suggested (Gordienko and Kudokotseva 1980). Biodegradation of PEG might pose an additional risk due to metabolite production. Chemically unsubstituted PPG is used in solvents for drugs and in paints, lubricants, inks, and cosmetics, but is mostly transformed to polyurethanes or surface-active agents. PBO is an oily material used in sizing agents, cleaning agents, and dispersants. PTMG is used exclusively as a constituent of polyurethane.

PEGs with different MWs have been produced and have been used in industrial and domestic applications for more than 60 years. Some of them are included as non-toxic and biodegradable segments of copolymers, and their larger parts are transformed into neutral detergents and liberated into streams after use.

Various types of PEG-degraders that are able to assimilate a variety of molecular sizes have been isolated since the first report of PEG 400 by Payne (1963). Although PEGs with MW higher than 1,000 were long considered to be bioresistant, those up to 20,000 or more have since been found to be biodegradable. PEGs with a high MW, from 4,000 to 20,000, are assimilated by a limited number of species: *Pseudomonas aeruginosa* (up to 20,000) (Haines and Alexander 1975), soil bacteria (up to 6,000) (Hosoya et al. 1978), *Pseudomonas stutzeri* (up to 13,500) (Obradors and Aguilar 1975), and *Sphingomonas* species (up to 20,000); the strains were originally identified as *Flavobacterium* species (Ogata et al. 1975). Sphingomonads include sphingolipids in their outer membranes instead of the lipopolysaccharides found in most Gram-negative bacteria. Various lipophilic xenobiotic-assimilating bacteria are included in this genus (Kawai 1999). Most recently, a Gram-positive actinomycete, *Pseudonocardia* sp. strain K1, originally isolated as a tetrahydrofuran degrader, was also found to grow on PEG 4,000 and 8,000 (Kohlweyer et al. 2000).

We have isolated various PEG-utilizing bacteria with various degradabilities towards PEG 400–20,000 (Ogata et al. 1975). Isolates able to degrade PEG 4,000 and 20,000 were identified as Sphingomonads, and based on the newest taxonomy, they have been renamed and designated type species of *Sphingopyxis macrogoltabida and Sphingopyxis terrae*, respectively (Takeuchi et al. 2001). Interestingly, *S. terrae* can grow on PEG as a symbiotic mixed culture with a concomitant associate (Kawai and Yamanaka 1986; Kawai 1996). Another focus of PEG degradation studies is the biochemical mechanism of degradation. Several reports have suggested different mechanisms (Kawai 2002), but the most probable metabolic pathway is an exogenous metabolic one based on repeated oxidation steps. PEG is oxidized by alcohol dehydrogenases linked with a dye or NAD. PEG-dehydrogenases (PEG-DHs) from PEG-utilizing Sphingomonads have been cloned and characterized as FAD-including alcohol dehydrogenases (Sugimoto et al. 2001; Ohta et al. 2006). PEG-aldehyde dehydrogenase was cloned from PEG-utilizing Sphingomonads and characterized as a NADP-containing nicotinoprotein PEG-aldehyde dehydrogenase (Ohta et al. 2005), the first report of a nicotinoprotein aldehyde dehydrogenase. The ether bond-splitting enzyme involved in the PEG metabolism was perhaps a glycolic acid oxidase or glycolic acid dehydrogenase active on carboxylated PEG (Yamanaka and Kawai 1991; Enokibra and Kawai 1997). All the metabolic enzymes included in PEG degradation have been localized in the membrane and are thought to work in the periplasm, in accordance with the fact that PEG and its metabolites were detected in the periplasmic fraction (unpublished data), suggesting that PEG is taken up into the periplasm and metabolized there. We cloned the genes involved in PEG degradation, and found that the *peg* operon consisted of five genes and was expressed by PEG through induction of an *araC*-type regulator (Charoenpanich et al. 2006; Tani et al. 2007, 2008), as shown in Fig. 16.2. This was the first report on the regulation of degradative genes by a macromolecule. Two genes coding PEG-DH and PEG-aldehyde dehydrogenase are involved in the *peg* operon. The role of other genes in the *peg* operon was suggested with regards to the PEG

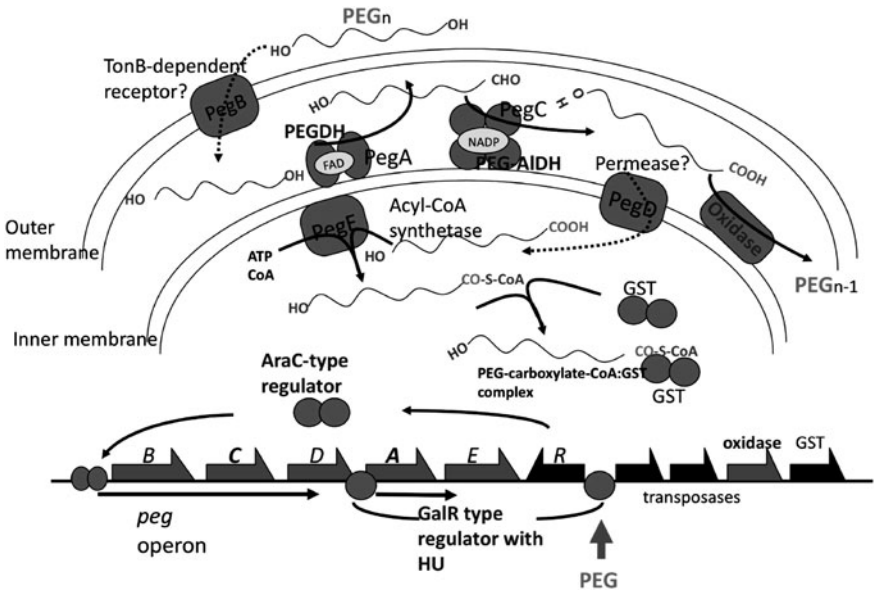


Fig. 16.2 The operonic structure of genes involved in PEG degradation and its regulation by PEG

metabolism, as summarized in Fig. 16.2 (Tani et al. 2007). A gene coding PEG-carboxylate dehydrogenase was detected in the downstream region of the *peg* operon, and was found to act as an ether bond-splitting enzyme (Somyoonsap et al. 2008). Anaerobic biodegradation of PEG has been well investigated as compared to other polymers. Schink's group reported that higher MW PEG (up to PEG 40,000) is degraded by anaerobes rather than by aerobes (Strass and Schink 1986; Schink and Stieb 1983). They proposed an anaerobic metabolic route for PEG, and suggested that acetaldehyde is produced from PEG by a diol dehydrorase-like enzyme (PEG acetaldehyde lyase), but they failed to purify the enzyme (Strass and Schink 1986; Frings et al. 1992). Schink's group also demonstrated conversion of 2-phenoxyethanol to phenol and acetaldehyde, in a way similar to a diol hydratase reaction, by a strictly anaerobic Gram-positive bacterium, *Acetobacterium* strain LuPhet1, but could not rule out an alternative pathway for the production of acetaldehyde (Speranza et al. 2002). Dwyer and Tiedje (1986) obtained a methanogenic consortia from sewage sludge that degraded ethylene glycol to PED 20,000. In addition, *Alcaligenes faecalis* var. *denitrificans* TEG-5, the first PEG-degrader able to degrade PEG under aerobic conditions, displayed PEG degradation under anaerobic nitrate-reducing conditions (Grant and Payne 1983).

In parallel with PEG degrading bacteria, we isolated PPG- and PTMG-utilizing bacteria (Kawai et al. 1977; Kawai and Moriya 1991). PPG with an MW of up to 4,000 was assimilated. Distinct degradation of PTMG was limited to oligomers up to octamer, since PTMG is insoluble in water and is used exclusively as a

constituent of polyurethanes, but oligomers up to octamer can be washed out with water as impurities from polymers, and are found in wastewater. The first attack on PPG and PTMG was considered to be dependent on dehydrogenases (Kawai and Moriya 1991; Tachibana et al. 2002). The presence of several different PPG dehydrogenases (PPG-DHs), localized in the membrane, the periplasm, and the cytoplasm respectively, has been suggested for PPG-utilizing *Stenotrophomonal maltophilia* (Tachibana et al. 2002) from which pyrroloquinoline quinone (PQQ)-dependent PPG-DH was purified and characterized as a type-I quinoprotein dehydrogenase, localized in the periplasm (Tachibana et al. 2003). Later, cytoplasmic NAD-dependent PPG-DH was characterized and hypothesized to work on low molecular sizes of PPG in the cytoplasm (Tachibana et al. 2008). This is different from the only membrane-bound PEG-DH suggested for PEG-degrading Sphingomonads. PPG might have more affinity with phospholipids, which are the main constituents of the cytoplasmic membrane, and oligomeric PPGs probably can traverse the cytoplasmic membrane and are metabolized in the cytoplasm (Kawai et al. 1985; Hu et al. 2008a). Oligomeric PPG might express genes related to PPG metabolism.

16.3.2 Polyvinyl Alcohol

Historically, PVA has been produced on an industrial scale by the hydrolysis of poly(vinyl acetate), since a vinyl alcohol monomer cannot exist due to tautomerization into acetoaldehyde. PVA are widely used due to its excellent physico-chemical properties, especially for fabric and paper sizing, fiber coating, adhesives, emulsion polymerization, films for packing and farming, and the production of poly(vinyl butyral). Maximum production of PVA amounted to about 1,250 kt in 2007 (http://www.sriconsulting.com/CEH/Public/Reports/580,1810), the top volume in the total volume of synthetic water-soluble polymers produced in the world, and consumption is expected to increase annually. Large quantities of PVA are poured into water systems each year, especially when used in paper and textile mills. PVA might be the only polyvinyl-type synthetic polymer that is biodegradable. The biodegradation of PVA has been reviewed by Matsumura (2002), Chiellini et al. (2003) and Kawai and Hu (2009). The history of PVA biodegradation goes back over 70 years, since the first report of degradation by *Fusarium lini* B (Nord 1936). Suzuki et al. (1973) reinitiated extensive studies of PVA biodegradation, followed by Watanabe et al. (1975). A variety of microorganisms with the ability to assimilate PVA have been reported. Most PVA-degraders are Pseudomonads or Sphingomonads, but they range across Gram-negative and Gram-positive bacteria and fungi (Kawai and Hu 2009). Some of them can degrade PVA as a mixed culture, due to different underlying mechanisms (Kawai 2010b). No anaerobic PVA-degrading microbe has yet been isolated, but river sediments and anaerobically preincubated-activated sludge have been found to degrade PVA (Matsumura et al. 1993). The anaerobic biodegradation rate of

PVA is low and is influenced by its MW, unlike the biodegradation of PVA under aerobic conditions.

The main metabolic route is based on two steps. The first is either (i) oxidation of two adjacent hydroxyl groups leading to β-diketone structures, or (ii) the oxidation of one hydroxyl group, yielding monoketone structures. Based on the products of the first step of PVA degradation, there are two possible pathways for the second step: either hydrolysis of β-diketone structures of oxidized PVA (oxiPVA) by a β-diketone hydrolase (oxiPVA hydrolase) or the aldolase reaction of the monoketone structures of oxiPVA. We cannot rule out the possibility of an aldolase reaction, but diketone structures are surely the main products of PVA degradation (Kawai and Hu 2009). Since diketone structures are non-enzymatically hydrolyzed, the oxidation process for the degradation of PVA is the most important. *Penicillium* sp. appears to utilize the metabolic pathway proposed above in the bacterial degradation of PVA (Qian et al. 2004).

The two-step degradation of PVA was confirmed by the genetic structure of the *pva* operon in *Sphingopyxis* sp. 113P3 (Klomklang et al. 2005), which consisted of three genes encoding oxiPVA hydrolase, PVA dehydrogenase (PVA-DH), and cytochrome *c*, as shown in Fig. 16.3. Cytochrome *c* has been suggested to be a natural electron acceptor for PVA-DH in vivo (Mamoto et al. 2008). PVA-DH is a member of the type-II quinohemoprotein alcohol dehydrogenases (Hirota-Mamoto et al. 2006), but the position of the amino acid sequences for the heme-binding domain and superbarrel domain found in this family are the reverse of those of the other members. This is considered significant in terms of the ability of PVA-DH to react with a macromolecule such as PVA. Unfortunately, a non-quinohemoprotein PVA oxidase has not yet been cloned. PVA-DH was reported to be constitutive, as opposed to PVA oxidase, which was PVA-inducible, but we found that the *pva* operon was constitutively expressed, although its expression was promoted with PVA (Hu et al. 2008b). We confirmed that PVA was taken up through the outer membrane (Hu et al. 2007b) and accumulated in the periplasm (unpublished data), where the three enzymes are located. How PVA regulates the expression of the *pva* operon and the size required for induction remains to be determined. In a megaplasmid, the presence of the *pva* operon is similar to that of a *peg* operon (Tani et al. 2007), which promotes the acquisition of degradation ability, resulting in a wide distribution of the operon among different species. In fact, the widely distributed PEG-DH gene (AB196775) of *S. macrogoltabida* strain 103 has been detected in newly isolated PEG-degraders, including *S. macrogoltabida*, *Stenotrophomonas maltohilia*, *Pseudomonas* sp., and *Sphingobium* sp., and shows 99% identity with the original (Hu et al. 2007a).

Although the TonB-dependent receptor-like gene and the permease-like gene are included in the *peg* operon, no gene that might be related to translocation of a polymeric compound is included in the *pva* operon (Hu et al. 2008b), although its expression is clearly promoted by the presence of PVA, which necessitates regulation of the *pva* operon by PVA or its metabolite. On the other hand, morphological changes in the cell surface occur upon exposure to PVA (Hu et al. 2007b). Hence, on the basis of PEG and PVA degradation studies,

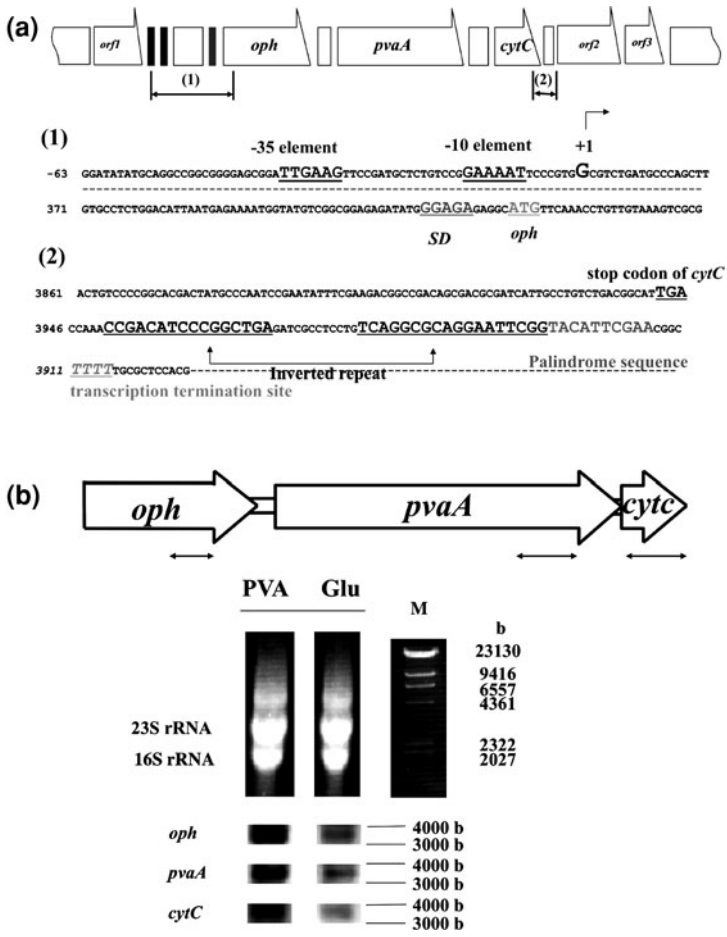


Fig. 16.3 The operonic structure of genes involved in PVA degradation and its expression. **a** The operonic structure of three genes; **b** the expression of three genes in PVA and glucose media. Symbols: *oph* oxiPVA hydrolase gene; *pvaA* PVA-DH gene; *cytC* cytochrome *c* gene

we can conclude that these operons are regulated by macromolecules and require a specific transport system for inducer macromolecules.

16.3.3 Polyacrylate

The biodegradation of polyacrylate was reviewed by Kawai and Hayashi (2002). This "acrylic polymer" commonly includes a variety of homo- and copolymers of acrylic and methacrylic acids and their esters. After World War II, the demand for

this family of acrylic polymers expanded rapidly, and numerous applications depended on the specific polymer structure, molecular weight, and composition. Among acrylic polymers, PAA is a linear homopolymer of acrylic acid and its salts. Due to the presence of a large number of carboxyl groups, PAA dissolves in water and has a large share of the water-soluble speciality polymer market. The polymer is currently used in detergent builders, pigment and filler dispersants, and flocculants in water treatment. After use, PAA is usually disposed off in sewage and water bodies, e.g., streams, rivers, and lakes. As has been described for PEG and PVA, molecular size significantly affects its susceptibility to biodegradation. The MW of commercially available PAA ranges from approximately 100 to several million. High MW PAA is predicted to be either non- or slightly biodegradable, but when practically applied, it is removed by precipitation and disposed off in its solid form by incineration or in a landfill. In addition, when exposed to UV light, high MW PAA in a dilute solution (less than 1%) is photochemically decomposed into smaller fragments that are biodegradable. Ozonization can also lower the MW of PAA. Hayashi (1998) concluded that even high MW PAA can be finally biodegraded after physico-chemical treatment. What, then, does MW have to do with the environment? By adsorption to sewage sludge and precipitation in the form of polymer-calcium complexes or by coagulation–flocculation with aluminium chloride or ferric chloride, more than 90% of the polymers with MWs of tens of thousands are removed from the water and 45–60% of the polymers with MWs of 3,000–4,000 are removed. Sand-column tests have shown that PAA with a MW greater than about 3,500 is strongly adsorbed to soil particles (Rittmann et al. 1992b). Therefore, biodegradability is a prerequisite for PAA with a MW less than about 3,500.

Matsumura et al. (1988) did the first study of the biodegradability of acrylic oligomers using activated sludge. They suggested that partial mineralization of PAA with an average MW of 2,000 or 4,500 occurs within 90 days. Hayashi et al. (1993) first reported the isolation of a bacterium (*Arthrobacter* sp.) from soil samples that is capable of assimilating the acrylic oligomers up to a heptamer. Kawai (1993) also reported three bacterial strains that assimilated acrylic trimer, and showed metabolic activity towards PAA (MW 1,000–4,500); they proposed an aerobic metabolic pathway for PAA degradation based on a β-oxidation form of metabolism (Kawai et al. 1994). PAA with a MW of less than 1,000 can be biodegraded to a considerable extent by activated sludges (Larson et al. 1997). Hayashi et al. (1994) isolated aerobic bacteria capable of degrading PAA of MW 4,000 from soils, including a single bacterium (*Alcaligenes* sp.) and a consortium of three bacteria (*Alcaligenes* sp., *Sphingomonas* sp., and *Mycoplana* sp.), and suggested that effective decomposition of high MW PAA can be achieved by a combination of physico-chemical treatment and microbial degradation. Rittmann et al. (1992a) found that soluble PAA with a MW of 1–100 kD could be mineralized to CO_2 with an anaerobic biomass formed in a sand column. More recently, Doser et al. (1997) reported that *Pseudomonas* sp. isolated from activated sludge was capable of utilizing high MW PAA (MW, 100 kD) as a carbon source. Iwahashi et al. (2003) found that a microbial consortium of several bacterial

species degraded PAA with an average MW of 2,100, and they proposed a metabolic pathway similar to the β-oxidation pathway identified by Kawai et al. (1994) with different metabolites. Hence, PAA of a MW lower than 3,500 is considered biodegradable.

16.3.4 Poly(amino acid)s and Polyamides

The degradation of poly(amino acid)s and polyamides was reviewed by Obst and Steinbüchel (2004). In nature, proteins and poly(amino acid)s exist as two different types of amino acid polymers. The most critical differences are (i) that the former is a random polymerization of 20 amino acids and the latter a polymer of a single amino acid, and (ii) the synthesis of proteins is ribosome-dependent and forms an α-amino-α-carboxyl peptide linkage while that of poly(amino acid)s is ribosome-independent and catalyzed by peptide synthetases (EC 5.3.2.-), and forms other linkages, including β- and γ-carboxyl groups as well as ε-amino groups. Theoretically, poly(amino acid)s can be biosynthesized from acidic and basic amino acids with a free amino or carboxyl group that is not used in peptide bonds, such as glutamic acid, aspartic acid, lysine, histidine, and arginine. Naturally occurring poly(amino acid)s include poly(γ-glutamic acid) (γ-PGA), poly(ε-L-lysine) (ε-PL), and cyanophycin (cyanophycin granule polypeptide (CGP)). The CGP molecular structure is related to that of PAS, and unlike synthetic PAS, it is a comb-like polymer with α-amino-α-carboxyl-linked L-aspartic acid residues representing the poly(α-L-aspartic acid) backbone and L-arginine residues bound to the β-carboxylic groups of aspartic acids. A charged polypeptide composed of repeated oligomeric arginine and histidine units has been identified in the ergot fungus *Verticillium kibiense* (Nishikawa and Ogawa 2004). Poly(amino acid)s occur in various molecular sizes and are generally polydisperse, whereas proteins are monodisperse. These poly(amino acid)s have a poly(ionic) nature and are generally water soluble; they can be used in many applications. PAS and nylons have been commercialized as synthetic polyamides. The former is used as a biodegradable detergent builder substituting for hardly biodegradable PAA. Nylon is a solid polymer used in films and fabrics, for example, and is non-biodegradable. However, oligomeric by-products from a factory producing nylon can be biodegraded, as described below.

PAS is not a naturally occurring poly(amino acid) and is chemically synthesized, principally by thermal polymerization. This produces a branched PAS comprising α and β-carboxy-linked poly(D/L-aspartic acid). PAS has the same chelating ability as PAA due to the presence of the same carboxyl groups on the polymer chain, and thus it is expected to be a suitable substitute for PAA due to its higher susceptibility to microbial degradation. Since branched PAS is less biodegradable than straight-chain PAS, development of it with little or no branching is expected. Recently, Soeda et al. (2003) found that α-poly(D/L-aspartic acid) with molecular weight of up to 3,700 Da can be synthesized from diethyl L-aspartate in

organic solvents using a *Bacillus subtilis* protease as catalyst. In nature, only aspartic acid-rich polypeptide sequences have been found. For example, these regulate the formation of calcite crystals in sea shells (Rusenko et al. 1991). In view of the structural similarity between CGP and PAS, alternative degradation mechanisms for CGP initiated by hydrolytic β-cleavage leading to the release of free arginine from CGP are desirable for the formation of straight-chain PAS, for which many technical applications are known, but this has not been reported. A PAS peptide comprising 20 aspartic acids is secreted by an engineered *B. subtilis*, WB600/pBE92 (Ornek et al. 2002).

Tabata et al. (2000) isolated *Pedobacter* sp. KP-2 and *Sphingomonas* sp. KT-1, which degrade high MW linear PAS completely to low MW products in their mixed culture. The former endogenously hydrolyzes PAS of masses between 5 and 15 kDa to aspartic oligomers, whereas the latter completely degrades exogenously PAS with masses below 5 kDa to aspartic acid monomers. PAS-hydrolyzing enzymes (hydrolase-1 and hydrolase-2) have been purified from cell extracts of strain KT-1 (Tabata et al. 2001; Hiraishi et al. 2003a, b). Hydrolase-1 specifically cleaves the bonds between the β, β-amide units of thermally synthesized PAS endogenously to aspartic oligomers. Hydrolase-2 exogenously hydrolyzes α-oligo(L-aspartic acid) to aspartic acid and shows similarity to a putative peptidase (Hiraishi et al. 2003a, 2004).

Nylons, synthetic polyamides, are one of the most successful commercialized plastics; they are widely used in producing stockings, fibers, carpeting, ropes, fishnets, and so on. They are barely biodegradable, but nylon oligomers occurring as by-products during the synthesis of nylon can be biodegraded by microorganisms. Linear and cyclic oligomers of ε-aminocaproic acid (by-products of nylon-6 manufacturing) are assimilated by *Flavobacterium* sp. KI72 and *Pseudomonas* sp. NK87 (Kinoshita et al. 1975; Kanagawa et al. 1989), which utilize them as their sole carbon and nitrogen sources. Three enzymes are associated with the degradation of the oligomers: (i) 6-aminohexanoate-cyclic-dimer hydrolase (NylA) (Kinoshita et al. 1977), (ii) 6-aminohexanoate-linear-dimer hydrolase (exo-type), which degrades the dimer-hexamer to 6-aminohexanoate (Nyl B) (Kinoshita et al. 1981), and (iii) an aminohexanoate-oligomer hydrolase (endo-type) (NylC), which is responsible for the cleavage of cyclic and linear oligomers with more than 3 subunits into linear dimers (Negoro et al. 1992; Kakudo et al. 1993, 1995). Genes for three hydrolases were encoded on one of the plasmids in strain K172 (Negoro et al. 1992; Negoro 2000). Negoro and co-workers have confirmed that nylon oligomer-degrading enzymes can be obtained through experimental evolution from a non-degrader (Prijambada et al. 1995), and they have found that a mutant of a carboxyesterase with a β-lactamase fold with weak activity has acquired greater affinity and catalytic efficiency for the substrate (Kawashima et al. 2009). They isolated novel alkanophilic nylon oligomer-degrading bacteria, *Agromyces* sp. KY5R and *Kocuria* sp. KY2, and found that the genetic organization of the nylon oligomer-degrading enzymes is similar to that of strain K172, albeit with some rearrangements (Yashuhira et al. 2007a, b). These results indicate that microorganisms rapidly evolve by random mutagenesis of existing genes, and that these

occasionally cause variations in the catalytic properties of the respective encoded enzymes and make possible adaptation to synthetic polymers newly introduced into the environment. However, the mutation rate is not the same for all synthetic polymers, since polyethylene or polypropylene are only slightly biodegradable, although they have been used for approximately the same period of time as nylon. Besides, a thermophilic bacterium, *Geobacillus thermocatenulatus*, has been suggested as a possible degrader of nylon-12 and 66 but not nylon-6 (Tomita et al. 2003a), but its enzymes have yet to be characterized.

16.4 Biodegradation of Plastics

Plastics are generally very resistant to environmental influences such as humidity and microbial attack (Müller et al. 2001), but during the past two decades several biodegradable plastics have been developed, and their uses have been gradually expanded and commercialized, for example, Apexa® (DuPont), Ecoflex® (BASF), EasterBio® (Eastman Chemicals), Bionole® (Showa Highpolymer), Matabee® (The Nippon Synthetic Chemical Industry), LACEA® (Mitsui Chemicals), Runale® (Nippon Shokubai), Novon® (Warner–Lanbert), and Nature Works® (Cargill Dow Polymers). Biodegradable plastics can be applied in single-use articles that can be disposed of by biological waste treatment such as composting or anaerobic digestion (Baere et al. 1994). It is notable that a large number of biodegradable plastics are categorized as polyesters. Many examples of plastic degradation have been documented. Here I introduce our work on the biodegradability of PEwax, aliphatic–aromatic-*co*-polyester, and PLA.

16.4.1 Polyethylene Wax

Polyethylene (PE) is regarded as a chemically inert polymer due to various factors, such as its long degradation time. Early studies of the biodegradation of PE indicated that the biodegradation of PE is affected by various factors: preliminary irradiation from a UV source, the presence of photodegradative enhancers, morphology and surface area, additives, and MW (Albertsson et al. 1987). By measuring $^{14}CO_2$ generation, they showed that the degradation of PE proceeded very slowly. Scot (1975) had concluded that an attack on PE by microorganisms is a secondary process. The first process in the degradation of PE is an oxidation process that reduces the MW of the molecule to the level required for biodegradation to occur. Based on this theory, he developed the so-called Scott-Gilead process (Scott and Gilead 1978) to enhance the oxidation of PE molecules. Potts et al. (1973) found that linear paraffin molecules (approximately below MW 500) are utilized by several microorganisms. Otake et al. (1995) reported remarkable degradation of low density PE thin films buried under soil for over 32 years,

but reported no data on molecular sizes. We have tested the microbial degradation of low-density PE capsules was enhanced by 3% w/w of Scott–Gilead system. The PE includes photoactivators, such as iron acetyl acetonate and nickel dibutyldithiocarbamate (Kawai et al. 1999). The capsules were kept outdoors for several years and were used as photodegraded PE (PDPE) that was fragmented perhaps through a pathway similar to that of the Norrish reactions (Al-Malaika et al. 1986). PDPE and commercial PEwax (MW = 1,290) were used as sole carbon and energy sources for the soil microorganisms (154 field soil samples) (Kawai et al. 1999). Several consortia grew on PDPE or PEwax, and were confirmed to have degraded by weight loss or gel permeation chromatography (GPC). Based on the GPC pattern, appreciable degradation was found in PEwax up to approximately 3,000 Da. On the other hand, manganese peroxidase produced by a white-rot fungus strain, IZU-154, also nonspecifically degraded PE as well as Nylon 66 (Deguchi et al. 1997).

16.4.2 Aliphatic–Aromatic Copolyester

Polyesters are classified into three groups; aliphatic, aromatic, and aliphatic-*co*-aromatic. There are many reports of enzymatic and microbial attack on aliphatic polyesters. The polyesters are gradually hydrolyzable in water and are susceptible to enzymatic attack by lipases in general, but their degradation rate is dependent on their chemical structure or the melting point. Aromatic polyesters, such as polyethylene terephthalate (PET), are practically non-biodegradable, although two papers reported that cutinases can hydrolyze PET with low crystallinity (Müller et al. 2005; Ronkvis et al. 2009). The addition of aliphatic groups to aromatic polyesters endows them with advantageous physico-chemical properties for practical use together with biodegradability characteristics. They can be used as a film or coating for disposable packaging of food (for example, bowls, plates, cups, sandwich wraps, and clamshell sandwich containers), and can be biodegraded in compost. Since the aromatic group is typically terephthalic acid, the physical properties of the plastics are determined by the type and content of the aliphatic groups. The biodegradation of aliphatic–aromatic copolyesters has been extensively studied by Müller et al. (2001). They used Ecoflex (a copolymer of 1,4-butane diol, dimethyl terephthalate, and adipic acid) as the target polyester. Biodegradation of Apexa® (formerly Biomax®, consisting of terephthalic acid, ethylene glycol, and an undisclosed component that ensures compostability) has also been studied in a bioreactor maintained at 58°C, inoculated with compost tea (Nagarajan et al. 2006) and in compost (Hu et al. 2008c).

Microorganisms able to colonize on Luria–Bertani agar plates containing the polyester (Apexa® 4026) at 50°C were directly isolated from composted films (Hu et al. 2010). Actinomycetes accounted for approximately 70% of the total isolates, and were categorized into the genera *Streptomyces*, *Thermobifida*, *Saccharomonospora*, and *Thermoactinomyces*. Members of the *Bacillus* group

accounted for approximately 30% of the total isolates, and were categorized into the genera *Bacillus*, *Ureibacillus*, and *Aneurinibacillus*, We selected *Thermobifida alba* AHK119 for further work, since it was the strongest degrader of the copolyester and polycaprolactone (PCL). *T. alba* AHK119 degraded the particles of Apexa 4026 and 4027 and produced terephthalic acid in the culture supernatant. We cloned a gene coding a 300-amino acid protein, Est119, which belongs to an esterase-lipase superfamily (serine hydrolase). The highly conserved -G-X-S-X-G- serine hydrolase sequence was defined as -G-H–S-M-G- in Est119, and Ser129, His207, and Asp175 were identified as a catalytic triad. The mature protein is a single polypeptide chain made up of 266 amino acids. The sequence encoding the mature Est119 protein was cloned into pQE80L to create expression vector pQE80L-*est119*. The recombinant protein harboring an N-terminal hexahistidine tag was expressed in *Eschericia coli* Rosetta-gami B(DE3). The transformed cells produced an approximately 30 kDa protein when induced with 0.1 mM isopropyl-*β*-D-thiogalactopyranoside. The purified Est119 is a monomeric protein with a molecular mass of 30 kDa. The enzyme shares 84% identity with a hydrolase for an aliphatic–aromatic copolyester from *Thermomonospora fusca* (now *Thermobifida fusca*) DSM43793 (Chen et al. 2008; Kleeberg et al. 2005). There are only two enzymes of the genus *Thermobifida* that work on aliphatic–aromatic copolyesters. Both *Thermobifida* strains are major Actinomycetes components within composts and might contribute to the biological recycling of aliphatic–aromatic copolyesters.

16.4.3 Poly(lactic acid)

Chemical synthesis of PLA dates back to 1932, when Carothers first synthesized PLA of approximately 3,000 Da. In the 1960s, PLA was found use in the medical field as a bio-absorbable material. Since the latter half of the 1980s, plastic waste has caused public concern due to its negative impact on the environment, and recently rising costs and the limited availability of crude oil has turned more attention towards alternative sources. This trend has returned attention to PLA as a bio-based material capable of replacing oil-based materials. PLA is chemically synthesized from lactic acid, a representative of fermentation products from plant resources, and hence is defined as a biomass plastic. The biodegradability of PLA has been established since the first report on enzymatic hydrolysis of PLA by William (1981), who described its feasibility for proteases and unfeasibility for esterases. Later, certain lipases and esterases were reported to be able to hydrolyze PLA, but they appear to be active only for low molecular weight polymers or poly(DL-lactate) (DL-PLA). There have also been many reports on the microbial assimilation of PLA, since Pranamuda et al. (1997) first isolated PLA-assimilating *Amycolatopsis* sp. strain HT-32. Tokiwa and Calabia (2006) concluded that most PLA-degrading microorganisms belong phylogenetically to the family *Pseudonocardiaceae* and related genera, such as *Amycolatopsis* and *Lentzea*, in which

proteinaceous materials promote the production of the PLA-degrading enzyme. PLA-degrading enzymes of PLA-assimilating microorganisms were purified from different strains of *Amycolatopsis* at about the same time by two groups (Pranamuda et al. 2001; Nakamura et al. 2001) and were characterized as proteases. Later, both groups cloned the genes (Matsuda et al. 2005; Tokiwa et al. 2003). It is notable that almost all the degradation tests have been carried out using poly(L-lactic acid) (PLLA), and that no information regarding the biodegradability of poly(D-lactic acid) (PDLA) is available, except for the fact that proteinase K hydrolyzed PLLA, but not PDLA (Reeve et al. 1994). Tomita et al. (2003b) isolated a thermophile, *Bacillus stearothermophilus*, which grew at 60°C on PLA as sole carbon source.

Actinomycetes, *Bacillus*, *Brevibacillus*, and *Geobacillus*, have been reported to be thermophilic degraders (Kawai 2010a). Since PLA is hydrolyzed at a relatively high rate at high temperatures (>50°C), the question whether the strain excretes a PLA degrading enzyme or utilizes hydrolyzed products depends on future characterization of their PDLA degrading enzymes. Mayumi et al. (2008) recently cloned three genes encoding PLA depolymerases based on a metagenome derived from the compost. One of them coded for a thermostable esterase homologous to *Bacillus* lipase and showed an ability to bind to DL-PLA powders with molecular masses lower than 20,000. Since the expressed enzyme had no activity on PLLA with molecular masses of approximately 130,000, the enzyme might be able to degrade depolymerized PLA products. It can be surmised that the same mechanism exists in other thermophilic enzymes. A fungus, *Tritirachium album* ATCC22563, also shows an ability to degrade PLLA, silk fibroin, and elastin; degradation is inducible with gelatin, suggesting the induction of a protease (Jarerat and Tokiwa 2001). However, the role of the fungus in degrading PLA in nature is doubtful, since the enzyme was not induced at all in the absence of gelatin.

The degradation of PLA is possible at 30°C, far lower than the glass transition temperature (Tg: approximately 55°C), and is difficult to explain based on the flexibility of the substrate molecular chain, but is understandable because PLA absorbs water and collapses the polymer block, which then becomes available for attack by microbes or enzymes.

Masaki et al. (2005) isolated *Cryptococcus* sp. S-2 for use in wastewater treatment, and found that the strain displayed strong lipase activity. They cloned the gene for a lipase and found that it had higher homology with cutinases (EC 3.1.1.74) than with lipases, which showed stronger degradation ability toward PLA than proteinase K.

Matsuda et al. (2005) confirmed that a recombinant PLA depolymerase from *Amycolatopsis* did not work on PCL or PHB. PHB depolymerase does not act on PLLA, a kind of hydroxyalkanoate (PHA), due to differences in the optical activities of the two substrates and in the carbon chain lengths of 2-hydroxyalkanoate and 3-hydroxyalkanoate. Commercially available lipases (esterases) act on DL-PLA, but not on optically active PLLA or PHB, poly(D-hydroxybutyrate) (Tokiwa and Jarerat 2004). Thus polyester-degrading enzymes are categorized into

three groups: PLA depolymerase, PHB depolymerase, and general polyester-degrading enzymes, which also have a variety of substrate specificities towards aliphatic and aliphatic-*co*-aromatic polyesters. PLA is considered to be one of the third type of polyesters, following synthetic polyesters and PHA, including PHB.

Using the recombinant purified PLA-degrading enzyme from *Amycolatopsis* sp. K104-1 and the recombinant purified cutinase like enzyme (CLE) from *Cryptococcus* sp. S-2 (Masaki et al. 2005), we examined enantioselectivity towards PLLA and PDLA (Kawai 2010a). The PLA-degrading enzyme was PLLA-specific. Together with a report on the enatioselectivity of proteinase K (Reeve et al. 1994) and the fact that proteases originally recognize the polymer of L-amino acids, we concluded that protease-type PLA depolymerases are PLLA-specific. On the other hand, CLE acted on both PLLA and PDLA, but the activity was higher on PDLA than on PLLA (it was PDLA-preferential). The enantioselectivity of crude enzymes can be a good indicator in predicting the type of enzyme, either protease or cutinase, which leads to successful cloning of enzyme genes based on the conserved regions of the various groups.

Commercially available true lipases did not act on PLLA or PDLA. True lipases have a lid covering an active site that leads to interfacial activation (Schimid and Verger 1988), but some lipases, esterases, and cutinases have neither a lid nor interfacial activation. To cover an active site with a lid completely, the size of the active site inlet cannot be too big. On the other hand, the inlet of PLA depolymerase must be big enough to accommodate a macromolecular PLA. Accordingly, lipase-type PLA depolymerases are probably not typical true lipases, but esterases (cutinases) without a lid, useful for interfacial activation, and probably possesses an active cavity big enough to accommodate a polymer substrate. Cutin is a rather large molecule with a complex structure.

16.5 Non-Metabolic Degradation

The first report on the degradation of synthetic polymeric materials by lignin-degrading fungi described the degradation of nylon-6 and -66 by white-rot fungi, including an isolate (IZU-154) and stock cultures (Deguchi et al. 1997); nylon-degrading activity is based on oxidation by manganese peroxidase and is closely related to the lignolytic activity of fungi (Deguchi et al. 1998). Larking et al. (1999) found that the degradation of PVA was promoted by a combination of treatments with Fenton's reagent followed by biological degradation, probably by laccase produced in the culture supernatant of the white-rot fungus *Pycnoporus cinnabarinus.* Another lignin-degrading white-rot fungus, *Phanerochaete chrysoporium,* excreted lignin peroxidase, which promoted the degradation of PVA chains through the formation of carbonyl groups as well as double bonds (Mejia et al. 1999). A substantial decrease (approximately 80%) in average MW was observed. Since the carbon chain of oxidized PVA is readily cleaved through non-enzymatic processes, strong oxidation of PVA either by an oxidizing enzyme

or by a Fenton reaction leads to cleavage of the main carbon chain and yields oligomeric materials. Oligomers are generally biodegradable, as are oligomeric ethylene, styrene, and isoprene, although their polymers are non-biodegradable (Kawai 1995). On the other hand, the brown-rot fungus *Gloeophyllum trabeum* secretes quinones (Jensen et al. 2001) that reduce Fe^{3+} and produce H_2O_2, resulting in an extracellular Fenton reaction degrading PEG. Thus, brown- and white-rot fungi can play a significant role in the recycling of materials in the environment, and their degradation-related enzymes have potential applications in the treatment of polymer wastes and wastewater.

16.6 Conclusion

The degradation of polymers was at first expected to be catalyzed solely by extracellular enzymes, given the assumption that macromolecules are never incorporated into cells, but this was disproved by the periplasmic degradation of PEG and PVA, as described above. These are surely incorporated into the periplasm through the outer membranes of Sphingomonads and are metabolized by periplasmic enzymes, although the mechanism of macromolecule uptake has not been well characterized. Many enzymes related to the microbial degradation of polymers have been elucidated genetically. They show unique features: PEG-DH creates a small branch in GMC flavoprotein oxidoreductases (Zamocky et al. 2004) and PEG-aldehyde dehydrogenase was the first nicotinoprotein aldehyde dehydrogenase. Ether bond-splitting enzymes are not catalyzed by ether bond-specific enzymes, but by various divergent enzymes such as monooxygenase, oxidase, dehydrogenase, hydrolase, and lyase. PVA-DHs form a unique group of quinohemoprotein dehydrogenases. Research on nylon oligomer-degrading enzymes has verified that xenobiotic polymer-degrading enzymes can readily evolve by spontaneous mutation from ancestor enzymes that originally recognized natural compounds analogous to the target polymers. A PEG-DH gene from a Sphingomonad has been distributed and conserved among different genera in the 35 years since PEG-degrading Sphingomonads were isolated in 1975 and a gene for PEG-DH was cloned in 2001, suggesting a significant role for a large plasmid harboring a PEG-degradative gene cluster in circulation for degradation ability among microorganisms. Since a *pva* operon is on a large plasmid of *Sphingopyxis* sp. strain 113P3, the PVA-DH gene and the operon structure have probably been distributed among microorganisms. This strongly suggests that the short history of xenobiotic polymers to date has been sufficient for degrading enzymes to evolve since the prototype enzymes existed and the microorganisms had to adapt to an environment contaminated by xenobiotics. In addition, megaplasmids must have sped up the distribution rate of degrading ability. The existence of Sphingomonads that degrade various xenobiotic polymers, such as PEG and PVA, via intracellular enzymes suggest that they have a means of taking macromolecules into the periplasm as well as metabolic enzymes adapted to respond to these

macromolecules. Polymer-degrading microorganisms and their enzymes for polymer degradation have been well described. Many examples of symbiotic polymer degradation appear to suggest that symbiotic degradation occurs in an ecosystem when new artificial compounds are introduced. At the same time, non-metabolic polymer degradation suggests that the ecosystem has the disposal potential and versatility in terms of new artificial compounds.

However, information is still limited with regard to the operonic structures of the genes related to degradation and to the regulation of individual genes and operons, except for those involved in PEG and PVA degradation. The *peg* operon is expressed in the presence of PEG, but expression is triggered by oligomers having the size of more than tetramer. The *pva* operon is constitutively expressed, but expression is remarkably enhanced by PVA. The size of PVA that triggers promotion of the *pva* operon remains to be determined. Since PVA-DH and oxidized PVA-hydrolyzing enzymes are periplasmic enzymes, it is likely that depolymerized oligomeric PVA can be incorporated into the cytoplasm where it promotes the expression of the *pva* operon. Extracellular PLA-degrading proteases from PLA-degrading microorganisms have been induced with proteinaceous materials. Since it is probably impossible for hard, solid PLA to penetrate cells, the microorganisms must have selected another strategy for degradation, one that employs extracellular excretion of proteases induced by proteinous materials including alanine, analogously to lactate. An aliphatic–aromatic-*co*-polyester-degrading enzyme from *T. alba* strain AHK119 was expressed constitutively (unpublished data), as the target polyester can never be incorporated into cells. The polyester-degrading enzymes belong to the lipase family and are distinguished from true lipases in the sense that they have no lid covering the active site, which is prerequisite to the interfacial activation, characteristic of true lipases. They must have acquired the ability to recognize and hydrolyze solid macromolecules, but the details of the mechanism are still under study. Thus, microorganisms have employed a variety of adaptation strategies in relation to novel artificial compounds in a short time. Although oligomeric structures are biodegradable, some polymers, such as PE, polystylene, and polyacrylonitrile, are not practically biodegradable. This suggests limitations in the biodegradation of solid polymers with regard to their physico-chemical properties, such as MW, Tg, and mp. In other words, we can design biodegradable polymers and non-biodegradable polymers based on their intended uses.

References

Albertsson AC, Andersson SO, Karlsson A (1987) The mechanism of biodegradation of polyethylene. Polym Degrad Stab 18:73–87

Al-Malaika S, Marogi AM, Scott G (1986) Mechanisms of antioxidant action: time-controlled photoantioxidaants for polyethylene based on soluble iron compounds. J Appl Polym Sci 31:685–698

Baere L, De Wilde B, Tillinger R (1994) Standard test methods for polymer degradation in solid waste treatment systems. In: Doi Y, Fukuda K (eds) Studies in polymer science-biodegradable plastics and polymers. Elsevier, The Netherlands, pp 323–330

Charoenpanich J, Tani A, Moriwaki N, Kimbara K, Kawai F (2006) Dual regulation of a polyethylene glycol degradative operon by AraC-type and GalR-type regulators in *Sphingopyxis macrogoltabida* strain 103. Microbiology 152:3025–3034

Chen S, Tong X, Woodard RW, Du G, Wu J, Chen J (2008) Identification and characterization of bacterial cutinase. J Biol Chem 283:25854–25862

Chiellini E, Corti A, D'Antone S, Solaro R (2003) Biodegradartion of poly(vinyl alcohol) based materials. Prog Polym Sci 28:963–1014

Deguchi T, Kakezawa M, Nishida T (1997) Nylon biodegradation by lignin-degrading fungi. Appl Environ Microbiol 63:329–331

Deguchi T, Kitaoka Y, Kakezawa M, Nishida T (1998) Purification and characterization of a nylon-degrading enzyme. Appl Environ Microbiol 64:1366–1371

Doser C, Zschocke P, Biedermann R, Sussmuth R, Trauter J (1997) Mikrobieller abbau von polyacrylsaureshlichten. Texilveredlung 32:245–249

Dwyer DF, Tiedje JM (1986) Degradation of ethylene glycol and polyethylene glycols by methanogenic consortia. Appl Environ Microbiol 52:852–856

Enokibara S, Kawai F (1997) Purification and characterization of an ether bond-cleaving enzyme involved in the metabolism of polyethylene glycol. J Ferment Bioeng 83:549–554

Frings J, Schramm E, Schink B (1992) Enzymes involved in anaerobic polyethylene glycol degradation by *Pelobacter venetianus* and *Bacteroides* strain PG1. Appl Environ Microbiol 58:2164–2167

Gordienko AD, Kudokotseva EV (1980) Study of the functional composition of mitochondria in cellular suspensions subject to a cryoprotectant solution. Kriobiol Kriomed 7:32–34

Grant MA, Payne WJ (1983) Anaerobic growth of *Alcaligenes faecalis* var. *denitrificans* at the expense of ether glycols and nonionic detergents. Biotechnol Bioeng 25:627–630

Haines J, Alexander M (1975) Microbial degradation of polyethylene glycols. Appl Microbiol 29:621–625

Hayashi T (1998) Microbial degradation of poly(sodium acrylate). Recent Res Dev Microbiol 2:335–349

Hayashi T, Mukouyama M, Sakano K, Tani Y (1993) Degradaion of a sodium acrylate oligomer by an *Arthrobacter* sp. Appl Environ Microbiol 59:1555–1559

Hayashi T, Nishimura H, Sakano K, Tani Y (1994) Microbial degradation of poly(sodium acrylate). Biosci Biotechnol Biochem 58:444–446

Herald DA, Keil K, Bruns DE (1989) Oxidation of polyethylene glycols by alcohol dehydrogenase. Biochem Pharmacol 38:73–76

Hiraishi T, Kajiyama M, Tabata K, Abe H, Yamato I, Doi Y (2003a) Biochemistry and molecular characterization of poly(aspartic acid) hydrolase-2 from *Sphingomonas* sp. KT-1. Biomacromolecules 4:1285–1292

Hiraishi T, Kajiyama M, Tabata K, Yamato I, Doi Y (2003b) Genetic analysis and characterization of poly(aspartic acid) hydrolase-1 from *Shphingomonas* sp. KT-1. Biomacromolecule 4:80–86

Hiraishi T, Kajiyama M, Yamato I, Doi Y (2004) Enzymatic hydrolyxis of α- and β-oligo (L-aspartic acid)s by poly(aspartic acid) hydrolases-1 and 2 from *Sphingomonas* sp. KT-1. Macromol Biosci 4:330–339

Hirota-Mamoto R, Nagai R, Tachibana S, Yasuda M, Tani A, Kimbara K, Kawai F (2006) Cloning and expression of the gene for periplasmic poly(vinyl alcohol) dehydrogenase from *Sphingomonas* sp. strain 113P3, a novel-type quinohaemoprotein alcohol dehydrogenase. Microbiology 152:1941–1949

Hosoya H, Miyazaki N, Sugisaki Y, Takanashi E, Tsurufuji M, Yamasaki M, Tamura G (1978) Bacterial degradation of synthetic polymers and oligomers with the special reference to the case of polyethylene glycol. Agric Biol Chem 42:1545–1552

Hu X, Fukutani A, Liu X, Kimbara K, Kawai F (2007a) Isolation of bacteria able to grow on both polyethylene glycol (PEG) and polypropylene glycol (PPG) and their PEG/PPG dehydrogenases. Appl Microbiol Biotechnol 73:1407–1413

Hu X, Mamoto R, Shimomura Y, Kimbara K, Kawai F (2007b) Cell surface structure enhancing uptake of polyvinyl alcohol (PVA) is induced by PVA in the PVA-utilizing *Sphingopyxis* sp. strain 113P3. Arch Microbiol 188:235–241

Hu X, Liu X, Tani A, Kimbara K, Kawai F (2008a) Proposed oxidative metabolic pathway for polypropylene glycol in *Sphingobium* sp. strain PW-1. Biosci Biotechnol Biochem 72:1115–1118

Hu X, Mamoto R, Fujioka Y, Tani A, Kimbara K, Kawai F (2008b) The *pva* operon is located on the megaplasmid of *Sphingopyxis* sp. strain 113P3 and is constitutively expressed, although expression is enhanced by PVA. Appl Micobiol Biotechnol 78:685–693

Hu X, Osaki S, Hayashi M, Kaku M, Katuen S, Kobayashi H, Kawai F (2008c) Degradation of a terephthalate-containing polyester by thermophilic actinomycetes and *Bacillus* species derived from composts. J Polym Environ 16:103–108

Hu X, Thumarat U, Zhang X, Tang M, Kawai F (2010) Diversity of polyester-degrading bacteria in compost and molecular analysis of a thermoactive esterase from *Thermobifida alba* AHK119. Appl Microbiol Biotechnol 87:729–771

Iwahashi M, Katsuragi T, Tani Y, Tsutsumi K, Kakiuchi K (2003) Mechanism for degradation of poly(sodium acrylate) by bacterial consortium No. L7–98. J Biosci Bioeng 95:483–487

Jarerat A, Tokiwa Y (2001) Degradation of poly(L-lactide) by a fungus. Macromol Biosci 1:136–140

Jensen KA Jr, Houman CJ, Ryan ZC, Hammel KE (2001) Pathways for extracellular Fenton chemistry in the brown rot basidiomycete *Gloeophyllum trabeum*. Appl Environ Microbiol 67:2705–2711

Kakudo S, Negoro S, Urabe I, Okada H (1993) Nylon oligomer degradation gene, *nylC* on plasmid pOAD2 from a *Flavobacerium* strain encodes endo-type 6-aminohecanoate oligomer hydrolase: purification and characterization of the *nylC* gene product. Appl Environ Microbiol 59:3978–3980

Kakudo S, Negoro S, Urabe I, Okada H (1995) Characterization of endotype 6-aminohexanoate-oligomer hydrolase from *Flavobacerium* sp. J Ferment Bioeng 80:12–17

Kanagawa K, Negoro S, Takada N, Okada H (1989) Plasmid dependence of *Pseudomonas* sp. strain NK87 enzymes that degrade 6-aminohexanoate-cyclic dimer. J Bacteriol 171:3181–3186

Kawai F (1987) The biochemistry of degradation of polyethers. CRC Crit Rev Biotechnol 6:273–307

Kawai F (1993) Bacterial degradation of acrylic oligomers and polymers. Appl Microbiol Biotechnol 39:382–385

Kawai F (1995) Breakdown of plastics and polymers by microorganisms. In: Fichter A (ed) Advances in biochemical engineering/biotechnology, vol 52. Springer, Germany, pp 151–194

Kawai F (1996) Bacterial degradation of a new polyester, polyethylene glycol-phthalate polyester. J Environ Polym Degrad 4:21–28

Kawai F (1999) Sphingomonads involved in the biodegradation of xenobiotic polymers. J Ind Microbiol Biotechnol 23:400–407

Kawai F (2002) Microbial degradation of polyethers. Appl Microbiol Biotechnol 58:30–38

Kawai F (2010a) Polylactic acid (PLA)-degrading micoorganisms and PLA depolymerases. In: Gross RA, Chen HN (eds) Green polymer chemistry: biocatalysts and biomaterials. American Chemical Society, USA, pp 405–414

Kawai F (2010b) The biochemistry and molecular biology of xenobiotic polymer degradation by microorganisms. Biosci Biotechnol Biochem 74:1743–1759

Kawai F, Hayashi T (2002) Biodegradation of polyacrylate. In: Matsumura S, Steinbüchel A (eds) Biopolymers, vol 9. Wiley-VCH, Germany, pp 299–321

Kawai F, Hu X (2009) Biochemistry of microbial polyvinyl alcohol degradation. Appl Microbiol Biotechnol 84:227–237

Kawai F, Moriya F (1991) Bacterial assimilation of polytetramethylene glycol. J Ferment Bioeng 71:1–5
Kawai F, Yamanaka H (1986) Biodegradation of polyethylene glycol by symbiotic mixed culture (obligate mutualism). Arch Microbiol 146:125–129
Kawai F, Hanada K, Tani Y, Ogata K (1977) Bacterial degradation of water-insoluble polymer (polypropylene glycol). J Ferment Technol 55:89–96
Kawai F, Okamoto T, Suzuki T (1985) Aerobic degradation of polypropylene glycol by *Corynebacterium* sp. J Ferment Technol 68:239–244
Kawai F, Igarashi K, Kasuya F, Fukui M (1994) Proposed mechanism for bacterial metabolism of polyacrylate. J Environ Polym Degrad 2:59–65
Kawai F, Shibata M, Yokoyama S, Maeda S, Tada K, Hayashi S (1999) Biodegradability of Scott–Gelead photodegradable polyethylene and polyethylene wax by microorganisms. Macromol Symp 144:85–99
Kawashima Y, Ohki T, Shibata N, Higuchi Y, Wakitani Y, Matsuura Y, Nakata Y, Takeo M, Kato D, Negoro S (2009) Moleccular design of a nylon-6 byproduct-degrading enzyme from a carboxylesterase with a β-lactamase fold. FEBS J 276:2547–2556
Kinoshita S, Kageyama S, Iba K, Yamada Y, Okada H (1975) Utilization of a cyclic dimer and linear oligomers of ε-aminocaproic acid by *Achromobacter guttatus* KI71. Agric Biol Chem 39:1219–1223
Kinoshita S, Negoro S, Muramatsu M, Bisaria VS, Sawada S, Okada H (1977) 6-Aminohexanoic acid cyclic dimer hydrolase: a new cyclic amide hydrolase produced by *Achromobacter guttatus* KI72. Europe J Biochem 80:489–490
Kinoshita S, Terada T, Tanigucchi T, Takene Y, Masda S, Matsunaga N, Okada H (1981) Purification and characterization of 6-aminohexanoic-acid-oligomer hydrolase of *Flavobacerium* sp. KI71. Europe J Biochem 116:547–551
Kleeberg I, Welzel K, VandenHeuvel J, Müller R-J, Deckwer W-D (2005) Characterization of a new extracellular hydrolase from *Thermobifida fusca* degrading aliphatic-aromatic copolyesters. Biomacromolecules 6:262–270
Klomklang W, Tani A, Kimbara K, Mamoto R, Ueda T, Shimao M, Kawai F (2005) Biochemical and molecular characterization of a periplasmic hydrolase for oxidized polyvinyl alcohol from *Sphingomonas* sp. strain 113P3. Microbiology 151:1255–1262
Kohlweyer U, Thiemer B, Schraeder T, Andreesen JR (2000) Tetrahydrofuran degradation by a newly isolated culture of *Pseudonocardia* sp. strain K1 FEMS. Microbiol Lett 186:301–306
Larking DM, Crawford RJ, Christie GBY, Linergan GT (1999) Enhanced degradation of polyvinyl alcohol by *Pycnoporus cinabarinus* after pretreatment with Fenton's reagent. Appl Environ Microbiol 65:1798–1800
Larson RJ, Bookland EA, Williams RT, Yocom KM, Saucy DA, Freeman MB, Swift G (1997) Biodegradation of acrylic acid polymers and oligomers by mixed microbial communities in activated sludge. J Environ Polym Degrad 5:41–48
Mamoto R, Hu X, Chiue H, Fujioka Y, Kawai F (2008) Cloning and expression of soluble cytochrome *c* and its role in polyvinyl alcohol degradation by polyvinyl alcohol-utilizing *Sphingopyxis* sp. strain 113P3. J Biosci Bioeng 105:147–151
Masaki K, Kamini NR, Ikeda H, Iefuji H (2005) Cutinase-like enzyme from the yeast *Cryptococcus* sp. strain S-2 hydrolyzes polylactic acid and other bioderadable plastics. Appl Environ Microbiol 71:7548–7550
Matsuda E, Abe N, Tamakawa H, Kaneko J, Kamio Y (2005) Gene cloning and molecular characterization of an extracellular poly(L-lactic acid) depolymerae from Amycolatopsis sp. strain K104–1. J Bacteriol 187:7333–7340
Matsumura S (2002) Biodegradation of poly(vinyl alcohol) and its copolymers. In: Matsumura S, Steinbüchel A (eds) Biopolymers, vol 9. Wiley-VCH, Germany, pp 329–361
Matsumura S, Maeda S, Takahashi J, Yoshikawa S (1988) Biodegradation of poly(vinyl alcohol) and poly(sodium acrylate)-*co*-(vinyl alcohol). Kobunshi Ronbunshu 45:317–324
Matsumura S, Kurita H, Shimokobe H (1993) Anaerobic biodegradability of polyvinyl alcohol. Biotechnol Lett 15:749–754

Mayumi D, Shigeno Y, Uchiyama H, Nomura N, Nakajima KT (2008) Identification and characterization of novel poly(DL-lactic acid) depolymerases from metagenome. Appl Microbiol Biotechnol 79:743–750

Mejia AI, Lucy Lopez BL, Mulet A (1999) Biodegradation of poly(vinyl alcohol) with enzymatic extracts of *Phaenerochaete chrysosporium*. Macromol Symp 148:131–147

Müller R, Kleeberg I, Deckwer W (2001) Biodegradation of polyesters containing aromatic constituents. J Biotechnol 86:87–95

Müller R-J, Schrader H, Profe J, Dresler K, Deckwer W-D (2005) Enzymatic degradation of poly(ethylene terephthalate): Rapid hydrolyse using a hydrolase from *T. fusca*. Macromol Rapid Commun 6:1400–1405

Nagarajan V, Singh M, Kane H, Khalili M, Bramucci M (2006) Degradation of a terephthalate-containing polyester by a thermophilic microbial consortium. J Polym Environ 14:281–287

Nakamura K, Tomita T, Abe N, Kamio Y (2001) Purification and characterization of an extracellular poly(L-lactic acid) depolymerase from a soil isolate, *Amycolatopsis* sp. strain K104–1. Appl Environ Microbiol 67:345–353

Negoro S (2000) Biodegradation of nylon oligomers. Appl Microbiol Biotechonol 54:461–466

Negoro S, Kakudo S, Urabe I, Okada H (1992) A new nylon oligomer degradation gene (*nylC*) on plasmid pOAD2 from *Flavobacterium* sp. J Bacteriol 174:7948–7953

Nishikawa M, Ogawa K (2004) Antimicrobial activity of a chelatable poly(arginyl-histidine) produced by the ergot fungus *Verticillium kibiense*. Antimicrob Agents Chemother 48:229–235

Nord FF (1936) Dehydrogenation ability of *Fusarium lini* B. Naturwissenschaften 24:793

Obradors N, Aguilar J (1975) Efficient biodegradation of high-molecular-weight polyethylene glycols by pure cultures of *Pseudomonas stutzeri*. Appl Environ Microbiol 57:2383–2388

Obst M, Steinbüchel A (2004) Microbial degradation of poly(amino acid)s. Biomacromolecules 5:1166–1176

Ogata K, Kawai F, Fukaya M, Tani Y (1975) Isolation of polyethylene glycols-assimilable bacteria. J Ferment Technol 53:757–761

Ohta T, Tani A, Kimbara K, Kawai F (2005) A novel nicotinoprotein aldehyde dehydrogenase involved in polyethylene glycol degradation. Appl Microbiol Biotechnol 68:639–646

Ohta T, Kawabara T, Nishikawa K, Tani A, Kimbara K, Kawai F (2006) Analysis of amino acid residues involved in catalysis of polyethylene glycol dehydrogenase from *Sphingopyxis terrae*, using three-dimensional molecular modeling-based kinetic characterization of mutants. Appl Environ Microbiol 72:4388–4396

Ornek D, Jayaraman A, Syrett BC, Hsu C-H, Mansfeld FB, Wood TK (2002) Pitting corrosion inhibition of aluminum 2024 by *Bacillus* biofilms secreting polyaspartate or γ-polyglutamate. Appl Microbiol Biotechnol 58:651–657

Otake Y, Kobayashi T, Asabe H, Murakami N, Ono K (1995) Biodegradation of low-density polyethylene, polystyrene, polyvinyl chloride, and urea formaldehyde resin buried under soil for over 32 years. J Appl Pol Sci 56:1789–1796

Payne WJ (1963) Pure culture studies of the degradation of detergent compounds. Biotechnol Bioeng 5:355–365

Potts JE, Clendinning RA, Achart WBA, Niegishi WD (1973) The biodegradability of synthetic polymers. Polym Sci Technol 3:61–79

Pranamuda H, Tokiwa Y, Tanaka H (1997) Polylactide degradation by an *Amycolatopsis* sp. Appl Environ Microbiol 63:1637–1640

Pranamuda H, Tsuchii A, Tokiwa T (2001) Poly(L-lactide)-degrading enzyme produced by *Amycolatopsis* sp. Macromol Biosci 1:25–29

Prijambada ID, Negoro S, Yomo T, Urabe I (1995) Emergence of nylon ligomer degradation enzymes in *Pseudomonas aeruginosa* PAO through experimental evolution. Appl Environ Microbiol 61:2020–2022

Qian D, Du G, Chen J (2004) Isolation and culture characterization of a new polyvinyl alcohol-degrading strain; *Penicillium* sp. WSH02–21. World J Microbiol Biotechnol 20:587–591

Reeve MS, McCarthy SP, Downew MJ, Gross RA (1994) Polylactide stereochemistry: effect on enzymatic degradability. Macromolecules 72:825–831

Rittmann BE, Benjamin H, Odencrantz JE, Sutfin JA (1992a) Biological fate of a polydisperse acrylate polymer in anaerobic sand-medium transport. Biodegradation 2:171–179

Rittmann BE, Sutfin JA, Benjamin H (1992b) Biodegradation and sorption properties of polydisperse acrylate polymers. Biodegradation 2:181–190

Ronkvis AM, Xie X, Lu W, Gross RA (2009) Cutinase-catalyzed hydrolysis of poly(ethylene terephthalate). Macromolecules 42:5128–5138

Roy AB, Curtis CG, Powell GM (1987) The metabolic sulphation of polyethylene glycols by isolated perfused rat and guinea-pig livers. Xenobiotica 17:725–732

Rusenko KW, Donachy JE, Weler AP (1991) Purification and characterization of a shell matrix phosphoprotein from the American oyster. In: Sikes CS, Wheeler AP (eds) Surface reactive peptides and polymers: discovery and commercialization. American Chemical Society, USA, pp 107–121

Schink B, Stieb M (1983) Fermentative degradation of polyethylene glycol by a strictly anaerobic, Gram-negative, nonsporeforming bacterium. Appl Environ Microbiol 45:1905–1913

Schmid RD, Verger R (1988) Lipases: interfacial enzymes with attractive applications. Angew Chem Int Ed 37:1608–1633

Scott G (1975) Biological recycling of polymers. Polym Age 6:54–56

Scott G, Gilead D (1978) British Patent 1, 586, 344

Soeda Y, Toshima K, Matsumura S (2003) Sustainable enzymatic preparation of polyaspartate using a bacterial protease. Biomacromolecules 4:196–203

Somyoonsap P, Tani A, Charoenpanich J, Minami T, Kimbara K, Kawai F (2008) Invovement of PEG-carboxylate dehydrogenase and glutathione *S*-transferase in PEG metabolism by *Sphingoyxis macrogoltabida* strain 103. Appl Microbiol Biotechnol 81:473–484

Speranza G, Mueller B, Orlandi M, Morelli CF, Manitto P, Schink B (2002) Mechanism of anaerobi ether leavage; conversion of 2-phenoxyethanol to phenol and acetaldehyde by *Acetobacterium* sp. J Biol Chem 277:11684–11690

Strass A, Schink B (1986) Fermentation of polyethylene glcol via acetaldehyde in *Pelobacter venetianus*. Appl Microbiol Biotechnol 25:37–42

Sugimoto M, Tanabe M, Hataya M, Enokibara S, Duine JA, Kawai F (2001) The first step in polyethylene glycol degradation by sphingomonads proceeds via a flavoprotein alcohol dehydrogenase containing flavin adenine dinucleotide. J Bacteriol 183:6694–6698

Suzuki T, Ichihara Y, Yamada M, Tonomura K (1973) Some characteristics of *Pseudomonas* O-3 which utilizes polyvinyl alcohol. Agric Biol Chem 37:747–756

Tabata K, Abe H, Doi Y (2000) Microbial degradation of poly(aspartic acid) by two isolated strains of *Pedobacter* sp. and *Sphingomonas* sp. Biomacromolecules 1:157–161

Tabata K, Kajiyama M, Hiraishi T, Abe H, Doi Y (2001) Purification and characterization of poly(aspartic acid) hydrolase from *Sphingomonas* sp. KT-1. Biomacromolecules 2:1155–1160

Tachibana S, Kawai F, Yasuda M (2002) Heterogeneity of dehyrrogenases of *Stenotrophomonas maltophilia* showing dye-linked activity with polypropylene glydols. Biosci Biotechnol Biochem 66:737–742

Tachibana S, Kuba N, Kawai F, Duine JA, Yasuda M (2003) Invovement of a quinoprotein (PQQ-containing) alcohol dehydrogenase in the degrdation of polypropylene glycols by the bacterium *Stenotrophomonas maltophilia*. FEMS Microbiol Lett 218:345–349

Tachibana S, Naka N, Kawai F, Yasuda M (2008) Purification and characterization of cytoplasmic NAD^+-dependent polypropylene glycol dehydrogenase from *Stenotrohomonas maltophilia*. FEMS Microbiol Lett 288:266–272

Takeuchi M, Hamana K, Hiraishi A (2001) Proposal of the genus *Sphingomonas sensu stricto* and three new genra, *Sphingobium*, *Novosphingobium* and *Spingopyxis*, on the basis of phylogenetic and chemotaxonomic analyses. Int J Syst Evol Microbiol 51:1405–1417

Tani A, Charoenpanich J, Mori T, Takeuchi M, Kimbara K, Kawai F (2007) Structure and conservation of a polyethylene glycol-degradative operon in sphingomonads. Microbiology 153:338–346

Tani A, Somyoonsap P, Minami T, Kimbara K, Kawai F (2008) Polyethylene glycol (PEG)-carboxylate-CoA synthetase is involved in PEG metabolism in *Sphingopyxis macrogoltabida* strain 103. Arch Microbiol 189:407–410

Tokiwa Y, Calabia BP (2004) Degradation of microbial polyesters. Biotechnol Lett 26:1181–1189

Tokiwa Y, Calabia BP (2006) Biodegradability an biodegradation of poly(lactide). Appl Microbiol Biotechnol 72:244–251

Tokiwa Y, Jarerat A (2004) Biodegradation of poly(L-lactide). Biotechnol Lett 26:771–777

Tokiwa Y, Jarerat A, Tsuchiya A (2003) Japanese Patent 2003-61676, 4 March 2003

Tomita K, Ideda N, Ueno A (2003a) Isolation and characterization of a thermophilic bacterium, *Geobacillus thermocatenulatus,* degrading nylon 12 and nylon 66. Biotechnol Lett 25:1743–1746

Tomita K, Tsuji T, Nakajima H, Kikuchi Y, Ikarashi K, Ikeda N (2003b) Degradation of poly (D-lactic acid) by a thermophile. Polym Degrad Stab 81:167–171

Watanabe Y, Moria M, Hamada N, Tsujisaka Y (1975) Formation of hydrogen peroxide by a polyvinyl alcohol degrading enzyme. Agric Biol Chem 39:2447–2448

Williams DF (1981) Enzymatic hydrolysis of polylactic acid. Eng Med 10:5–7

Yamanaka H, Kawai F (1991) Purification and characterization of a glycolic acid (GA) oxidase active toward diglycolic acid (DGA) produced by DGA-utilizing *Rhodococcus* sp. no. 431. J Ferment Bioeng 71:83–88

Yasuhira K, Tanaka Y, Shibata H, Kawashima Y, Ohara A, Kato D, Takeo M, Negoro S (2007a) 6-Aminohexanoate oligomer hydrolases from the alkanophilic bacteria *Agromyces* sp. strain KY5R and *Kocuria* sp. strain KY2. Appl Environ Microbiol 73:7099–7102

Yasuhira K, Uedo Y, Takeo M, Kato D, Negoro S (2007b) Genetic organization of nylon-oligomer-degrading enzymes from alkalophilic bacterium, *Agromyces* sp. KY5R. J Biosci Bioeng 104:521–524

Zamocky M, Hallberg M, Ludwig R, Divne C, Haltrich D (2004) Ancestal gene fusion in cellobiose dehydrogenases reflects a specific evolution of GMC oxidoreductases in fungi. Gene 338:1–14

Chapter 17
Microbial Degradation of Alkanes

S. N. Singh, B. Kumari and Shweta Mishra

17.1 Introduction

Petroleum hydrocarbons are introduced into the environment due to their extensive use as fuels and chemicals. Besides, leaks and accidental spills occur often during exploration, production, refining, transport and storage of petroleum and petroleum products which used to add an additional burden of hydrocarbons to soils and water systems. The technologies commonly used for soil remediation of petroleum hydrocarbons include mechanical burying, evaporation, dispersion and washing. These remedial measures are not only cost intensive and time consuming, but also not very effective. On the other hand, bioremediation leads to complete mineralization of organic compounds into CO_2 and water by indigenous micro-organisms and hence a preferred choice also being eco-friendly and cost-effective.

Anthropogenic hydrocarbon contamination of soil is a global issue throughout the industrialised world (Macleod et al. 2001; Brassington et al. 2007). In England and Wales alone, 12% of all serious contamination incidents in 2007 were hydrocarbon related. Soil acts as a repository for many hydrocarbons, which is a serious concern due to their adverse impact on human health and environmental persistence for a long time (Jones et al. 1996; Semple et al. 2001).

Alkanes are a major fraction (>50%) of the crude oil depending upon the oil source. Alkanes are saturated hydrocarbons and chemically very inert as apolar molecules (Labinger and Bercaw 2002). They may be classified as linear (*n*-alkanes), cyclic (cyclo-alkanes) or branched (iso-alkanes) and found in three states: gaseous (C1–C4), liquid (C5–C16) and solid (>C17) (Fig. 17.1). Although

S. N. Singh (✉) · B. Kumari · S. Mishra
Environmental Sciences Division, CSIR-National Botanical Research Institute, Lucknow 226 001, India
e-mail: drsn06@gmail.com

S. N. Singh (ed.), *Microbial Degradation of Xenobiotics*, Environmental Science and Engineering, DOI: 10.1007/978-3-642-23789-8_17,

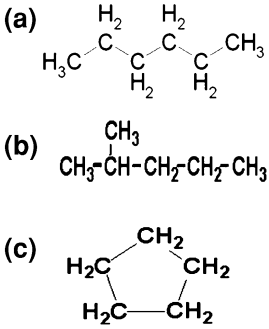


Fig. 17.1 Examples of linear; *n*-Hexane (**a**) branched; Iso-hexane (**b**) and cyclic alkanes; Cyclopentane (**c**)

highly inflammable, alkanes are less reactive as organic compounds. They are highly essential for modern life, but their inertness poses serious ecological problems when released to the environment. However, microbes have developed effective strategies involving specific enzymes and metabolic pathways to use *n*-alkanes as a carbon source. Thus, microbes have the capability to degrade alkanes and convert them to easily metabolizable substrates.

17.2 Microbial Degradation of Alkanes

Due to lack of functional groups as well as very low water solubility, aliphatic hydrocarbons exhibit both, low chemical reactivity and bioavailability for microorganisms. However, some microorganisms possess the metabolic capacity to use these compounds as carbon and energy sources for their growth (Berthe-Corti and Fetzner 2002).

A number of microbes including bacteria, fungi and yeasts have been reported to degrade alkanes using them as the source of carbon and energy (van Beilen et al. 2003; Wentzel et al. 2007). Bacteria with alkane degradation ability have also versatile metabolism to use other compounds in addition to alkanes as source of carbon (Margesin et al. 2003; Haryama et al. 2004). Use of bacteria in the degradation of alkane compounds has been extensively studied by Haryama et al. (2004). Many microbes have been reported for the degradation of aliphatic compounds, such as *Arthrobacter* sp., *Acinetobacter* sp., *Candida* sp., *Pseudomonas* sp., *Rhodococcus* sp., *Streptomyces* sp., *Bacillus* sp., *Aspergillus japonicus*, *Arthrobacter* sp., *Acinetobacter* sp., etc. In addition, some bacterial species are reported as highly specialized in degrading hydrocarbons and hence called hydrocarbonoclastic bacteria. They play a key role in the removal of hydrocarbons from the polluted environments (Head et al. 2006; Yakimov et al. 2007). Schneiker et al. (2006) found a marine bacterium (*Alcanivorax borkumensis*) capable of assimilating both linear or branched alkanes, but unable to metabolize aromatic hydrocarbons. *Alcanivorax dieselolei*, a *g*-proteobacterium, is also a member of the hydrocarbonoclastic bacteria and cannot assimilate sugars

or amino acids as sources of energy and carbon. But it can utilize some organic acids and alkanes. Notably, the spectrum of alkanes utilized by *A. dieselolei* (C5–C36) (Liu and Shao 2005) is substantially broader than those of most other previously described alkane degraders (van Beilen and Funhoff 2007). Other alkane degrading bacterial genera are *Thalassolitus* (Yakimov et al. 2004), *Oleiphilus* (Golyshin et al. 2002), *Bacillus*, *Geobacillus* (Marchant et al. 2006), *Thermus* (Meintanis et al. 2006) and *Oleispira* (Yakimov et al. 2003).

Acinetobacter sp. was found to be capable of utilizing *n*-alkanes of chain length C10–C40 as a sole source of carbon (Throne-Holst et al. 2007). Other bacterial genera, namely, *Gordonia*, *Brevibacterium*, *Aeromicrobium*, *Dietzia*, *Burkholderia* and *Mycobacterium* isolated from petroleum contaminated soil were proven to be potential degraders of hydrocarbons (Chaillan et al. 2004). Hexadecane degradation was observed by the bacteria, such as *Pseudomonas putida*, *Rhodococcus erythroplotis* and *Bacillus thermoleovorans* (Abdel-Megeed et al. 2010) and two bacterial strains; *Flavobacterium* sp. ATCC39723 and *Arthrobacter* sp. (Steiert et al. 1987). Hexadecane (HXD) is present in the aliphatic fraction of crude oil and is one of the major components of diesel (Chenier et al. 2003). Volke-Sepulveda et al. (2003) demonstrated that HXD biodegradation by *Aspergillus niger* was considerably higher in SSF (Solid state fermentation) than in submerged fermentation. Complete HXD conversion was achieved at a C/N ratio of 29 under SSF conditions (Stroud et al. 2008)**.** *Desulfatibacillum alkenivorans* AK-01 is a mesophilic sulfate-reducer isolated from estuarine sediment which utilizes C13–C18 alkanes, 1-alkenes (C15 and C16) and 1-alkanols (C15 and C16) as growth substrates.

Thermophilic alkane degrading bacterium, *Goebacillus thermoleovorans* (previously *Bacillus thermoleovorans*) B23 was reported from a deep-subsurface oil reservoir in Japan (Kato et al. 2001). This strain effectively degraded alkanes at 70°C with the carbon chain longer than dodecane (C12). Since tetradecanoate and hexadecanoate or pentadecanoate and heptadecanoate were accumulated as degradation intermediates of hexadecane or heptadecane degradation, respectively, it indicated that the strain B23 degraded alkanes by a terminal oxidation pathway, followed by *β*-oxidation pathway. Recently, another long chain alkane degrading *Geobacillus thermodenitrificans* NG80-2 was also isolated from a deep sub -surface oil reservoir and its complete genome sequence was determined (Feng et al. 2007).

Some organisms adapted to cold environment are capable of degrading high molecular weight petroleum hydrocarbons. Whyte et al. (1998) reported that *Rhodococcus* sp. strain Q15 was able to degrade alkanes up to *n*-C21 as well as some branched alkanes in diesel, and could also grow on dotriacontane (*n*-C32). *Rhodococcus* strains capable of growing on eicosane (*n*-C20) have been reported by Bej et al. (2000). Studies on petroleum biodegradation in soils from cold regions have reported that lower-molecular weight *n*-alkanes and unsubstituted aromatic hydrocarbons are biodegraded preferentially over the relatively higher-molecular weight *n*-alkane compounds, isoalkanes, alkylated aromatic hydrocarbons, isoprenoids and the branched and cyclic hydrocarbons (Sanscartier et al. 2009).

Besides, many yeasts and fungi, are also known to thrive on alkanes (van Beilen et al. 2003). Among fungal genera, *Amorphoteca*, *Neosartorya*, *Talaromyces* and *Graphium* and yeast genera, *Candida*, *Yarrowia* and *Pichia*, isolated from oil-contaminated soil were found potential degraders of petroleum of petroleum hydrocarbons (Chaillan et al. 2004). Singh (2006) has reported a group of fungi, namely *Aspergillus*, *Cephalosporium* and *Pencillium* to be high degraders of crude oil hydrocarbons. Among yeast species, *Candida lipolytica*, *Rhodotrula mucilaginosa*, *Geotrichum* sp. and *Trichosporam mucoides* isolated from contaminated water were capable to degrade petroleum compounds effectively (Boguslawska-Was and Dabrowski 2001). New genera containing alkane degraders are constantly being identified, leading to a better understanding of ecosystems.

17.2.1 Uptake of n-Alkanes

Alkanes are insoluble in water. The solubility of alkanes depends largely on the molecular weight. With the increase in molecular weight, the solubility decreases in water (Eastcott et al. 1988). Hydrocarbons with a chain length C12 and above are virtually water insoluble. It is still not very clear how alkanes enter the cells of bacteria. The uptake mechanism depends on the bacterial species, the molecular weight of alkane and physico-chemical environment (Wentzel et al. 2007). Low molecular weight alkanes are sparingily soluble in water to ensure a sufficient mass transfer to bacterial cell, while high molecular weight (medium and long chain *n*-alkanes) alkanes find their accessibility to cell either by adherence or by a surfactant-mediated process. This is the reason that alkane degrading bacteria produce diverse surfactants which facilitate the emulsification of hydrocarbons (Ron and Rosenberg 2002). Noordman and Janssen (2002) have reported an increase in the uptake of alkanes in presence of biosurfactants, such as hexadecane in cultures, however, their role in soils and other environments is still not very evident (Holden et al. 2002).

In addition, biosurfactants may also facilitate cell mobility and adhesion to surfaces or biofilms (Boles et al. 2005). They also shield bacterial cells from direct exposure to toxic substances (Kang and Park 2009). Depending on the solubility, the alkanes may be arranged as follow: linear alkanes > branched alkanes > cyclic alkanes with regard to their susceptibility to microbial degradation.

17.2.2 Aerobic Degradation of Alkanes

Aerobic alkane degraders activate alkane molecules using O_2 as a reactant. The alkane-activating monooxygenase overcomes the low reactivity of the hydrocarbon by producing reactive oxygen species. Oxidation of methane leads to formation of

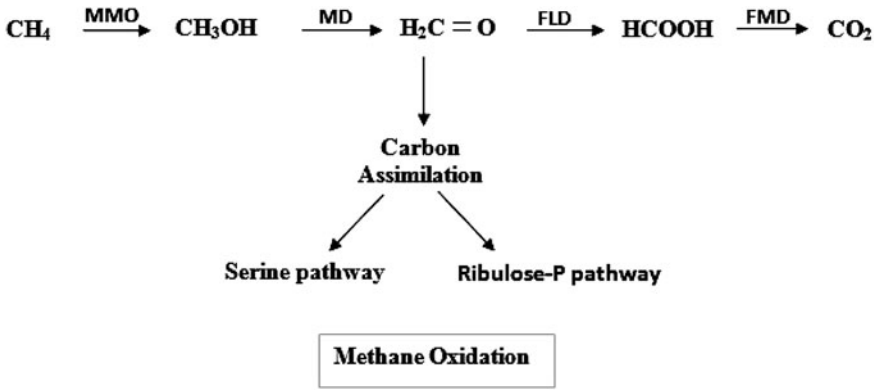


Fig. 17.2 Aerobic pathways of methane oxidation (after Rojo 2009)

methanol which is subsequently transformed to formaldehyde and then to formic acid (Fig. 17.2). This compound either gets converted to CO_2 or assimilated for biosynthesis of other organic compounds either by the ribulose monophosphate pathway or by the serine pathway depending upon the organism (Lieberman and Rosenzweig 2004). The complete degradation of hydrocarbons mainly occurs under aerobic conditions (Riser-Robert 1998). This process involves several steps as illustrated in Fig. 17.3: (1) Accessibility of chemicals to microbes having degradation ability. Since hydrocarbons are insoluble in water, their degradation essentially requires biosurfactants which are produced by bacteria. (2) Activation and incorporation of oxygen is the vital reaction catalysed by oxygenase and peroxidase. (3) Peripheral degradation pathways which convert hydrocarbons into intermediates of the tricarboxylic acid cycle (TCA) and (4) Biosynthesis of cell biomass from the central precursor metabolites i.e. acetyl-CoA, succinate and pyruvate, sugars are required for various biosynthesis and gluconeogenesis for growth.

Degradation of *n*-alkanes is initiated by the oxidation of a terminal methyl group to render a primary alcohol, which gets further oxidized to the corresponding aldehyde, and finally converted into a fatty acid. Fatty acids are conjugated to CoA and further processed by β-oxidation to generate acetyl-CoA (Wentzel et al. 2007) (Fig. 17.4). However, in some cases, both ends of the alkane molecule are oxidized through ω-hydroxylation of fatty acids at the terminal methyl group (ω position), rendering an ω-hydroxy fatty acid that is further converted into a dicarboxylic acid and processed by β oxidation (Coon 2005). Sub-terminal oxidation of *n*-alkanes has also been reported (Kotani et al. 2007). The product generated a secondary alcohol which is converted to the corresponding ketone, and then oxidized by a Baeyer–Villiger monooxygenase to render an ester. The ester is hydrolysed by an esterase, generating an alcohol and a fatty acid. Both terminal and sub-terminal oxidation can co-exist in some microorganisms.

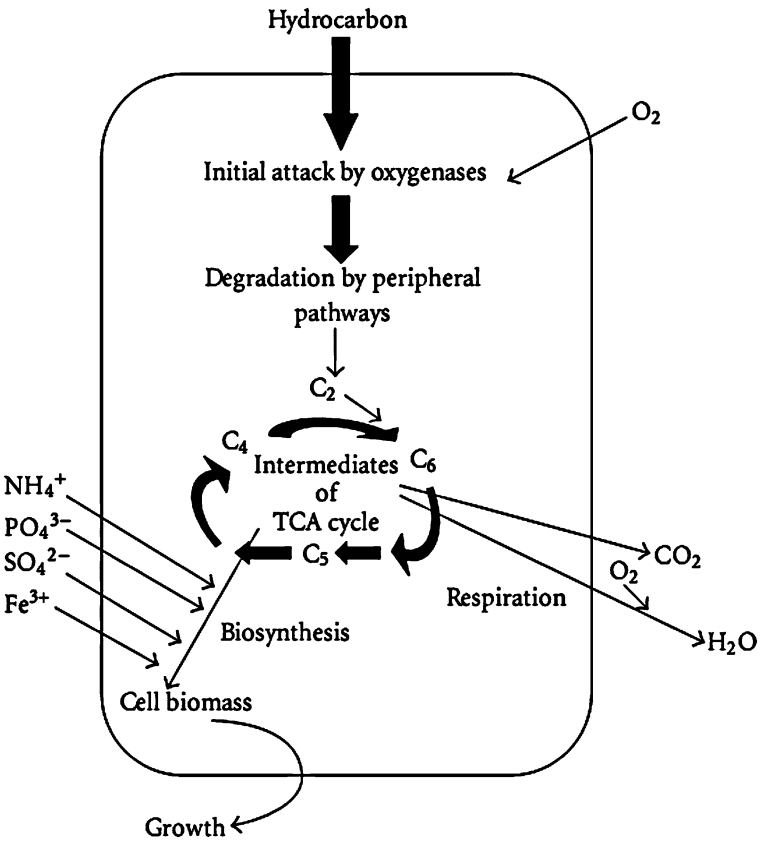


Fig. 17.3 Process of microbial aerobic degradation of hydrocarbons associated with growth process (after Fritsche and Hofrichter 2000)

Some strains of *Pseudomonas* are able to utilize alkanes as the sole carbon and energy source (Stanier et al. 1966). The initial pathway of alkane oxidation is the following:

$$R{-}CH_3 \rightarrow R{-}CH_2OH \rightarrow R{-}CHO \rightarrow R{-}COOH$$

This pathway has been established by simultaneous adaptation experiments (Heringa et al. 1961) and chromatographic analysis of the products of alkane oxidation (Thijsse and van der Linden 1963). *Acinetobacter* spp. can split a hydrocarbon at the number of ten position, forming hydroxyl acids. The initial steps appear to involve terminal attack to form carboxylic acid, sub-terminal dehydrogenation at the number ten position to form an unsaturated acid, and splitting of carbon chain to form a hydroxyl acid and alcohol. Highly branched isoprenoid alkanes, such as Pristane, have been found to undergo ω-oxidation with the formation of dicarboxylic acids as the major degradative pathway.

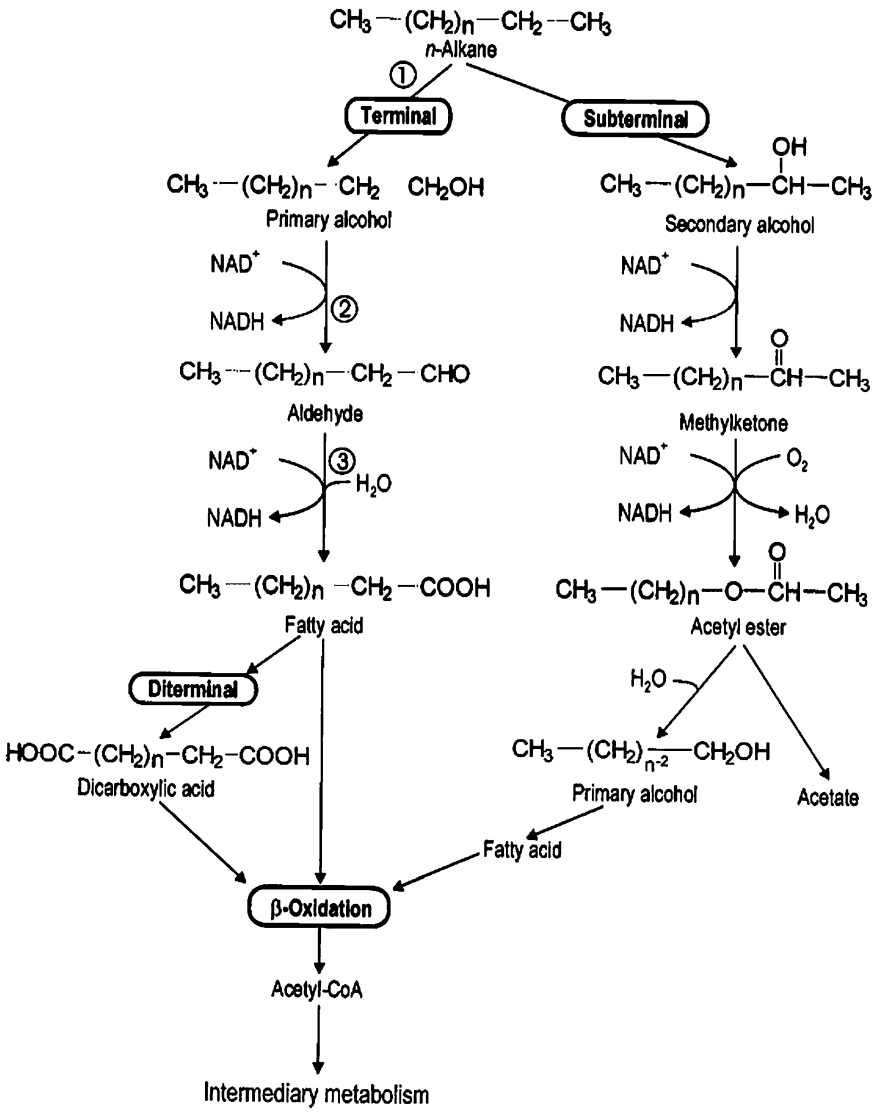


Fig. 17.4 Aerobic pathways of *n*-alkane degradation (after Fritsche and Hofrichter 2000)

Methyl branching increases the resistance of hydrocarbons to microbial attack. Methyl branching at β-oxidation requires an additional strategy, such as α-oxidation, ω-oxidation or β alkyl group removal (Atlas 1981). *Acremonium* spp. oxidize ethane to ethanol by NADPH dependent monooxygenase, which is subsequently oxidized to acetaldehyde and acetic acid. Acetate, thus formed, is assimilated into cellular carbon via reverse tricarboxylic acid cycle and glyoxalate

bypass. Similarly, a number of propane and butane utilizers have been reported that are also capable of growth on long chain alkanes, such as *n*-dodecane and *n*-hexadecane.

Long chain hydrocarbons (C10–C18) can be used rapidly by many high G + C Gram-positive bacteria, but only a few bacteria can oxidize C2–C8 hydrocarbons. Degradation of *n*-alkanes requires activation of the inert substrates by molecular oxygen with the help of oxygenases by three possible ways that are associated with membranes:

1. Monooxygenase attacks at the end producing alkan-1-ol:

$$R{-}CH_3 + O_2 + NAD(P)H + H^+ \rightarrow R{-}CH_2OH + NAD(P)^+ + H_2$$

2. Dioxygenase attack produces hydroperoxides, which are reduced to yield also alkan-1-ol:

$$\begin{aligned} R{-}CH_3 + O_2 &\rightarrow R{-}CH_2OOH + NAD(P)H + H^+ \\ &\rightarrow R{-}CH_2OH + NAD(P)^+ + H_2O \end{aligned}$$

3. Rarely, subterminal oxidation at C_2 by monooxygenase yields secondary alcohols.

Brevibacterium erythrogenes can use 2-methylundecane as substrate for growth by a combination of ω- and β-oxidation. *Arthrobacter* sp. has been reported to metabolize squalene (C30-multiple, methyl branched compound) to geranylacetone, which is accumulated in the medium as it cannot be further metabolized. Similarly, *Corynebacterium* sp. and *B. erythrogenes* have been shown to degrade pristane (2,6,10,14-tetramethyl pentadecane) involving ω-oxidation, followed by β-oxidation, yielding propionyl-CoA and acetyl-CoA units alternately.

17.2.3 Anaerobic Degradation of n-Alkanes

Apart from aerobic oxidation, anaerobic degradation also plays an important role in the recycling of hydrocarbons in the environment. Alkanes are also degraded through anaerobic process as reported by various workers (Callaghan et al. 2009; Higashioka et al. 2009). There are two known pathways of anaerobic *n*-alkanes degradation (Fig. 17.5). First pathway is the alkane addition to fumarate, and second is through putative pathways (So et al. 2003). Fumarate addition proceeds via terminal or sub-terminal addition of the alkanes to the double bond of fumarate, resulting in the formation of alkyl succinate which is further degraded via carbon skeleton rearrangement and β-oxidation. Alkane addition to fumarate has been documented for denitrifying bacteria (Wilkes et al. 2002), sulphate reducing consortia (Kniemeyer et al. 2007) and sulphate reducing bacteria (Callaghan et al. 2006; Kniemeyer et al. 2007). *Azoarcus* sp. HxN1, a denitrifying bacterium, uses C6–C8 alkanes, while *Desulfobacterium* Hdx3 metabolizes C12–C20 alkanes (reviewed in Widdel and Rabus 2001).

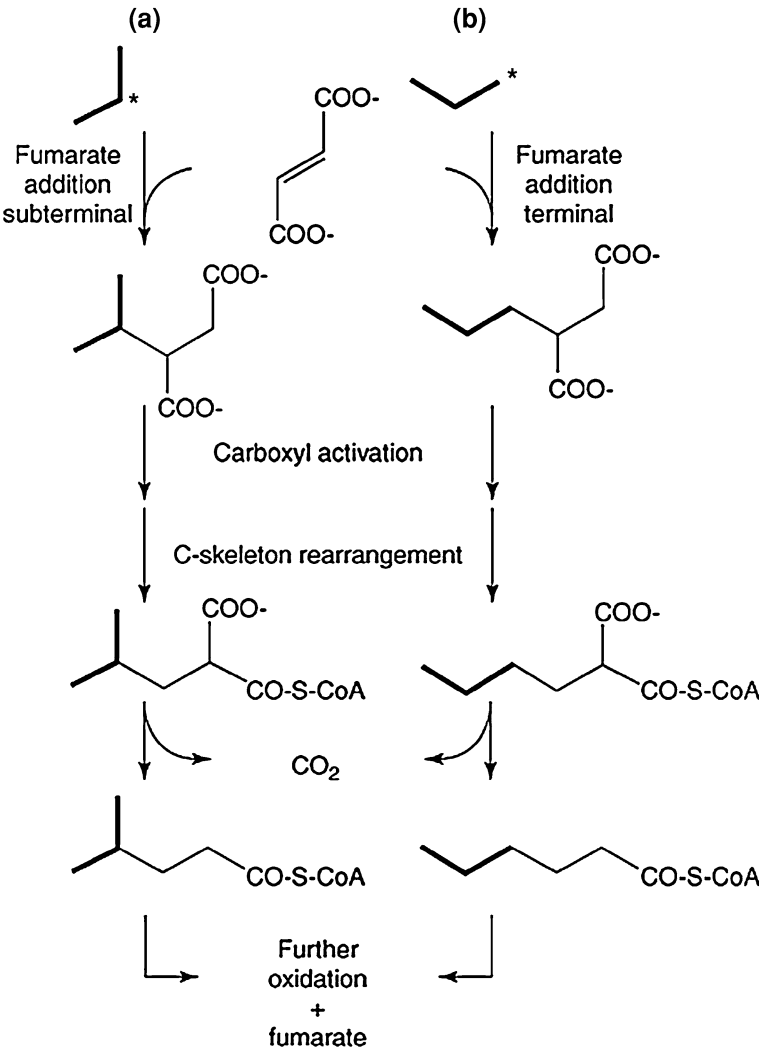


Fig. 17.5 Anaerobic activation of short chain alkanes by furarate addition. The formed methylalkylsuccinates are activated by binding with acetyl-coenzyme A (CoA), which yields a thioester that undergoes C-skeleton rearrangement, followed by decarboxylation and β-oxidation. **a** Activation of the secondary carbon in propane. **b** Activation of the primary carbon in propane, which requires more energy. * indicates the position of the radical carbon (after Kniemeyer et al. 2007)

Zedelius et al. (2011) studied alkane degradation under anaerobic conditions by a nitrate reducing bacterium to find out involvement of electron acceptor in substrate activation. Three bacterial isolates (HXN1, OcN1, HdN1) which were able to grow under aerobic conditions by coupling alkane oxidation to CO_2 with NO_3^- reduction to N_2, were compared for alkane metabolism (Fig. 17.6). Out of which,

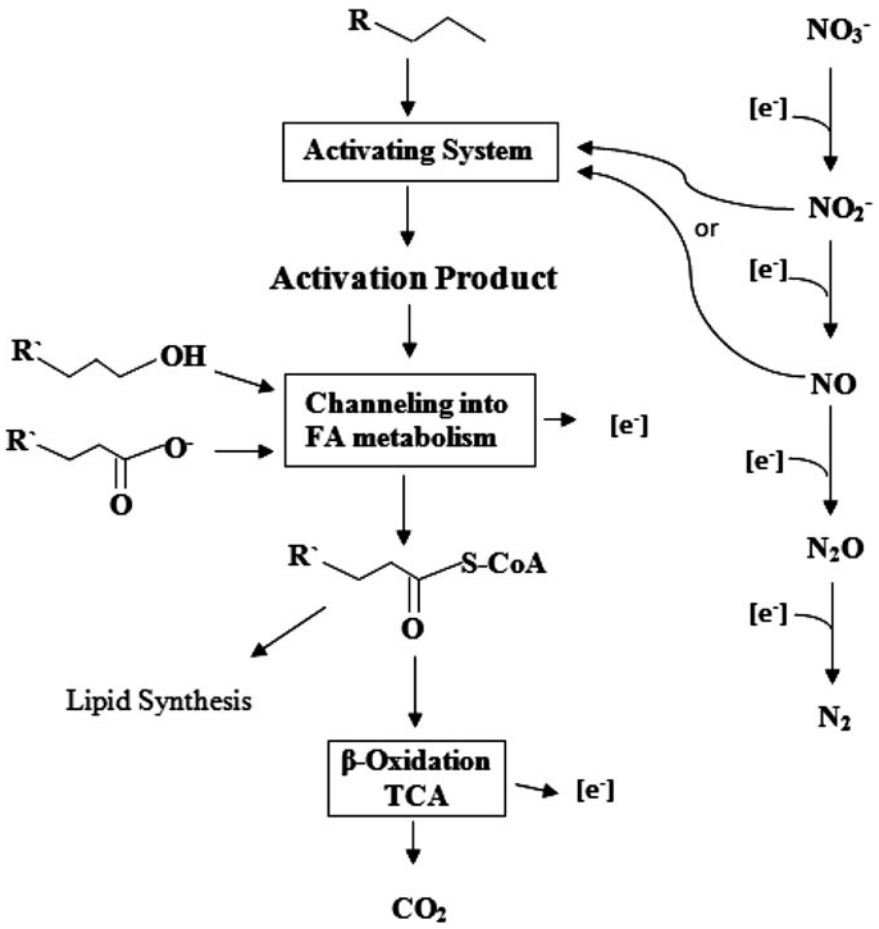


Fig. 17.6 Hypothetical involvement of denitrification intermediates in alkane activation. A small proportion of NO_2^- or NO is deviated from the respiratory chain for alkane activation. They may be used for activation indirectly (by yielding O_2 that is used by alkane monooxygenase; or by giving rise to another reactive factor or enzyme centre) or directly (as co-reactants introducing a polar group). The alkyl residue R′ may or may not be identical with the original residue R (depending on the activation mechanism and alkane C-atom being attacked). FA, fatty acid; TCA, tricarboxylic acid cycle (after Zedelius et al. 2011)

two strains HXN1 and OcN1 (both Betaproteobacteria) metabolized C6–C8 and C8–C12 alkanes, respectively. Both of them activated alkanes anaerobically in a fumarate-dependent reaction yielding alkylsuccinates as evidenced by metabolite and gene analyses. However, strain HdN1 was unique. It belonged to Gammaproteobacteria and utilized alkanes in the range of C6–C30. It also did not indicate fumarate-dependent alkane activation. While HXN1 and OcN1 grew on alkanes and NO_3^-, NO_2^- or N_2O added to medium, strain HDN1 oxidized alkanes only with NO_3^- or NO_2^- but not with N_2O. Since N–O species are the strong oxidants, these

strains may not activate alkane under the conditions of sulphate reduction or methanogenesis and allow a special mode of alkane activation.

Squalane (2,6,10,15,19,23-hexamethyltetracosane) is susceptible to microbial degradation and *Actinomycetes*, in particular, and those belonging to the genus *Mycobacterium*, are potent degraders of this multibranched saturated hydrocarbons. The putative pathway demonstrated that after the conversion of squalane to a dioic acid as one of the first intermediates, two propionyl- coA and acetyl-CoA molecules are oxidatively removed by β-oxidation route to form 3,7,11-trimethyldodecandioic acid as intermediate by a pathway analogous to that for degradation of the multiple branched alkane pristane (2,3,10,14-tetramethylpentadecane) (Berekaa and Steinbüchel 2000).

17.2.4 Non-Conventional Dissimilation Pathway

Sakai et al. (1996) observed a non-conventional dissimilation pathway in *Acinetobacter* sp. M1 in which *n*-alkanes are postulated to be converted to acid:

$$\mathrm{R{-}CH_3} \rightarrow \mathrm{RCH_2OOH} \rightarrow \mathrm{RCO(O)OH} \rightarrow \mathrm{RCHO} \rightarrow \mathrm{RCOOH} \quad \text{(Finnerty 1988)}.$$

However, there is little information available on the enzymes involved in the postulated pathway, particularly at the first step. They identified an enzyme—a flavoprotein which needed O_2 and Cu^{2+} for expression of its activity, but did not require NAD(P)H as a coenzyme. The enzyme reaction yielded hydroperoxide and the enzyme involved in *n*-alkane oxidation is likely to be a dioxygenase. Further, the postulated pathway is supported by the following observations: (1) *n*-alkane monooxygenase activity not detected, (2) low activity of fatty alcohol dehydrogenase, (3) induction of NAD(P)H-dependent long chain fatty aldehyde dehydrogenase in *n*-alkane grown cells.

Meng et al. (1996) isolated three kinds of enzymes designated A, B and C found in the cytoplasm of *n*-alkane grown *Acinetobacter* sp. M1, that catalyzed dioxygenation of *n*-alkanes to the corresponding *n*-alkyl hydroperoxides. Purified enzyme A consisted of four identical subunits having a molecular mass and strongly inhibited by several iron-chelating agents. Enzymes B and C were more active towards relatively short *n*-alkanes (C12–C16) where as enzyme A oxidized solid *n*-alkanes with the most preferable substrate being Tetracosane C24.

17.3 Oil Alkanes

Alkanes are the most important fraction of crude oil. The anaerobic degradation of alkanes is today of great significance for the oil industry. It is well established that microbial activities associated with oil reservoirs led to the decrease of oil quality, making refining more costly and recovery more difficult (Head et al. 2003).

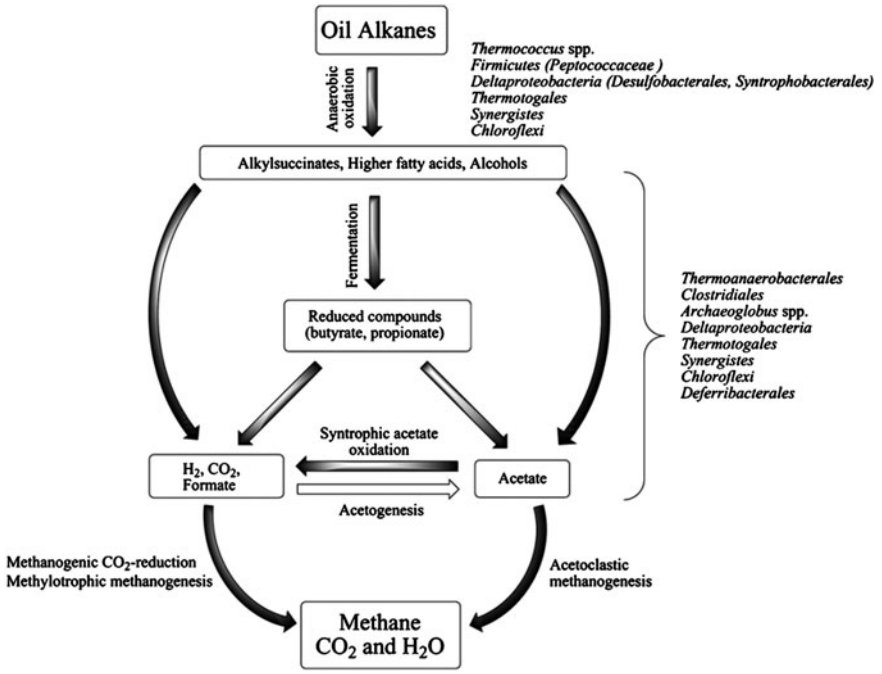


Fig. 17.7 Presumptive methanogenic degradation of oil alkanes (after Mbadinga et al. 2011)

Because of presence of microbial communities mainly dominated by anaerobes, the oil reserves are referred as 'geo bioreactors', in which fermentative, syntrophic, suthdogenic and methanogens are responsible for removal of alkanes from the saturated hydrocarbon fraction (Jones et al. 2008; Wang et al. 2010). Moreover, biogenic CH_4 production is the result of microbial degradation of oil alkanes. Since world demand for methane is likely to increase many folds in coming decades, the methanogenic conversion of oil alkanes to CH_4 is seen as a future solution for world increasing demand of energy (Fig. 17.7).

17.4 Enzymes Involved in Alkane Degradation

Ayala and Torres (2004) have indicated the involvement of three major enzymes in the degradation of alkanes; Methane monooxygenase (MMO), Alkane hydroxylase (Alk) and Cytochrome P450 monooxygenase (Fig. 17.8).

17.4.1 Methane Monooxygenase

Methane monooxygenase is expressed in microorganisms to use CH_4 as energy source and found in methanotrophs in two forms pMMO (particulate Methane

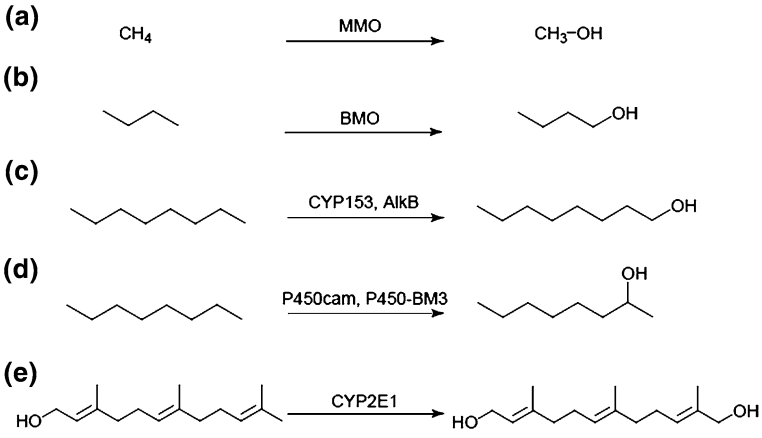


Fig. 17.8 **a** Methane to methanol by Methane Monooxygenase (MMO), **b** Butane to 1- butanol by Butane Monooxygenase (BMO), **c** Octane to 1-Octanol, **d** Octane to 2-Octanol, **e** Farnesol to 1-hydroxyfarnel (after van Beilen and Funhoff 2005)

monooxygenase) and sMMO (soluble Methane monooxygenase). While pMMO is a membrane-bound protein produced by all methanotrophs, sMMO is expressed by a subset of methanotrophs. pMMO is an iron–copper protein, produced under conditions of copper sufficiency (Nguyen et al. 1994) where as sMMO is an iron-containing enzyme produced only under Cu-depleted sites (Murrell et al. 2000b). sMMO is comprised of three components; an oxygenase, a reductase and a coupling protein (Fox et al. 1989). The NADH-dependent oxidation reaction catalysed by sMMO is reflected in Fig. 17.9. Both sMMO and Alk are characterized by the presence of diiron cluster in the hydroxylase component. The metallic center activates dioxygen during the oxidation of substrates. However, in sMMO, the diiron cluster is bridged by carboxylic residues, similar to the diiron centers of proteins, such as ribonucleotide reductase R2, stearoyl-ACP-9 desaturase and other monooxygenases, such as alkene monooxygenases, phenol monooxygenases and toluene monooxygenases (Leahy et al. 2003). sMMO shows a wide range of substrate specificity, including alkenes, aromatic, alicyclic and hetrocyclic compounds where as pMMO mediates the oxidation of a small group of alkanes (Murell et al. 2000a). Four different reaction mechanisms of sMMO for hydrocarbon hydroxylation have been suggested: (1) hydrogen atom abstraction from the substrate followed by radical recombination (Fox et al. 1990), (2) cation formation by electron abstraction from the substrate radical intermediate generated in first step followed by reaction with metal bound hydroxide (Jin et al. 2001), (3) direct insertion of the oxygen atom into the C–H bond (Valentine et al. 1997) and (4) cation formation on the substrate by transfer of a protonated oxygen from a hydroperoxy intermediate (derived from O_2), followed by loss of water (Choi et al. 1999).

Similar to sMMO, butane monooxygenase (BMO) is a non-heme iron monooxygenase and it can hydroxylate C2–C9 alkanes (Dubbels et al. 2007).

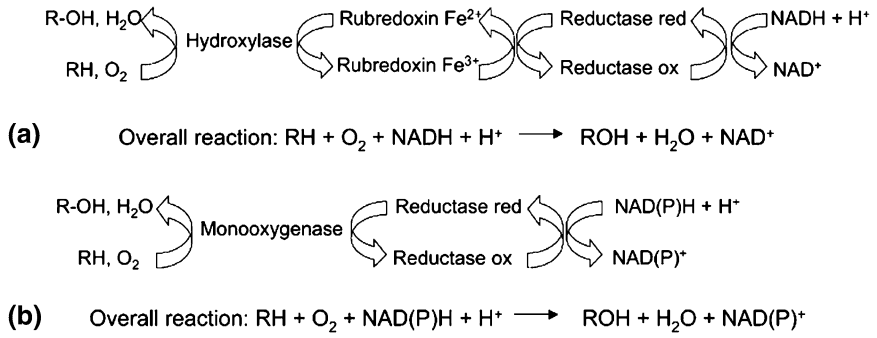


Fig. 17.9 Steps involved in the oxidation reaction catalysed by alkane hydroxylase (**a**) and methane monooxygenase and cytochrome P450 monooxygenase (**b**) (after Ayala and Torres 2004)

Chaperonin-like protein, BmoG is required for proper assembly of BMO (Kurth et al. 2008). However, its specificity is towards producing the terminal alcohols, unlike sMMO that produces sub-terminal alcohols.

Recently, a unique alkane monooxygenase that belongs to luciferase family was reported for *G. thermodenitrificans* (Li et al. 2008). Kato et al. (2009) reported that three novel membrane proteins, superoxide dismutase, catalase, and acyl-CoA oxidase in *G. thermoleovorans* B23 which were previously reported only in yeast, such as *C. tropicalis* (Shimizu et al. 1979), Activities of these enzymes were dramatically increased in the cells of *G. thermoleovorans* B23 when they were grown on alkanes.

17.4.2 Alkane Hydroxylase

This enzyme is three component monooxygenase, comprising a hydroxylase, a rubredoxin and rubredoxin reductase (Shanklin et al. 1997). The hydroxylase component is membrane-bound, while both rubredoxin and rubredoxin reductase components are soluble and cytoplasmic proteins. This enzymatic complex is able to oxidize medium and long chain linear alkanes using reducing equivalents from NADH or NADPH.

AlkB, an integral membrane protein, carries out a terminal hydroxylation of *n*-alkane (Kok et al. 1989). The electrons needed to carry out this step are delivered to AlkB via a rubredoxin reductase (AlkT) and two rubredoxins (AlkF and AlkG) (van Beilen et al. 2002). The resulting alcohol is further converted to a fatty acid via a pathway involving an alcohol dehydrogenase (AlkJ), an aldehyde dehydrogenase (AlkH) and an acyl-CoA synthetase (AlkK), that enters the β oxidation pathway (van Beilen et al. 2001). The histidine residues are required for activity in the members of this family (Shanklin et al. 1994). There is a

conserved NYXEHYG(L/M) motif in all identified alkane hydroxylases (Smits et al. 2002). This motif has been proposed as a signature for membrane-bound alkane hydroxylases (Smits et al. 2002).

Although crystal structure of Alk is not known, it is believed to have six transmembrane segments and a catalytic site that faces the cytoplasm. The active site includes four His-containing sequence motives that are conserved in other hydrocarbon monooxygenases which chelate two iron atoms (Shanklin et al. 1994). The diiron cluster allows the O_2-dependent activation of the alkane through a substrate radical intermediate (Shanklin et al. 1997; Bertrand et al. 2005). One of the O_2 atoms is transferred to the terminal methyl group of the alkane, rendering an alcohol, while the other one is reduced to H_2O by electrons transferred by the rubredoxin. Oxidation is regio- and stereospecific (van Beilen et al. 1995).

Baptist et al. (1963) have identified an enzyme system from *Pseudomonas putida* PpG6 grown on alkanes which is capable of oxidizing octane to octanoic acid, and the properties of the enzyme complex, which catalyzes the initial hydroxylation reaction, have been extensively studied (Mckenna and Coon 1970). In vitro, this hydroxylase complex is also capable of omega-oxidizing fatty acids (Mckenna and Coon 1970). This suggests that the oxidation of alkane and fatty acid chains might occur from both ends in strains with a functional hydroxylase.

The AlkB protein from *Pseudomonas putida* GPo1 is presently the best characterized Alk (van Beilen et al. 1994). It catalyses the first step of alkane degradation with the help of two electron transfer proteins, rubredoxin (AlkG) and rubredoxin reductase (AlkT) (van Beilen et al. 1994). Over the past decade, alkB-like hydroxylase genes have been detected in a wide range of alkane degrading bacteria, including *a*-, *b*- and *g*-proteobacteria; as well as in some high G + C content Gram-positive bacteria (Smits et al. 2002). Many of these contain more than one alkB homologue, such as *Pseudomonas aeruginosa* PAO1 (alkB1 and alkB2), *Rhodococcus erythropolis* Q15 (alkB1-4) and *Acinetobacter* sp. M1 (alkMa and alkMb).

The enzymes, that oxidize alkanes larger than C20, seem to be totally different. For example, *Acinetobacter* sp. M1, which can grow on C13–C44 alkanes, contains a soluble, Cu^{2+} -dependent Alk that is active on C10–C30 alkanes. It has been proposed to be a dioxygenase that generates *n*-alkyl hydroperoxides to render the corresponding aldehydes (Tani et al. 2001). A different *Acinetobacter* strain, DSM 17874, has been found to contain a flavin-binding monooxygenase, named AlmA, which oxidizes C20 to >C32 alkanes (Throne-Holst et al. 2007). Genes homologous to almA have been identified in several other long chain *n*-alkane degrading strains, including *Acinetobacter* sp. M1 and *A. borkumensis* SK2. A different long chain alkane hydroxylase, named LadA, has been characterized in *Geobacillus thermodenitrificans* NG80-2 (Feng et al. 2007). It oxidizes C15–C36 alkanes, generating primary alcohols. Its crystal structure has shown that it is a two-component flavin-dependent oxygenase belonging to the bacterial luciferase family of proteins (Li et al. 2008).

17.4.3 Cytochrome P450 Monooxygenase

These enzymes are heme proteins and catalyze the hydrocarbons using NAD(P)H as cofactor. They usually consist of two components; hydroxylase and reductase (Sono et al. 1996). These enzymes are usually membrane-bound and have a multi-component nature (Ayala and Torres 2004).

The molecular mechanisms of oxygen activation for some metalloenzymes are well investigated. Heme-oxygenases, such as CYP, hydroxylate inert hydrocarbon substrates by using a high-valent oxoiron(IV) porphyrin π-cation-radical intermediate similar to peroxidase compound I (Groves 2005). The consensus mechanism for oxygen activation and transfer involves a hydrogen atom abstraction-oxygen rebound pathway (Groves 2003, 2005). Hydroxylation of the very unreactive C–H bond of methane by non-heme diiron enzyme sMMO has many similarities to the P450 mechanism (Kopp and Lippard 2002; Newcomb et al. 2002).

Cytochrome P450 monooxygenase (CYP), present in certain strains of yeast *Candida*, is able to convert >C12 alkane by α, ω-oxidation to the corresponding dicarboxylic acids. The ω-oxidation of the alkane to alcohol is first reaction to be catalyzed by a hydroxylase complex composed of a CYP monooxygenase and NADPH and CYP oxireductase. Further oxidation to the acid is catalysed by fatty alcohol oxidase and a fatty aldehyde dehydrogenase (Gallo et al. 1973). Vatsyayan et al. (2008) studied the cytochrome P450 monooxygenase activity in the cells of *Aspergillus terreus* MTCC6324 and found that CYP catalysis of *n*-Hexadecane had followed both terminal and sub-terminal oxidations. The activity was localized in cytosol of *n*-hexadecane grown cells. CYP activity was obtained only when NADH was used as co-factor. No other compounds checked, such as NAD, NADP, NADPH, FMN, FAD and $FADH_2$, could serve as co-factor of the enzyme. Size of isolated enzyme was closer to that reported for *Fusamarium oxysporum* i.e. 118 kDa (Nakayama et al. 1996). The presence of secondary alcohol oxidase in mitochondrial fraction indirectly supports the existence of *n*-alkanes sub-terminal oxidation. van Beilin and Funhoff (2005) reported the sub-terminal oxidation of long chain alkane by bacteria and yeast.

In addition to these enzymes, other catabolic enzymes are also reported from the different microorganism as shown in Table 17.1.

17.5 Recombinant Bacteria for Alkane Degradation

Due to multi-component nature, recombinant production of CYP450 is difficult, but CYP BM-3 is readily expressed in *E. coli* (Peter et al. 2003).

Rothen et al. (1998) constructed a plasmid with gene coding for the three enzymes; alkane hydroxylase, alcohol dehydrogenase and aldehyde dehydrogenase simultaneously. The plasmid was inserted into an *E. coli* strain unable to

Table 17.1 Different enzymes involved in alkane degradation (van Beilen et al. 2003)

Enzymes	Microrganism	Substrate	Reference
sMMO	*Methylococcus capsulatus* *Methylisinus trichosporum* OB3b	C1–C10	Baik et al. (2003)
pMMO	All methanotrophs	C1–C5	Leieberman and Rosenzweig (2004)
Propane monooxygenase	*Pseudomonas butanovora* (ATCC 43655)	C2–C8	Kotani et al. (2003)
Butane monooxygenase	*Gordonia* sp. TYP	C3 and C13–C22	Sluis et al. (2002)
AlkB	*Acinetobacter*, *Alcanivorax*, *Burkholderia*, *Mycobacterium*, *Pseudomonas*, *Rhodococcus* etc.	C5–C16	Smits et al. (2002)
Cytochrome P450 (CYP153) monooxygenase	*Sphingomonas* sp. HXN-200, *Mycobacterium* sp. HXN1500 *Acinetobacter* sp. EB104	C4–C16	Maier et al. (2001)
Cytochrome P450 (CYP52) monooxygenase	*Candida maltosa*, *Candida tropicalls*, *Yarrowia lipolytica*	C10–C16	Craft et al. (2003)

metabolize fatty acids. The recombinant bacteria were able to oxidize octane to its corresponding carboxylic acid.

Glieder et al. (2002) produced a mutant 139-3 that was capable to catalyze the oxidation of medium chain alkanes. This mutant has the fastest known enzyme for alkane hydroxylation (more than 17 times faster than the MMO or Alk enzymatic systems).

A plasmid having three components of Alk system was introduced to a *Pseudomonas* lacking the alcohol dehydrogenase. Now the recombinant bacteria were able to transform C7–C11 alkanes to their corresponding alcohols (Bosetti et al. 1992).

Throne-Holst et al. (2007) constructed alkMa, alkMb and alkMa/alkMb disruption mutants of *Acinetobacter venetianus* 642. Single and double mutants were able to grow on *n*-alkanes (>C20).

17.6 Genes Involved in Alkane Degradation

The organization of the genes involved in alkane oxidation differs significantly among alkane degrading bacteria (van Beilen et al. 2003). The alkane degradation genes encoded by the OCT plasmid of *P. putida* GPo1 are clustered in two operons, and this pathway has clearly been transferred horizontally to many bacteria (van Beilen et al. 2001). When several alkane hydroxylases coexist in a single strain, they are normally located at different sites in the chromosome. Moreover, the regulators that control the expression of alkane degradation genes may or may not map adjacent to the genes they regulate. Therefore, the degree of clustering of alkane degradation genes is variable among bacterial strains.

Expression of the genes involved in the initial oxidation of alkanes is tightly controlled. A specific regulator assures that the pathway genes are expressed only in the presence of the appropriate alkanes. In addition, superimposed to this specific regulation, there are several mechanisms that modulate the induction of the pathway genes according to cell needs. The known specific regulators, that induce alkane degradation genes in response to alkanes, belong to different families, such as the LuxR/MalT, the AraC/XylS, the GntR or other non-related families of regulators. The *A. borkumensis* AlkS transcriptional regulator is believed to activate expression of the gene coding for the AlkB1 Alk and of downstream genes in response to alkanes (van Beilen et al. 2004; Schneiker et al. 2006). In a proteomic study, this regulator appeared associated to the membrane fraction, rather than to the cytoplasmic fraction (Sabirova et al. 2006). Some bacterial strains contain only one alkane hydroxylase, as is the case for the well-characterized alkane degrader *P. putida* GPo1. However, many other strains have several alkane degradation systems, each one being active on alkanes of a certain chain length or being expressed under specific physiological conditions. For example, *Acinetobacter* sp. strain M1 contains two AlkB related alkane hydroxylases, named AlkMa and AlkMb, which are differentially regulated depending on the alkane present in the medium. Expression of AlkMa, which is controlled by the AlkRa regulator, is induced by alkanes having a very long chain length (>C22), while that of AlkMb is induced by AlkRb in the presence of C16–C22 alkanes (Tani et al. 2001). *A. borkumensis* has two AlkB like alkane hydroxylases and three genes coding for cytochromes P450 believed to be involved in alkane oxidation (van Beilen et al. 2004; Schneiker et al. 2006). In addition, *A. borkumensis* seems to have other uncharacterized genes involved in the oxidation of branched alkanes and phytane (Schneiker et al. 2006). Finally, a gene similar to *Acinetobacter* sp. DSM 17874 almA, which oxidizes alkanes of very long chain length, has been predicted in *A. borkumensis* SK2 (Throne-Holst et al. 2007). Expression of all these alkane oxidation genes should be differentially induced according to the substrate present under each circumstance. The three *A. borkumensis* genes coding for similar cytochromes P450 of the CYP153 family are believed to participate in alkane degradation (Schneiker et al. 2006). Cytochrome P450-1 maps adjacent to other genes involved in the oxidation of alkanes. Cytochrome P450-2 is identical to P450-1,

and highly homologous to P450-3. Proteomic profiling analyses revealed that P450-1 and/or P450-2, which cannot be differentiated with this technique, are expressed in cells grown with either pyruvate or hexadecane as the carbon source, although expression was higher in alkane-grown cells (Sabirova et al. 2006). As P450-1 is probably co-transcribed with other adjacent genes that are upregulated by hexadecane, it is likely that expression of P450-1 is induced by hexadecane but, not that of P450-2 and P450-3. A gene coding for a transcriptional regulator of the AraC family maps close to P450-1.

Certain plasmids play an important role in adaptation of natural microbial populations to oil and other hydrocarbons. Some of the microbial catabolic pathways responsible for the degradation, including the alk (C5–C12 *n*-alkanes), nah (naphthalene) and xyl (toluene) pathways, have been extensively characterized and are generally located on large catabolic plasmids (Gary et al. 1990), but many reports describe and characterize microorganisms that can catabolize both aliphatic and aromatic hydrocarbons (Foght et al. 1990). Several environmental isolates of *Acinetobacter* sp. and *Alcaligenes* sp. (Lal and Khanna 1996), *Arthrobacter* sp. (Efroymson and Alexander 1991) and two *Rhodococcus* strains (Malachowsky et al. 1994) have been found to degrade both alkanes and naphthalene.

Vomber and Klinner (2000) used gene probe derived from alkB gene of *Pseudomonas aleovorans* ATCC 29347 to test the ability to assimilate short/medium chain of 54 bacetrial strains belonging to 37 species. The derived amino acid sequence of the alkB-amplificate of *Pseudomonas aureofaciens* showed high homology (95%) with AlkB from *P. oleovorans*. AlkB gene disruptants were not able to grow on decane.

17.7 Environmental Factors Regulating Biodegradation of Alkanes

Additional factors that influence the degradation process included soil pH, moisture and organic matter content and hydrocarbon aqueous solubility, octanol water partitioning coefficient and structure (Leahy and Colwell 1990; Ramírez et al. 2008). Effective biodegradation is dependent upon optimal biological (microbial functionality and biomass size), chemical (bioavailability and nutrients) and physical (water holding capacity) parameters (Towell et al. 2011).

17.7.1 Structure and Physical State

n-alkanes of intermediate chain length (C10–C24) are degraded most rapidly. Short chain alkanes (<C9) are toxic to many microorganisms, but being volatile, they are generally lost rapidly to the atmosphere. Higher chain length alkanes are generally resistant to biodegradation. Branching in alkanes generally reduces the rate of biodegradation. The bioavailability of hydrocarbons, which is largely a

function of concentration and physical state, hydrophobicity, sorption onto soil particles, volatilization and solubility of hydrocarbons, greatly affects the extent of biodegradation.

Water solubility of decane d10 is 0.052 mg/l, but the solubility of octadecane is almost tenfold less (0.006 mg/l). The water solubility of butane (C4) is 61.4 mg/l, but very toxic to cells. Short chain alkanes are toxic to microorganisms, because their increased water solubility results in increased uptake of the alkanes.

17.7.2 Temperature

Merin and Bucala (2007) reported that increase in temperature made the biological membranes to have more fluid due to increased vibrational activity to the fatty acid chains in the phospholipids bilayer. The increase in the rate of fluidity helps in increasing the rate of substance uptake from a cell's surrounding medium. Biodegradation of hydrocarbon has been shown to occur over a wide range of temperature from 0°C to as high as 70°C, though, in general optimum degradation occurs in the mesophilic temperature range. It also affects the solubility of hydrocarbon and enzyme activity. The stability of the enzyme CYP P450 monooxygenase in *Aspergillus terreus* MTCC6324 ranges between 25–40°C, maximum being at 25°C (Vatsayayan et al. 2008).

17.7.3 Nutrients

van Hamme et al. (2003) reported that nitrogen and phosphorus contents greatly affect the microbial degradation of hydrocarbons. They further stated that adjustment of the ratios of N and P by their addition in the form of slow releasing fertilizers stimulated the biodegradation of hydrocarbons. Österberg et al. (2006) found accelerated biodegradation of *n*-alkanes in aqueous solution by the addition of fermented whey. Bulking agents, such as compost, will enhance metabolism of organic contaminants because they provide extra nutrients, additional carbon sources and assist in retaining moisture content of the pile.

The increase in C/N ratios reduced the hexadecane (HXD) biodegradation. Limitation of microbial growth and metabolism in polluted soils can be related to the low concentration of inorganic nutrients, such as nitrogen, phosphorous and potassium, producing high C/N, C/P and C/K ratios (Volke-Sepulveda et al. 2006).

17.7.4 Oxygen

Hydrocarbons being highly reduced substrates, require an electron acceptor, with molecular oxygen being the most common. Though most studies have shown biodegradation of hydrocarbon to be an aerobic process, anaerobic biodegradation

of hydrocarbons has also been reported. In the absence of molecular oxygen, nitrate, iron, bicarbonate, nitrous oxide and sulfate, have been shown to act as an alternate electron acceptor during anaerobic hydrocarbon degradation.

17.7.5 pH

pH is not of much significance in marine environments since it is well buffered at about pH 8.5, but soil pH varies widely and pH between 7 and 8 has been found to support optimum degradation of alkanes in soils/sediments.

17.7.6 Surfactants

Surfactants are amphiphilic compounds, that reduce surface and interfacial tensions by accumulating at the interface of immiscible fluids or of a fluid and a solid, increase the contact surface areas of insoluble compounds, leading to increased mobility, bioavailability and subsequent biodegradation.

17.7.6.1 Synthetic Surfactants

The use of chemical surfactants has been extensively studied by various authors (Suchanek et al. 2000; Stortini et al. 2009). Chrzanowski et al. (2006) performed biodegradation studies of a model mixture of hydrocarbonyl dodecane and hexadecane (1:1 w/w) by applying different surfactants like: lecithin extracted from soyabeans, rhamnolipids from *Pseudomonas aeruginosa*, saponin, lutensol GD 70, Triton X-100 and Tween 20 with different concentration 150, 300 and 600 mg l^{-1} for 7 days. *Candida maltosa* was found capable to degrade hydrocarbons by a maximum of 92.7% in case of saponin (300 mg l^{-1}), followed by 90.3% in case of saponin (150 mg l^{-1}) and 80.9% with rhamnolipid (150 mg l^{-1}).

Surfactants have been also reported to increase the uptake and assimilation of alkanes such as hexadecane in liquid cultures (Beal and Betts 2000; Noordman and Janssen 2002), but their usefulness in soils and other situations is less evident (Holden et al. 2002). Surfactants produced by microorganisms probably have other roles as well, such as facilitating cell motility on solid surfaces (Caiazza et al. 2005), or the adhesion/detachment to surfaces or biofilms (Boles et al. 2005).

17.7.6.2 Biosurfactant

Microorganisms are grouped to endo- and exo-type ones, based on biosurfactant accumulation. Endo-type biosurfactants are bound up with the wall surface of

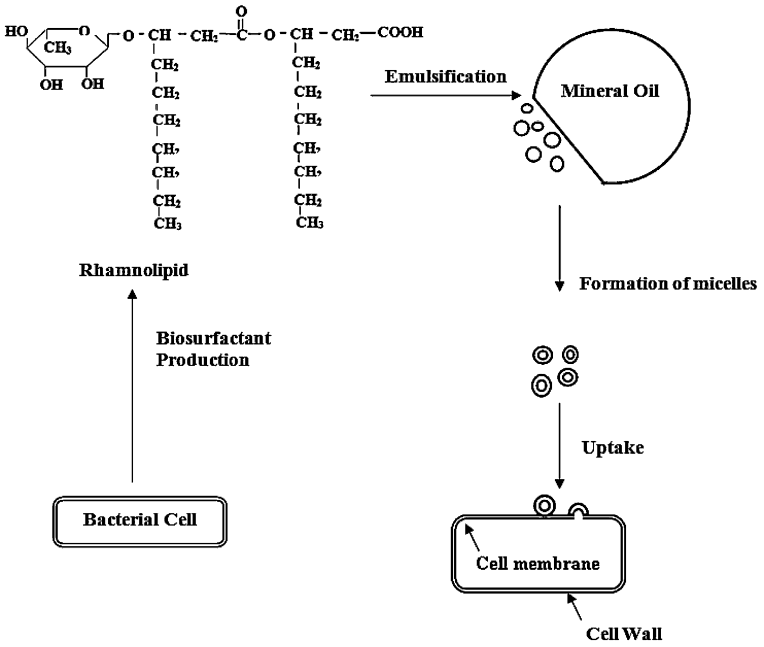


Fig. 17.10 Involvement of biosurfactants in the uptake of hydrocarbons and the emulsifying effect of a rhamnolipid produced by *Pseudomonas* spp. within the oil–water interphase and the formation of micelles (after Fritsche and Hofrichter 2000)

the microorganism cell and, as a rule, constitute components liposomally active (Al Tahhan et al. 2000). Exo-type biosurfactants are excreted into medium by cell to provide substrate access to cell surface, due to emulsion or suspension production in liquid medium (Deziel et al. 1996). Biosurfactants are very diverse in their chemical composition. They include glycolipids, lipopeptides, lipoproteins, phospholipids, fatty acids and polymeric surfactants (Rosenberg and Ron 1999).

Biosurfactants are organic molecules consisting of a hydrophilic moiety and act as emulsifying agents by decreasing the surface tension and forming micelles. The uptake mechanism of hydrocarbons and emulsification by rhamnolipid produced by the bacteria has been demonstrated in Fig. 17.10.

Microbial surfactants have advantages over synthetic surfactants due to lower toxicity, higher biodegradability and environmental compatibility (Cameotra and Makkar 2004). It may be produced cost effectively under *ex-situ* conditions and *in-situ* production may be stimulated at the site of contamination and can be recovered and recycled (Moran et al. 2000).

Bushnell and Haas (1941) were among the first to demonstrate bacterial production of biosurfactants. Based on molecular weight, microbial surfactants are classified in two groups (Hua et al. 2010). Glycolipids and lipopeptides are counted under low molecular-weight surfactants, whereas emulsa, alasan, biodispersan

and extracellular or cell membrane-bound bioemulsifiers (exopolysaccharide; EPS) are high molecular weight compounds. Maximum study was done with rhamnolipids produced by *Pseudomonas aeruginosa* (Rahman et al. 2002). It was shown that rhamnolipid extracts lipopolysaccharides (LPS) from cells of *Pseudomonas*, thereby increasing the hydrophobicity of the cell surface and promoting attachment of the cells to hydrocarbon droplets (Al-Tahhan et al. 2000). It is suggested that greater attachment stimulates hexadecane degradation (Al-Tahhan et al. 2000) while it was found that it inhibits octadecane degradation due to flocculation of the cells. Christova et al. (2004) reported that *Renibacterium salomininarum* 27BN also produced biosurfactant of glycolipid. It secretes two rhamnolipids RLL and RRLL from *Pseudomonas aeruginosa* when grown on hexadecane (2%) as sole source. At the end of 192 h, only $9.3 \pm 2.1\%$ residual hexadecane was obtained in cultures incubated with the whole cell.

Biosurfactant activities can be determined by measuring the changes in surface and interfacial tensions, stabilization or destabilization of emulsions and hydrophilic–lipophilic balance. The hydrophilic–lipophilic balance is directly related to the length of the hydrocarbon chain of fatty acids (Desai and Banat 1997). They are often good emulsifiers; the emulsions they form are more stable than the emulsions obtained by synthetic surfactants (Desai and Desai 1993).

In addition to solubility enhancement, EPS shields bacterial cells from direct exposure to toxic substances (Gutierrez et al. 2009). Ron and Rosenberg (2001) found that EPS alters the hydrophobicity by exposure of hydrophobic phospholipids tails of cells (Al-Tehhan et al. 2000). EPS, in case of biodegradation of petroleum hydrocarbon, was first reported by Watanabe and Takahashi (1997) in *Pseudomonas* sp. SLI and SLK. *Halomonas* spp. (Martinez-Checa et al. 2007) and marine *Enterobacter cloacae* (Iyer et al. 2006) have also been reported for the production of EPS. Iyer et al. (2006) found that emulsion of EPS produced by *Enterobacter cloacae* (EPS 71a) with hexane was stable up to 10 days between pH 2 and 10 in presence of NaCl in the range of 5–50 mg ml^{-1} at 35–37°C. Hua et al. (2010) found that EPS, secreted by *Enterobacter cloacae* strain TU during growing on *n*-hexadecane as the sole carbon source, composed of glucose and galactose with molecular weight of 12.4 ± 0.4 kDa. Kumar et al. (2007) observed the reduction of interfacial tension by EPS produced by *Planococcus maitriensis* Anita I for hexane and found that this EPS contained carbohydrate (12.06%), protein (24.4%), uronic acid (11%) and sulphate (3.03%).

17.8 Conclusion

Researches carried out on microbial degradation have provided new insights into the mechanism of alkane degradation. However, many aspects of degradation are still not very clear, particularly incorporation of alkanes into the microbial cell. A few new enzymes have been recently found which metabolize long chain

alkanes. Although there are indications for existence of new alkane hydroxylases, but they have been not yet characterized. We are still curious to know why there are several alkane hydroxylases with similar substrate specificities. Regulation and expression of genes coding for alkane degradation pathways are still not very clear. Elucidation of these pathways is very important to design bioremediation strategies for enhancing degradation of alkanes in the contaminated sites. Besides, recombinant and functionally improved strains have to be developed to enhance the process of biodegradation of oil hydrocarbons at contaminated sites.

References

Abdel-Megeed A, Al-Harbi N, Al-Deyab S (2010) Hexadecane degradation by bacterial strains isolated from contaminated soils. Afr J Biotech 9(44):7487–7494

Al-Tahhan RA, Sandrin TR, Bodonr AA, Maier RM (2000) Rhamnolipid-induced removal of lipopolysaccharide from *Pseudomonas aeruginosa*: effect on cell surface properties and interaction with hydrophobic substrates. Appl Environ Microbiol 66:3263–3268

Atlas RM (1981) Microbial degradation of petroleum hydrocarbons: an environmental perspective. Microbiol Rev 45(1):180–209

Ayala M, Torres E (2004) Enzymatic activation of alkanes: constraints and prospective. Appl Catal J 272(1–2):1–13

Baik MH, Newcomb M, Friesner RA, Lippard SJ (2003) Mechanistic studies on the hydroxylation of methane by methane monooxygenase. Chem Rev 103:2385–2419

Baptist JN, Gholson RK, Coon MJ (1963) Hydrocarbon oxidation by a bacterial enzyme system. I. Products of octane oxidation. Biochim Biophys Acta 69:40–47

Beal R, Betts WB (2000) Role of rhamnolipid biosurfactants in the uptake and mineralization of hexadecane in *Pseudomonas aeruginosa*. J Appl Microbiol 89:158–168

Bej AK, Saul D, Aislabie J (2000) Cold-tolerant alkane-degrading *Rhodococcus* species from Antarctica. Polar Biol 23(2):100–105

Berekaa MM, Steinbüchel A (2000) Microbial Degradation of the Multiply Branched Alkane 2,6,10,15,19,23-Hexamethyltetracosane (Squalane) by *Mycobacterium fortuitum* and *Mycobacterium ratisbonense*. Appl Environ Microbiol 66(1):4462–4467

Berthe-Corti L, Fetzner S (2002) Bacterial metabolism of *n*-alkanes and ammonia under oxic, suboxic and anoxic conditions. Acta Biotechnol 22:299–336

Bertrand E, Sakai R, Rozhkova-Novosad E, Moe L, Fox BG, Groves JT, Austin RN (2005) Reaction mechanisms of non-heme diiron hydroxylases characterized in whole cells. J Inorg Biochem 99:1998–2006

Bogusławska-Was E, Dąbrowski W (2001) The seasonal variability of yeasts and yeast-like organisms in water and bottom sediment of the Szczecin Lagoon. Intl J Hyg Environ Health 203(5–6):451–458

Boles BR, Thoendel M, Singh PK (2005) Rhamnolipids mediate detachment of *Pseudomonas aeruginosa* from biofilms. Mol Microbiol 57:1210–1223

Bosetti A, van Beilen JB, Preusting H, Lageveen RG, Witholt B (1992) Production of primary aliphatic alcohols with a recombinant *Pseudomonas* strain, encoding the alkane hydroxylase enzyme system. Enzym Microb Technol 14:702–708

Brassington KJ, Hough RL, Paton GI, Semple KT, Risdon GC, Crossley J, Hay I, Askari K, Pollard SJT (2007) Weathered hydrocarbon wastes: a risk management primer. Crit Rev Environ Sci Technol 37:199–232

Bushnell LD, Haas HF (1941) The utilization of certain hydrocarbons by microorganisms. J Bacteriol 41:653–673

Caiazza NC, Shanks RM, O'Toole GA (2005) Rhamnolipids modulate swarming motility patterns of *Pseudomonas aeruginosa*. J Bacteriol 187:7351–7361

Callaghan AV, Gieg LM, Kropp KG, Suflita JM, Young LY (2006) Comparison of mechanisms of alkane metabolism under sulfate-reducing conditions among two isolates and a bacterial consortium. Appl Environ Microbiol 72:4274–4282

Callaghan AV, Tierney M, Phelps CD, Young LY (2009) Anaerobic biodegradation of *n*-hexadecane by a nitrate-reducing consortium. Appl Environ Microbiol 75:1339–1344

Cameotra SS, Makkar RS (2004) Recent application of biosurfactant as biological and immunological molecules. Curr Opin Microbiol 7:262–266

Chaillan F, Le Fleche A, Bury E, Phantavonga Y, Grimontb P, Saliotc A, Oudot J (2004) Identification and biodegradation potential of tropical aerobic hydrocarbon-degrading microorganisms. Res Microbiol 155(7):587–595

Chenier MR, Beaumier D, Roy R, Driscoll BT, Lawrence JR, Greer CW (2003) Impact of seasonal variations and nutrient inputs on nitrogen cycling and degradation of hexadecane by replicated river biofilms. Appl Environ Microbiol 69:5170–5177

Choi S-Y, Eaton PE, Kopp DA, Lippard SJ, Newcomb M, Shen R (1999) Cationic species can be produced in soluble methane monooxygenase-catalyzed hydroxylation reactions; radical intermediates are not formed. J Am Chem Soc 121:12198–12199

Christova N, Tuleva B, Lalchev Z, Jordanova A, Jordanov B (2004) Rhamnolipid biosurfactants produced by *Renibacterium salmoninarum* 27BN during growth on *n*-Hexadecane. Z Naturforsch C 59(1–2):70–74

Chrzanowski Ł, Kaczorek E, Olszanowski A (2006) The ability of *Candida maltosa* for hydrocarbon and emulsified hydrocarbon degradation polish. J Environ Stud 15(1):47–51

Coon MJ (2005) Omega oxygenases: nonheme-iron enzymes and P450 cytochromes. Biochem Biophys Res Commun 338:378–385

Craft DL, Madduri KM, Eshoo M, Wilson CR (2003) Identification and characterization of the CYP52 family of *Candida tropicalis* ATCC 20336, important for the conversion of fatty acids and alkanes to a, v-dicarboxylic acids. Appl Environ Microbiol 69:5983–5991

Desai J, Banat I (1997) Microbial production of surfactants and their commercial potential. Microbiol Mol Biol Rev 61:47–64

Desai J, Desai A (1993) Production of biosurfactants. In: Kosaric N (ed) Biosurfactants: production, properties, applications. Marcel Dekker, New York, pp 65–97

Deziel E, Paquette G, Villemur R, Lepine F, Bisaillon J-G (1996) Biosurfactant production by a soil *Pseudomonas* strain growing on polycyclic aromatic hydrocarbons. Appl Environ Microbiol 62:1908–1912

Dubbels BL, Sayavedra-Soto LA, Arp DJ (2007) Butane monooxygenase of *Pseudomonas butanovora*: purification and biochemical characterization of a terminal-alkane hydroxylating diiron monooxygenase. Microbiology 153:1808–1816

Eastcott L, Shiu WY, Mackay D (1988) Environmentally relevant physical–chemical properties of hydrocarbons: a review of data and development of simple correlations. Oil Chem Pollut 4:191–216

Efroymson RA, Alexander M (1991) Biodegradation by an Arthrobacter species of hydrocarbons partitioned into an organic solvent. Appl Environ Microbiol 57:1441–1447

Feng L, Wang W, Cheng J, Ren Y, Zhao G, Gao C, Tang Y, Liu X, Han W, Peng X, Liu R, Wang L (2007) Genome and proteome of long-chain alkane degrading *Geobacillus thermodenitrificans* NG80–2 isolated from a deep-subsurface oil reservoir. Proc Natl Acad Sci U S A 104:5602–5607

Finnerty WR (1988) Lipids of *Acinetobacter*. In: Proceedings of the world conference biotechnology fats and oils industry pp 184–188

Foght JM, Fedorak PM, Westlake DWS (1990) Mineralization of [14C] hexadecane and [14C] phenanthrene in crude oil: specifi city among bacterial isolates. Can J Microbiol 36:169–175

Fox BG, Froland WA, Dege JE, Lipscomb JD (1989) Methane monooxygenase from Methylosinus trichosporium OB3b. Purification and properties of a three-component system with high specific activity from a type II methanotroph. J Biol Chem 264(17):10023–10033

Fox BG, Borneman JG, Wackett LP, Lipscomb JD (1990) Haloalkene oxidation by the soluble methane monooxygenase from *Methylosinus trichosporium* OB3b: mechanistic and environmental implications. Biochemistry 29(27):6419–6427

Fritsche W, Hofrichter M (2000) Aerobic degradation by microorganisms. In: Rehm HJ, Reed G (eds) Biotechnology 11b, Environmental Processes II. Wiley-VCH, Weinheim

Gallo MJ, Bertrand JC, Roche B, Azoulay E (1973) Alkane oxidation in *Candida tropicalis*. Biochim Biophys Acta (BBA)—Lipids Lipid Metab 296(3):624–638

Gary S, Sayler , Hooper SW, Layton AC, Henry King JM (1990) Catabolic plasmids of environmental and ecological significance. Microbial Ecol 19:1–207

Glieder A, Farinas ET, Arnold FH (2002) Laboratory evolution of soluble, self-sufficient, highly active alkane hydroxylase. Nat Biotechnol 20:1135–1139

Golyshin PN, Chernikova T, Abraham WR, Lünsdorf H, Timmis KN, Yakimov MM (2002) Oleiphilaceae fam. nov., to include Oleiphilus messinensis gen. nov., sp. nov., a novel marine bacterium that obligately utilizes hydrocarbons. Int J Syst Evol Microbiol 52:901–911

Groves JT (2003) The bioinorganic chemistry of iron in oxygenases and supramolecular assemblies. Proc Natl Acad Sci U S A 100:3569–3574

Groves JT (2005) Models and mechanisms of cytochrome P450 action. Kluwer Academic/Plenum, New York

Gutiérrez T, Leo VV, Walker GM, Green DH (2009) Emulsifying properties of a glycoprotein extract produced by a marine *Flexibacter* species strain TG382. Enzyme Microb Technol 45:53–57

Harayama S, Kasai Y, Hara A (2004) Microbial communities in oil-contaminated seawater. Curr Opin Biotechnol 15:2005–2014

Head IM, Jones DM, Larter SR (2003) Biological activity in the deep subsurface and the origin of heavy oil. Nature 426:344–352

Head IM, Jones DM, Roling WF (2006) Marine microorganisms make a meal of oil. Nat Rev Microbiol 4:173–182

Heringa JW, Huybregste R, van der Linden AC (1961) *n*-alkane oxidation by a Pseudomonas. Formation and,-oxidation of intermediate fatty acids. Antonie van Leeuwenhoek. J Microbiol Serol 27:51–58

Higashioka Y, Kojima H, Nakagawa T, Sato S, Fukui M (2009) A novel *n*-alkane-degrading bacterium as a minor member of p-xylene-degrading sulfate-reducing consortium. Biodegradation 20:383–390

Holden PA, LaMontagne MG, Bruce AK, Miller WG, Lindow SE (2002) Assessing the role of *Pseudomonas aeruginosa* surface-active gene expression in hexadecane biodegradation in sand. Appl Environ Microbiol 68:2509–2518

Hua X, Wu Z, Zhang H, Lu D, Wang M, Liu Y, Liu Z (2010) Degradation of hexadecane by *Enterobacter cloacae* strain TU that secrets an exopolysaccharide as a bioemulsifer. Chemosphere 80:951–956

Iyer A, Mody K, Jha B (2006) Emulsifying properties of a marine bacterial exopolysaccharide. Enzyme Microb Technol 38:220–222

Jin Y, Lipscomb JD (2001) Desaturation reactions catalyzed by soluble methane monooxygenase. J Biol Inorg Chem 6:717–725

Jones KC, Alcock RE, Johnson DL, Northcott GL, Semple KT, Woolgar PJ (1996) Organic chemicals in contaminated land: analysis, significance and research priorities. Land Contam Reclam 3:189–197

Jones DM, Head IM, Gray ND, Adams JJ, Rowan AK, Aitken CM, Bennett B, Huang H, Brown A, Bowler BFJ, Oldenburg T, Erdmann M, Larter SR (2008) Crude-oil biodegradation via methanogenesis in subsurface petroleum reservoirs. Nature 451:176–180

Kang YS, Park W (2009) Protection against diesel oil toxicity by sodium chloride induced exopolysaccharide in Acinetobacter sp. strain DR1. J Biosci Bioeng 109:118–123

Kato T, Haruki M, Imanaka T, Morikawa M, Kanaya S (2001) Isolation and characterization of long-chain-alkane degrading *Bacillus thermoleovorans* from deep subterranean petroleum reservoirs. J Biosci Bioeng 91:64–70

Kato T, Miyanaga A, Kanaya S, Morikawa M (2009) Alkane inducible proteins in *Geobacillus thermoleovorans* B23 BMC. Microbiology 9:60

Kniemeyer O, Musat F, Sievert SM, Knittel K, Wilkes H, Blumenberg M, Michaelis W, Classen A, Bolm C, Joye SB, Widdel F (2007) Anaerobic oxidation of short-chain hydrocarbons by marine sulfate-reducing bacteria. Nature 449:898–902

Kok M, Oldenhuis R, van der Linden MPG, Raatjes P, Kingma J, van Lelyveld PH, Witholt B (1989) *Pseudomonas oleovorans* alkane hydroxylase gene sequence and expression. J Biol Chem 264:5435–5441

Kopp D, Lippard SJ (2002) Soluble methane monooxygenase: activation of dioxygen and methane. Curr Opin Chem Biol 6:568–576

Kotani T, Yamamoto T, Yurimoto H, Sakai Y, Kato N (2003) Propane monooxygenase and NAD(+)-dependent secondary alcohol dehydrogenase in propane metabolism by *Gordonia* sp. strain TY-5. J Bacteriol 185:7120–7128

Kotani T, Yurimoto H, Kato N, Sakai Y (2007) Novel acetone metabolism in a propane-utilizing bacterium, *Gordonia* sp. strain TY-5. J Bacteriol 189:886–893

Kumar AS, Mody K, Jha B (2007) Evaluation of biosurfactant/bioemulsifier production by a marine bacterium. Bull Environ Contam Toxicol 79:617–621

Kurth EG, Doughty DM, Bottomley PJ, Arp DJ, Sayavedra-Soto LA (2008) Involvement of BmoR and BmoG in *n*-alkane metabolism in *Pseudomonas butanovora*. Microbiol 154(1):129–147

Labinger JA, Bercaw JE (2002) Understanding and exploiting C–H bond activation. Nature 417:507

Lal B, Khanna S (1996) Degradation of crude oil by *Acinetobacter calcoaceticus* and *Alcaligenes odorans*. J Appl Bacteriol 81:355–362

Leahy JG, Colwell RR (1990) Microbial degradation of hydrocarbons in the environment. Microbiol Rev 54(3):305–315

Leahy JG, Batchelor PJ, Morcomb SM (2003) Evolution of the soluble diiron monooxygenases. FEMS Microbiol Rev 27(4):449–479

Li L, Liu X, Yang W, Xu F, Wang W, Feng L, Bartlam M, Wang L, Rao Z (2008) Crystal structure of long-chain alkane monooxygenase (LadA) in complex with coenzyme FMN: unveiling the longchain alkane hydroxylase. J Mol Biol 376:453–465

Lieberman RL, Rosenzweig AC (2004) Biological methane oxidation: regulation, biochemistry, and active site structure of particulate methane monooxygenase. Crit Rev Biochem Mol Biol 39:147–164

Liu C, Shao Z (2005) Alcanivorax dieselolei sp. nov., a novel alkane-degrading bacterium isolated from sea water and deep-sea sediment. Int J Syst Evol Microbiol 55(3):1181–1186

Macleod JA, Morriss AWJ, Semple KT (2001) The role of microorganisms in ecological risk assessment of hydrophobic organic contaminants in soils. Adv App Microbiol 48:172–212

Maeng JH, Sakai Y, Ishige T, Tani Y, Nobuo N (1996) Diversity of dioxygenases that catalyze the first step of oxidation of long-chain *n*-alkanes in *Acinetobacter* sp. M-l. FEMS Microbiol Lett 141:177–182

Maier T, Foerster H-H, Asperger O, Hahn U (2001) Molecular characterization of the 56-kDa CYP153 from *Acinetobacter* sp. EB104. Biochem Biophys Res Commun 286:652–658

Malachowsky K, Phelps TJ, Teboli AB, Minnikin DE, White DC (1994) Aerobic mineralization of trichloroethylene, vinyl chloride and aromatic compounds by *Rhodococcus* sp. Appl Environ Microbiol 60:542–548

Marchant R, Sharkey FH, Banat IM, Rahman TJ, Perfumo A (2006) The degradation of *n*-hexadecane in soil by *Thermophilic geobacilli*. FEMS Microbiol Ecol 56(1):44–54

Margesin R, Labbe D, Schinner F, Greer CW, Whyte LG (2003) Characterization of hydrocarbon-degrading microbial populations in contaminated and pristine alpine soils. Appl Environ Microbiol 69:3085–3092

Martínez-Checa F, Toledo FL, EI Mabrouki K, Quesada E, Calvo C (2007) Characteristics of bioemulsifier V2–7 synthesized in culture media added of hydrocarbons: chemical composition, emulsifying activity and rheological properties. Bioresour Technol 98:3130–3135

Mbadinga SM, Wang LY, Zhou L, Liu JF, Gu JD, Mua BZ (2011) Microbial communities involved in anaerobic degradation of alkanes. Intl Biodeter Biodegrad 65:1–13

McKenna EJ, Coon MJ (1970) Enzymatic w-oxidation. IV. Purification and properties of the w-hydroxylase of *Pseudomonas oleovorans*. J Biol Chem 245:3882–3889

Meintanis C, Chalkou KI, Kormas KA, Karagouni AD (2006) Biodegradation of crude oil by thermophilic bacteria isolated from a volcano island. Biodegradation 17:3–9

Merino J, Bucala V (2007) Effect of temperature on the release of hexadecane from soil by thermal treatment. J Hazarad Mat 143:455–461

Moran AC, Olivera N, Commendatore M, Esteves JL, Sineriz F (2000) Enhancement of hydrocarbon waste biodegradation by addition of a biosurfactant from *Bacillus subtilis* O9. Biodegradation 11:65–71

Murrell JC, Gilbert B, McDonald IR (2000a) Molecular biology and regulation of methane monooxygenase. Arch Microbiol 173:325–332

Murrell JC, McDonald IR, Gilbert B (2000b) Regulation of expression of methane monooxygenases by copper ions. Trends Microbiol 8:221–225

Nakayama N, Takemae A, Shoun H (1996) Cytochrome 450 foxy, a catalytically self-sufficient fatty acid hydroxylase of the fungus *Fusarium oxysporum*. J Biochem 119:435–440

Newcomb M, Shen R, Lu Y, Coon MJ, Hollenberg PF, Kopp DA, Lippard SJ (2002) Evaluation of norcarane as a probe for radicals in cytochrome P450- and soluble methane monooxygenase-catalyzed hydroxylation reactions. J Am Chem Soc 124:6879–6886

Nguyen HHT, Schiemke AK, Jacobs SJ, Hales BJ, Lidstrom ME, Chan SI (1994) The nature of the copper ions in the membranes containing the particulate methane monooxygenase from *Methylococcus capsulatus* (Bath). J Biol Chem 269:14995–15005

Noordman WH, Janssen DB (2002) Rhamnolipid stimulates uptake of hydrophobic compounds by *Pseudomonas aeruginosa*. Appl Environ Microbiol 68:4502–4508

Österberg TL, Jonsson A, Lundström US (2006) Accelerated biodegradation of *n*-alkanes in aqueous solution by the addition of fermented whey. Intl Biodeter Biodegrad 57(3):190–194

Peters MW, Meinhold MW, Glieder MA, Arnold FH (2003) Regio- and enantioselective alkane hydroxylation with engineered cytochromes P450 BM-3. J Am Chem Soc 125:13442–13450

Rahman KSM, Thahira-Rahmana J, Lakshmanaperumalsamy P, Banat IM (2002) Towards efficient crude oil degradation by a mixed bacterial consortium. Biores Technol 85(3): 257–261

Ramírez ME, Zapiéna B, Zegarraa HG, Rojasc NG, Fernández LC (2008) Assessment of hydrocarbon biodegradability in clayed and previous weathered polluted soils. Intl Biodeter Biodegrad 63:347–353

Riser-Roberts E (1998) Remediation of petroleum contaminated soils. CRC Press, Boca Raton

Rojo F (2009) Degradation of alkanes by bacteria. Environ Microbiol 11(10):2477–2490

Ron EZ, Rosenberg E (2001) A review of natural roles of biosurfactants. Environ Microbiol 3:229–236

Ron EZ, Rosenberg E (2002) Biosurfactants and oil bioremediation. Curr Opin Biotechnol 13:249–252

Rosenberg E, Ron E (1999) High- and low-molecular-mass microbial surfactants. Appl Microbiol Biotechnol 52:154–162

Rothen SA, Sauer M, Sonnleitner B, Witholt B (1998) Biotransformation of octane by *E. coli* HB101[pGEc47] on defined medium: octanoate production and product inhibition. Biotechnol Bioeng 58:356–365

Sabirova JS, Ferrer M, Regenhardt D, Timmis KN, Golyshin PN (2006) Proteomic insights into metabolic adaptations in Alcani-vorax borkumensis induced by alkane utilization. J Bacteriol 188:3763–3773

Sakai Y, Maeng JH, Kubota S, Tani A, Tani Y, Kato N (1996) A non-conventional dissimilation pathway for long chain *n*-alkanes in *Acinetobacter* sp. M-1 that starts with a dioxygenase reaction. J Ferment Bioeng 81(4):286–291

Sanscartier D, Zeeba B, Koch I, Reimer K (2009) Bioremediation of diesel-contaminated soil by heated and humidified biopile system in cold climates. Cold Reg Sci Technol 55(1):167–173

Schneiker S, dos Santos VAPM, Bartels D, Bekel T, Brecht M, Buhrmester J, Chernikova TN, Denaro R, Ferrer M, Gertler C, Goesmann A, Golyshina OV, Kaminski F, Khachane AN, Lang S, Linke B, McHardy AC, Meyer F, Nechitaylo T, Pühler A, Regenhardt D, Rupp O, Sabirova JS, Selbitschka W, Yakimov MM, Timmis KN, Vorhölter FJ, Weidner S, Kaiser O, Golyshin PN (2006) Genome sequence of the ubiquitous hydrocarbon-degrading marine bacterium *Alcanivorax borkumensis*. Nat Biotechnol 24:997–1004

Semple KT, Reid BJ, Fermor TR (2001) Review of composting strategies to treat organic pollutants in contaminated soils. Environ Pollut 112:269–283

Shanklin J, Whittle E, Fox BG (1994) Eight histidine residues are catalytically essential in a membrane-associated iron enzyme, stearoyl-CoA desaturase, and are conserved in alkane hydroxylase and xylene monooxygenase. Biochemistry 33:12787–12794

Shanklin J, Achim C, Schmidt H, Fox BG, Munck E (1997) Mossbauer studies of alkane omega-hydroxylase: evidence for a diiron cluster in an integral-membrane enzyme. Proc Natl Acad Sci U S A 94:2981–2986

Shimizu S, Yasui K, Tani Y, Yamada H (1979) Acyl-CoA oxidase from *Candida tropicalis*. Biochem Biophys Res Commun 91:108–113

Singh H (2006) Mycoremediation: fungal bioremediation. Wiley-Interscience, New York

Sluis MK, Sayavedra , Soto LA, Arp DJ (2002) Molecular analysis of the soluble butane monooxygenase from *Pseudomonas butanovora*. Microbiology 148:3617–3629

Smits THM, Balada SB, Witholt B, van Beilen JB (2002) Functional analysis of alkane hydroxylases from gram-negative and gram-positive bacteria. J Bacteriol 184:1733–1742

So CM, Phelps CD, Young LY (2003) Anaerobic transformation of alkanes to fatty acids by a sulfate-reducing bacterium, strain Hxd3. Appl Environ Microbiol 69:3892–3900

Sono M, Roach MP, Coulter ED, Dawson JH (1996) Heme-containing oxygenases. Chem Rev 96:2841–2887

Stanier RY, Palleroni NJ, Doudoroff M (1966) The aerobic pseudomonads: a taxonomic study. J Gen Microbiol 43:159–271

Steiert JG, Pignatello JJ, Crawford RL (1987) Degradation of chlorinated phenols by a pentachlorophenol degrading bacterium. Appl Environ Microbiol 53:907–910

Stortini AM, Martellini T, Del Bubba M, Lepri L, Capodaglio G, Cincinelli A (2009) *n*-alkanes, PAHs and surfactants in the sea surface microlayer and sea water samples of the Garlache Inlet sea (Antarctica). Microchem J 92:37–43

Stroud JL, Paton GI, Semple KT (2008) Linking chemical extraction to microbial degradation of ^{14}C-hexadecane in soil. Environ Pollut 156(2):474–481

Suchanek M, Kostal J, Demnerova K, Kralova B (2000) Use of sodium dodecyl sulphate for stimulation of biodegradation of *n*-alkanes without residual contamination by the surfactant. Intl Biodeter Biodegrad 45(1–2):27–33

Tani A, Ishige T, Sakai Y, Kato N (2001) Gene structures and regulation of the alkane hydroxylase complex in *Acinetobacter* sp. strain M-1. J Bacteriol 183:1819–1823

Thijsse GJE, van der Linden AC (1963) Pathways of hydrocarbon dissimilation by a *Pseudomonas* as revealed by chloramphenicol. Antonie van Leeuwenhoek J Microbiol Serol 29:89–100

Throne-Holst M, Wentzel A, Ellingsen TE, Kotlar HK, Zotchev SB (2007) Identification of novel genes involved in long-chain *n*-alkane degradation by *Acinetobacter* sp. strain DSM 17874. Appl Environ Microbiol 73(10):3327–3332

Towell MG, Bellarby J, Paton GI, Simon CF, Pollard JT, Semple KT (2011) Mineralisation of target hydrocarbons in three contaminated soils from former refinery facilities. Environ Pollut 159:515–523

Valentine AM, Wilkinson B, Liu KE, Komar-Panicucci S, Priestly ND, Williams PG, Morimoto H, Floss HG, Lippard SJ (1997) Tritiated chiral alkanes as substrates for soluble methane monooxygenase from *Methylococcus capsulatus* (Bath): probes for the mechanism of hydroxylation. J Am Chem Soc 119:1818–1827

van Beilen JB, Funhoff EG (2005) Expanding the alkane oxygenase toolbox: new enzymes and applications. Curr Opin Biotechnol 16:308–314

van Beilen JB, Funhoff EG (2007) Alkane hydroxylases involved in microbial alkane degradation. Appl Microbiol Biotechnol 74:13–21

van Beilen JB, Wubbolts MG, Witholt B (1994) Genetics of alkane oxidation by *Pseudomonas oleovorans*. Biodegradation 5:161–174

van Beilen JB, Wubbolts M, Qi C, Nieboer M, Witholt B (1995) Effects of two-liquid-phase systems and expression of *alk* genes on the physiology of alkane-oxidizing strains. In: Nakazawa T, Furukawa K, Haas D, Silver S (eds) Molecular Biology of Pseudomonads, ASM Press, Washington, pp 35–47

van Beilen JB, Panke S, Lucchini S, Franchini AG, Rothlisberger M, Witholt B (2001) Analysis of the *Pseudomonas putida* alkane degradation gene cluster and flanking insertion sequence: evolution and regulation of the *alk* genes. Microbiology 147:1621–1630

van Beilen JB, Neuenschwander M, Smits THM, Roth C, Balada SB, Witholt B (2002) Rubredoxins involved in alkane oxidation. J Bacteriol 184:1722–1732

van Beilen JB, Li Z, Duetz WA, Smits THM, Witholt B (2003) Diversity of alkane hydroxylase systems in the environment. Oil Gas Sci Technol 58:427–440

van Beilen JB, Marin MM, Smits TH, Rothlisberger M, Franchini AG, Witholt B, Rojo F (2004) Characterization of two alkane hydroxylase genes from the marine hydrocarbonoclastic bacterium *Alcanivorax borkumensis*. Environ Microbiol 6:264–273

van Hamme JD, Ward OP (2003) Recent advances in petroleum microbiology. Microbiol Mole Biol Rev 67:649

Vatsyayan P, Kumar AK, Goswami P, Goswami P (2008) Broad substrate Cytochrome P450 monooxygenase activity in the cells of *Aspergillus terreus* MTCC 6324. Biores Technol 99(1):68–75

Volke-Sepulveda TL, Gutierrez-Rojas M, Favela-Torres E (2003) Biodegradation of hexadecane in liquid and solid-state fermentations by *Aspergillus niger*. Biores Technol 87:81–86

Volke-Sepùlvda T, Gutièrrez-Rojas M, Favela-Torres E (2006) Biodegradation of high concentrations of hexadecane by Aspergillus niger in a solid-state system: Kinetic analysis. Biores Technol 94(14): 1583–1591

Vomberg A, Klinner V (2000) Distribution of alk gene with in *n*-alkane degrading bacteria. J Appl Microbiol 89:339–348

Wang LY, Mbadinga SM, Li H, Liu JF, Yang SZ, Mu BZ (2010) Anaerobic degradation of petroleum hydrocarbons and enlightenment of the prospects for microbial bio-gasification of residual oil. Microbiology 37:96–102

Watanabe HK, Takahashi N (1997) Isolation and characterization of slime-producing bacteria capable of utilizing petroleum hydrocarbons as a sole carbon source. J Ferment Bioeng 84:528–531

Wentzel A, Ellingsen TE, Kotlar HK, Zotchev SB, Throne-Holst M (2007) Bacterial metabolism of long-chain *n*-alkanes. Appl Microbiol Biotechnol 76:1209–1221

Whyte LG, Hawari J, Zhou E, Bourbonniere L, Inniss WE, Greer CW (1998) Biodegradation of variable-chain-length alkanes at low-temperatures by a psychrotrophic *Rhodococcus* sp. Appl Environ Microbiol 64:2578–2584

Widdel F, Rabus R (2001) Anaerobic biodegradation of saturated and aromatic hydrocarbons. Curr Opin Biotechnol 12:259–276

Wilkes H, Rabus R, Fischer T, Armstroff A, Behrends A, Widdel F (2002) Anaerobic degradation of *n*-hexane in a denitrifying bacterium: further degradation of the initial intermediate (1-methylpentyl)succinate via C-skeleton rearrangement. Arch Microbiol 177: 235–243

Yakimov MM, Giuliano L, Gentile G, Crisafi E, Chernikova TN, Abraham WR, Lünsdorf H, Timmis KN, Golyshin PN (2003) *Oleispira antarctica* gen. nov., sp. nov., a novel hydrocarbonoclastic marine bacterium isolated from Antarctic coastal sea water. Int J Syst Evol Microbiol 53:779–785

Yakimov MM, Giuliano L, Denaro R, Crisafi E, Chernikova TN, Abraham WR, Luensdorf H, Timmis KN, Golyshin PN (2004) *Thalassolituus oleivorans* gen. nov., sp nov., a novel marine bacterium that obligately utilizes hydro-carbons. Int J Syst Evol Microbiol 54:141–148

Yakimov MM, Timmis KN, Golyshin PN (2007) Obligate oil-degrading marine bacteria. Curr Opin Biotechnol 18:257–266

Zedelius J, Rabus R, Grundmann O, Werner I, Brodkorb D, Schreiber F, Ehrenreich P, Behrends A, Wilkes H, Kube M, Reinhardt R, Widde F (2011) Alkane degradation under anoxic conditions by a nitrate-reducing bacterium with possible involvement of the electron acceptor in substrate activationemi. Environ Microbiol Rep 3(1):125–135

Index

S. N. Singh (ed.), *Microbial Degradation of Xenobiotics*,
Environmental Science and Engineering, DOI: 10.1007/978-3-642-23789-8,