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Shree Nath Singh *Editor*

Microbial Degradation of Xenobiotics

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Shree Nath Singh
Editor

Microbial Degradation of Xenobiotics

 Springer

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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

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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

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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 polychlorophenols 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. chlorophenicum* 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). *Desulfobacterium 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; Bisailon 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. chlorophenicum* 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. chlorophenicum* L-1, the complete degradation pathway has been determined, which is different from that of *M. chlorophenicum* (Cai and Xun 2002). *S. chlorophenicum* L-1 is isolated from a PCP-contaminated soil and it is able to completely mineralize PCP to CO₂ and chlorine (Saber and Crawford 1985). Genetic approaches were initially used to study the metabolic pathway in *S. chlorophenicum* 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. chlorophenicum* 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. chlorophenicum* 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. chlorophenicum* 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 O₂. 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

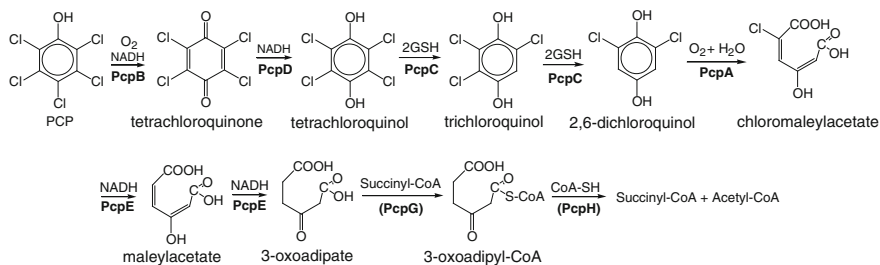


Fig. 1.1 PCP degradation pathway of *S. chlorophenolicum* L-1. PcpB, PCP 4-monooxygenase; PcpD, tetrachloroquinone reductase; PcpC, tetrachloroquinol reductive dehalogenase; PcpA, 2,6-dichloroquinol 1,2-dioxygenase; PcpE, chloromaleylacetate reductase; PcpG, 3-oxoadipate: succinyl-CoA transferase; and 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 tetrafluoro-*p*-hydroxybenzoate, the direct product is a quinone, which is chemically reduced by NADH to produce trifluoro-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 $5 \mu\text{M Fe}^{2+}$) (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 *Lactobacillus*) 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 transiently 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. chlorophenicum* (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. chlorophenicum* 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. chlorophenicum* 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. chlorophenicum* 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

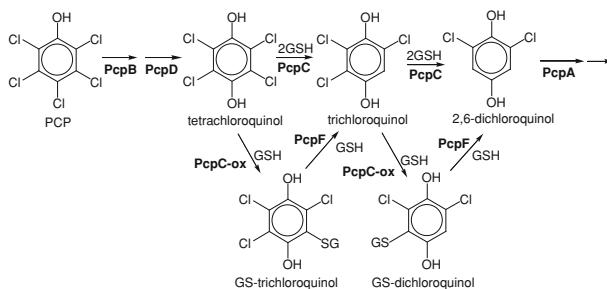


Fig. 1.2 The maintenance role of PcpF in PCP degradation by *S. chlorophenolicum* L-1. PcpC-ox oxidatively damaged PcpC; PcpF, GS-(chloro)quinol reductase

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 analysis 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

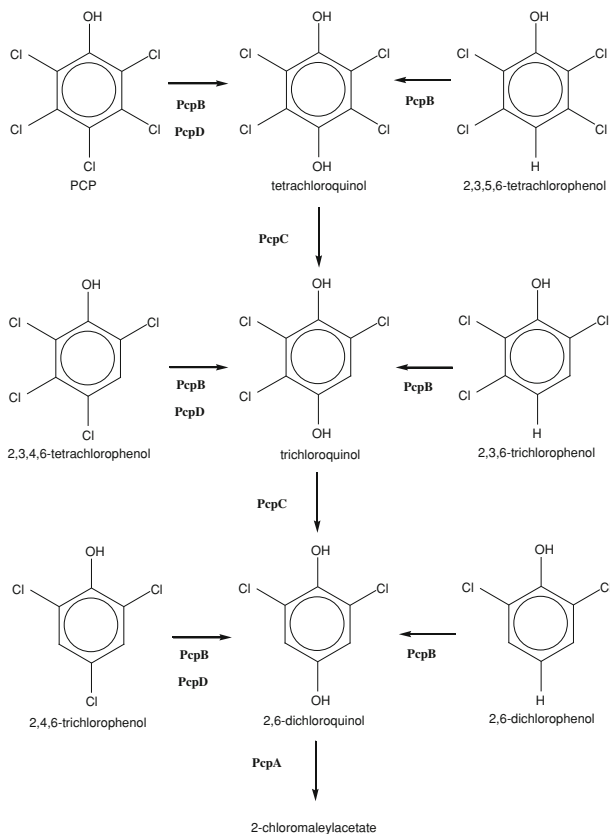


Fig. 1.3 The versatility of the PCP-degrading enzymes for the degradation of polychlorophenols. *S. chlorophenolicum* L-1 degrades several polychlorophenols as long as the 2,6-positions are occupied with chlorines. The combined actions of PcpB, PcpD, PcpC, and PcpA are responsible for the degradation. 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; Tirola 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-monoxygenase 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-monoxygenase (TcpA) is partially purified from JMP134 cell extracts and characterized as a reduced flavin adenine dinucleotide (FADH₂)-dependent monoxygenase. The corresponding gene is identified by PCR using primers designed from conserved regions of FADH₂-dependent monoxygenases, and a gene cluster (*tcpABC*) has been determined. *tcpA* encodes the FADH₂-dependent 2,4,6-TCP 4-monoxygenase, 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-Monoxygenase (TcpA)

Recombinant TcpA, purified from *E. coli*, transforms 2,4,6-TCP to 6-chlorohydroxyquinol when FADH₂ 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 monoxygenases 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

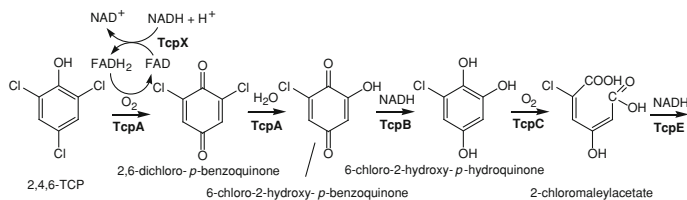


Fig. 1.4 2,4,6-TCP degradation pathway of *C. necator* JMP134. TcpA, 2,4,6-TCP 4-monoxygenase; TcpB, quinone reductase; TcpC, 6-chlorohydroxyquinol 1,2-dioxygenase; and TcpD, chloromaleylacetate reductase

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 FADH₂-dependent monooxygenases. *E. coli* 4-hydroxyphenylacetate 3-monoxygenase (Galán et al. 2000; Xun and Sandvik 2000), TcpA (Louie et al. 2002), chlorophenol 4-monoxygenase (TftD) (Gisi and Xun 2003), and phenol 2-monoxygenase (Kirchner et al. 2003) are the early reported FADH₂-dependent monooxygenases. Sequence analysis suggests that a group of aromatic compound-hydroxylating enzymes are FADH₂-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 FADH₂-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 FADH₂-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 FADH₂ to FADH₂-dependent monooxygenases, TcpX is expected to be the flavin reductase that provides TcpA with FADH₂ (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 FADH₂ is unstable in the presence of O₂, 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 FADH₂ generated by other flavin reductases. In fact, TcpA is characterized with a general *E. coli* flavin reductase (Fre) that provides FADH₂ 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 FMNH₂ (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.

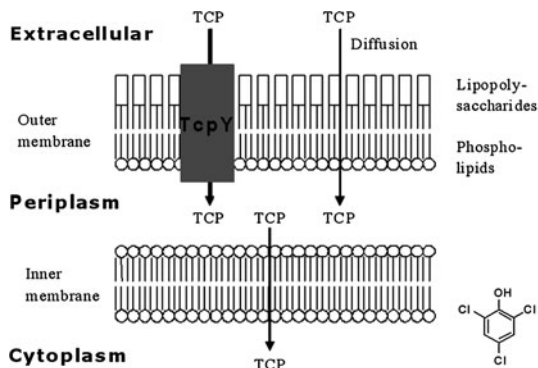
1.3.2.5 6-Chlorohydroxyquinol 1,2-Dioxygenase (TcpC)

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 FADH₂ 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 lipopolysaccharides 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-trichlorophenol into *C. necator* (Belchik et al. 2010). Except *tcpY*, the inactivation of other genes in the *tcpRXABCDYD* 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 FADH₂ 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 FADH₂ 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

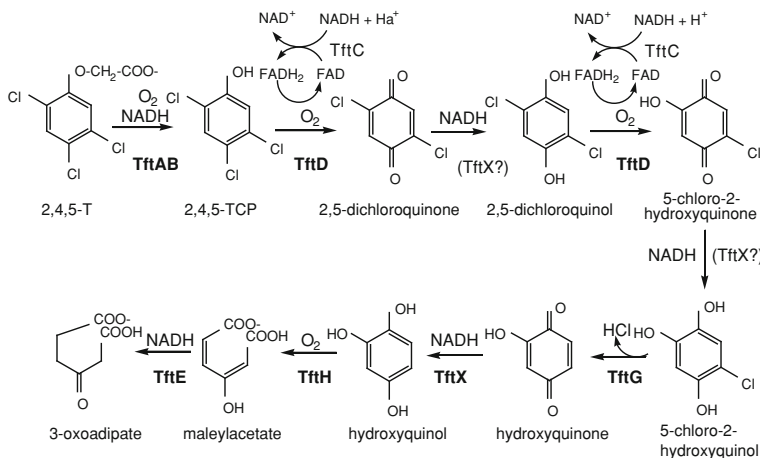


Fig. 1.6 2,4,5-Trichlorophenoxyacetate degradation pathway of *B. cepacia* AC1100. TftAB, 2,4,5-T terminal oxygenase; TftD, 2,4,5-TCP 4-monooxygenase; TftC, NADH:FAD oxidoreductase; TftX, quinone reductase; TftG, 5-chlorohydroxyquinol dehydrochlorinase; TftH, hydroxyquinol 1,2-dioxygenase; and TftE, 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 α₂β₂ structure, and each αβ 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 αβ 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 O₂. 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 FADH₂-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 FADH₂. TftC is highly homologous to TcpX that provides TcpA with FADH₂ for 2,4,6-TCP oxidation. TftD is an FADH₂-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

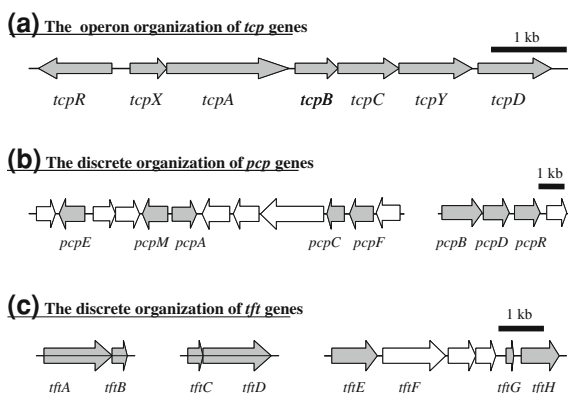
The biochemistry of polychlorophenol degradation has revealed several dechlorination mechanisms. First, oxygenolytic dechlorination is quite common for the three metabolic pathways. Molecular oxygen is involved in the dechlorination and a chlorine is replaced by a hydroxyl group with the oxygen atom derived from O₂. 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. chlorophenicum*, 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 H₂O. 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 (Tirola 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*, *pcpC*, *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.

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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

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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 these oxidations may serve as redox mediators for LiP-catalyzed reactions effectively extending the substrate

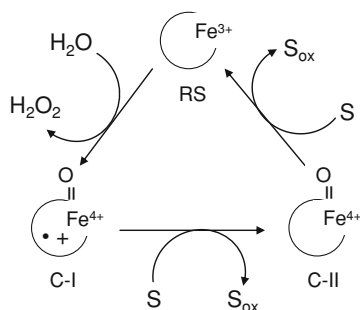


Fig. 2.1 Catalytic cycle of ligninolytic peroxidases (LiP, MnP, and VP). The enzyme resting state (RS, containing Fe^{3+}) undergoes oxidation by hydrogen peroxide to yield Compound I (C-I, containing Fe^{4+} -oxo and porphyrin cation radical). Reduction of C-I in two one electron steps results in the formation of Compound II (C-II, containing $\text{Fe}^{4+} = \text{O}$ 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. chrysosporium* 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 ($\text{CO}_2^{\bullet-}$). 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	<i>T. versicolor</i> , <i>B. adusta</i> , <i>G. lucidum</i> , <i>C. maxima</i> , <i>P. ostreatus</i> , <i>T. pubescens</i>	Cañas and Camarero (2010), Thurston (1994), Hildén et al. (1994)
Lignin peroxidase	Extracellular, most basidiomycetes	Two electron oxidation of the enzyme by H ₂ O 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	<i>P. chrysosporium</i> , <i>P. sordida</i> , <i>P. radiata</i> , <i>P. tremellosa</i> , <i>T. versicolor</i> , <i>B. adusta</i>	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 ₂ O 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	<i>P. chrysosporium</i> , <i>P. sordida</i> , <i>C. subvermispora</i> , <i>P. radiata</i> , <i>D. squelens</i> , <i>P. rivulosus</i>	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	<i>P. eryngii</i> , <i>P. ostreatus</i> , <i>B. adusta</i> , <i>Trametes</i> 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 O₂ 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 O₂-dependent oxidation of a variety of phenolic compounds and do not require H₂O₂ 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 ($E'_{o} \sim 1.2$ V, pH 3), followed by MnP ($E'_{o} \sim 0.8$ V, pH 4.5) and laccase ($E'_{o} \sim 0.79$ 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).

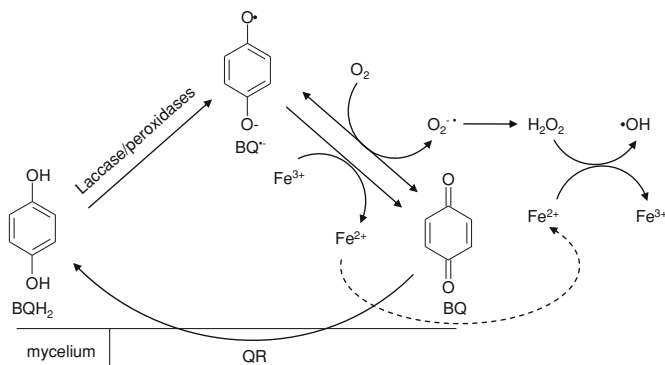


Fig. 2.2 Reaction scheme involved in the production of hydroxyl radical by white rot fungi via quinone redox cycling (Gomez-Toribio et al. 2009a, b). 1,4-benzoquinone (BQ) is reduced by quinone reductase (QR) producing hydroquinone (BQH₂), which is oxidized by any of the lignin modifying enzymes to semiquinones (BQ^{•-}). The production of superoxide anion radicals (O₂^{•-}) by BQ^{•-} autooxidation is mainly catalyzed by Fe³⁺, that is reduced to Fe²⁺. Fenton's reagent formation is accomplished by O₂^{•-} dismutation to H₂O₂

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 O₂, semiquinones are autooxidized to regenerate the quinone, while O₂ is reduced to superoxide anion radical (O₂^{•-}) (Guillen et al. 1997). In this strategy, the incubation of the mycelium with quinones and ferric ion (Fe³⁺) generates the required Fenton's reagent (Fe²⁺ and H₂O₂) to produce hydroxyl radical via reduction of Fe³⁺ to Fe²⁺ by semiquinone radical and spontaneous dismutation of O₂^{•-} to H₂O₂ (Fig. 2.2). H₂O₂ and Fe²⁺ react to produce •OH and Fe³⁺. 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, O₂ or Fe³⁺ 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 ($\text{CO}_2^{\bullet-}$), which was shown to dechlorinate chloro-organic compounds, similar to that described above for LiP, when incubated with veratryl alcohol and $\text{CO}_2^{\bullet-}$ (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 H₂O₂-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, H₂O₂, 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 [¹⁴C] TCE as the substrates showed that most of the TCE was transformed to ¹⁴CO₂. 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

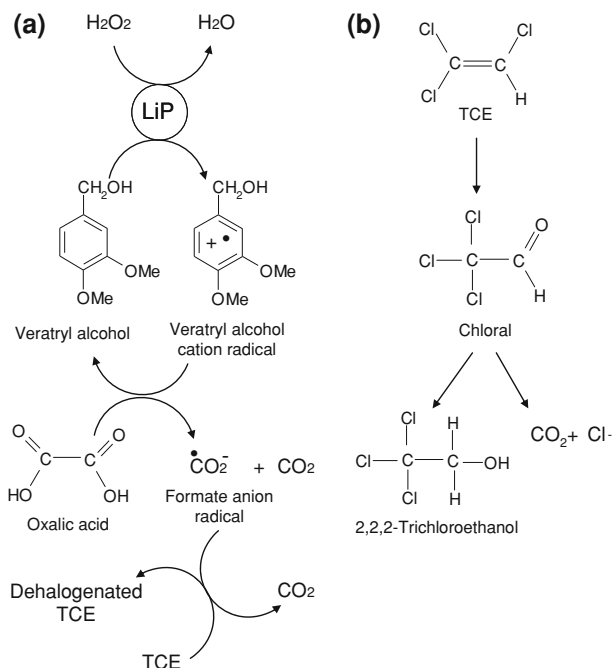


Fig. 2.3 Mechanisms of trichloroethylene (TCE) degradation by white rot fungi. **a** Proposed reductive dechlorination of TCE by LiP of *P. chrysosporium* using oxalate as the electron donor (Khindaria et al. 1995). **b** Oxidation of TCE catalyzed by cytochrome P450 system in *T. versicolor* (Marco-Urrea 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	<i>P. chrysosporium</i>	Mineralization of [¹⁴ C]2-CBP.	Thomas et al. (1992)
2,5-diCBP	<i>T. versicolor</i>	Identification of dichlorobenzenes, chlorophenols and alkylated benzenes as intermediates.	Koller et al. (2000)
4,4'-diCBP	<i>P. chrysosporium</i> <i>Phanerochaete</i> sp. MZ142	Identification of metabolites 2-OH- and 3-OH-diCBP in <i>Phanerochaete</i> sp. MZ142 and 3-OH- and 4-OH-diCBP in <i>P. chrysosporium</i> ; and 3-methoxy-4,4'-diCBP, 4-CBZ, 4-CBZH, and 4-CBZOH in both.	Kamei et al. (2006a)
2,4',5-triCBP	<i>P. chrysosporium</i> <i>P. chrysosporium</i> , <i>B. adusta</i> <i>T. versicolor</i> , <i>P. ostreatus</i>	Mineralization of [¹⁴ C]4,4'-diCBP. Identification of CBZ and CBZOH as metabolites. Mineralization of [¹⁴ C]2,4',5-triCBP: <i>T. versicolor</i> > <i>B. adusta</i> > <i>P. ostreatus</i> > <i>P. chrysosporium</i> . No correlation established between degradation, mineralization, and peroxidase production.	Dietrich et al. (1995) Beaudette et al. (1998)
2,2',4,4'-tetraCBP	<i>T. versicolor</i>	Mineralization of [¹⁴ C]2,4,5-triCBP was stimulated by the surfactant Triton X-100.	Beaudette et al. (2000)
3,3',4,4'-tetraCBP	<i>P. chrysosporium</i> <i>P. brevispora</i>	Mineralization of [¹⁴ C]2,2',4,4'-tetraCBP. Identification of 5-methoxy-3,3',4,4'-tetraCBP as metabolite.	Thomas et al. (1992) Kamei et al. (2006b)
	<i>P. chrysosporium</i> <i>P. chrysosporium</i> <i>P. chrysosporium</i>	Low mineralization of [¹⁴ C]3,3',4,4'-tetraCBP (0.8%). Low mineralization of [¹⁴ C]3,3',4,4'-tetraCBP (1.1%). Low mineralization of [¹⁴ C]3,3',4,4'-tetraCBP (1.4%).	Dietrich et al. (1995) Bumpus et al. (1985) 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	<i>P. brevispora</i>	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	<i>P. brevispora</i>	Three metabolites identified: 5-methoxy-, 5'-methoxy-pentaCBP and 4-methoxy-tetraCBP.	Kamei et al. (2006b)
3,3',4,4',5-pentaCBP	<i>P. brevispora</i>	Identification of 5'-methoxy-pentaCBP as metabolite.	Kamei et al. (2006b)
3,3',4,4',5,5'-hexaCBP	<i>P. brevispora</i>	Identification of 5'-methoxy-pentaCBP.	Kamei et al. (2006b)
2,2',4,4',5,5'-hexaCBP	<i>P. chrysosporium</i>	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. chrysosporium* 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

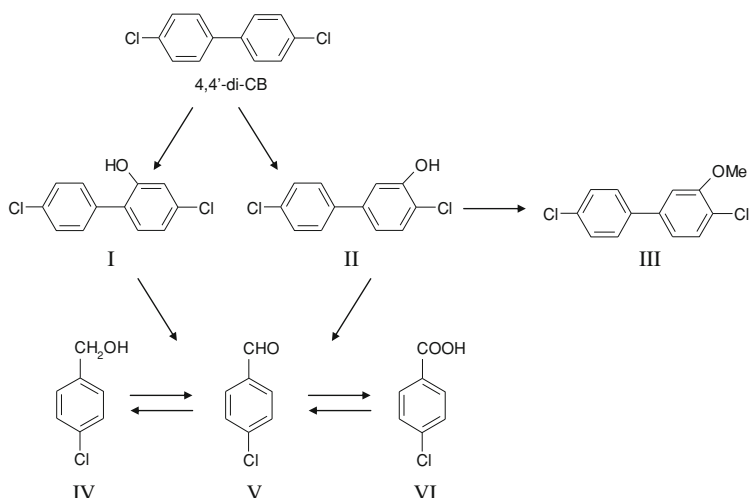


Fig. 2.4 Pathway for 4,4'-dichlorobiphenyl (4,4'-diCBP) degradation by *Phanerochaete* sp. MZ142 (Kamei et al. 2006a). Symbols: I: 2-hydroxy-4,4'-dichlorobiphenyl, II: 3-hydroxy-4,4'-dichlorobiphenyl, III: 3-methoxy-4,4'-dichlorobiphenyl, IV: 4-chlorobenzyl alcohol, V: 4-chlorobenzaldehyde, VI: 4-chlorobenzoic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

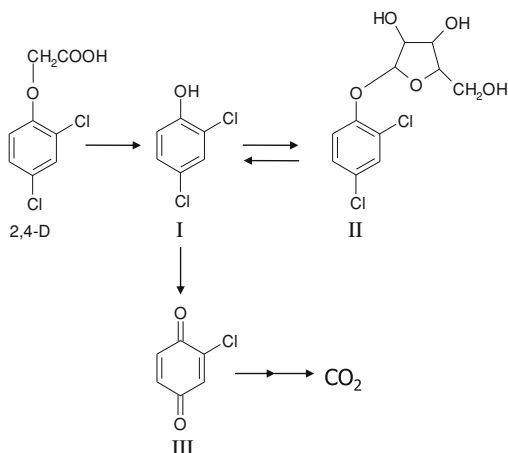
from PCB-contaminated sites. Unlike PCBs, hydroxylated PCBs are transformed by laccase. As a general rule, degradation rate constants of hydroxylated PCBs by purified laccases decreased with increase in the degree of chlorination, which is positively correlated with the ionization potential of the molecules (Keum and Li 2004; Fujihira 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; Fujihira 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 dimers (Fujihira 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 transformation 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 ¹⁴C-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 [¹⁴C]-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

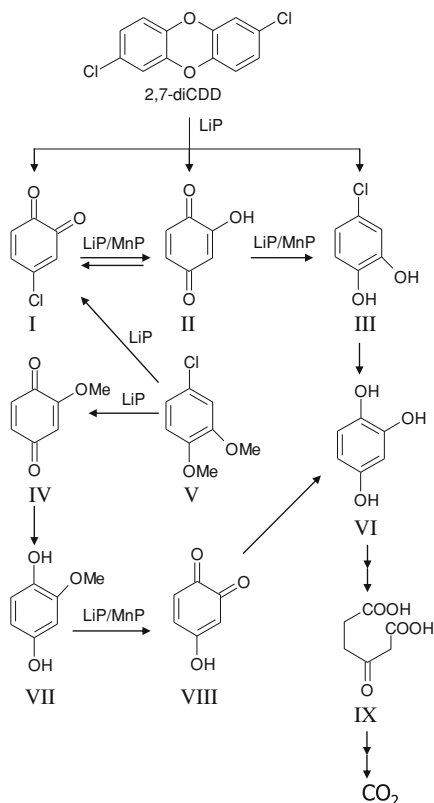


Fig. 2.6 Pathway for degradation of 2,7-dichlorodibenzo-p-dioxin (2,7-diCDD) by *P. chrysosporium* (Valli et al. 1992). Symbols: I: 4-chloro-1,2-benzoquinone, II: 2-hydroxy-1,4-benzoquinone, III: 4-chlorocatechol, IV: 2-methoxy-1,4-benzoquinone, V: 4-chloroveratrole, VI: 1,2,4-trihydroxybenzene, VII: 2-methoxyhydroquinone, VIII: 4-hydroxy-1,2-benzoquinone, IX: β -keto adipic 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. lindneri* 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 intermediates (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

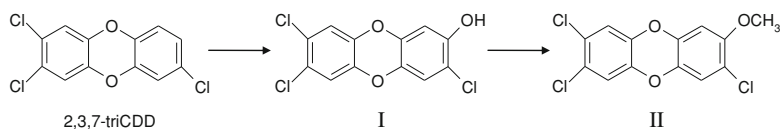


Fig. 2.7 Degradation pathway of 2,3,7-trichlorodibenzo-*p*-dioxin (2,3,7-triCDD) by *Phlebia lindtneri* (Kamei and Kondo, 2005). Symbols: I: monohydroxy-triCDD, II: monomethoxy-triCDD

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)
2-monoCDD	Cyt P450	Yeast clones expressing individual cyt P450s of the fungus show metabolism towards 1-monoCDD.	Kasai et al. (2010)
	LiP	LiP transformed 2-monoCDD to the corresponding cation radical as immediate product.	Hammel et al. (1986)
2,7-diCDD	<i>P. chrysosporium</i>	Identification of metabolites and elucidation of enzymatic mechanisms shown in Fig. 2.6.	Valli et al. (1992)
	<i>P. lindmeri</i>	Identification of one hydroxylated metabolite.	Mori and Kondo (2002b)
	<i>P. lindmeri</i> , <i>Phlebia</i> 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)
	<i>P. stypticus</i>	Identification of metabolite III (Fig. 6); cyt P450 involvement in the initial oxidation of 2,7-diCDD.	Sato et al. (2002)
	<i>P. lindmeri</i>	Identification of hydroxylated, methoxylated and dimethoxylated metabolites.	Kamei and Kondo (2005)
	<i>Phlebia</i> 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	<i>P. lindmeri</i>	Identification of hydroxylated and methoxylated metabolites.	Kamei and Kondo (2005)
	<i>Phlebia</i> spp.	Degradation of substrate.	Kamei et al. (2005)
	<i>P. chrysosporium</i>	Low mineralization of [¹⁴ C]2,3,7,8-tetraCDD (2%).	Bumpus et al. (1985)
	<i>P. sordida</i>	Identification of 4,5-chlorocatechols as metabolite in <i>P. sordida</i> . This strain does not produce LiP.	Takada et al. (1996)

Table 2.3 (continued)

Compound	Fungus/enzyme	Comments	Reference
2,3,7,8-tetraCDD	<i>P. chrysosporium</i> <i>P. brevispora</i>	Crude enzymes of MnP did not oxidize CDDs. Identification of monohydroxylated, monomethoxylated, dimethoxylated metabolites and 3,5-dichlorocatechol. Possible role of cyt P450	Takada et al. (1996) Kamei et al. (2005)
1,3,6,8-tetraCDD	<i>Bjerkandera</i> spp. <i>P. brevispora</i>	Degradation of substrate. Examination of the bioremediation potential in different types of soils.	Manji and Ishihara (2004) Kamei et al. (2009)
1,2,6,7-TetraCDD	<i>P. lindneri</i>	Identification of two hydroxylated and one monomethoxylated metabolites.	Kamei and Kondo (2005)
1,2,3,7,8-pentaCDD	<i>Phlebia</i> spp.	Based on degradation.	Kamei et al. (2005)
1,2,3,4,7,8-hexaCDD	<i>P. sordida</i>	Based on degradation.	Takada et al. (1996)
1,2,3,4,6,7,8-heptaCDD	<i>P. chrysosporium</i>		
1,2,3,4,6,7,8,9-octaCDD	<i>P. sordida</i>	Identification of tetrachlorocatechol as metabolite in <i>P. sordida</i> . This strain does not produce LiP.	Takada et al. (1996)
	<i>P. chrysosporium</i>	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 (~30%) whereas only a smaller portion of monochlorobenzene, *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*

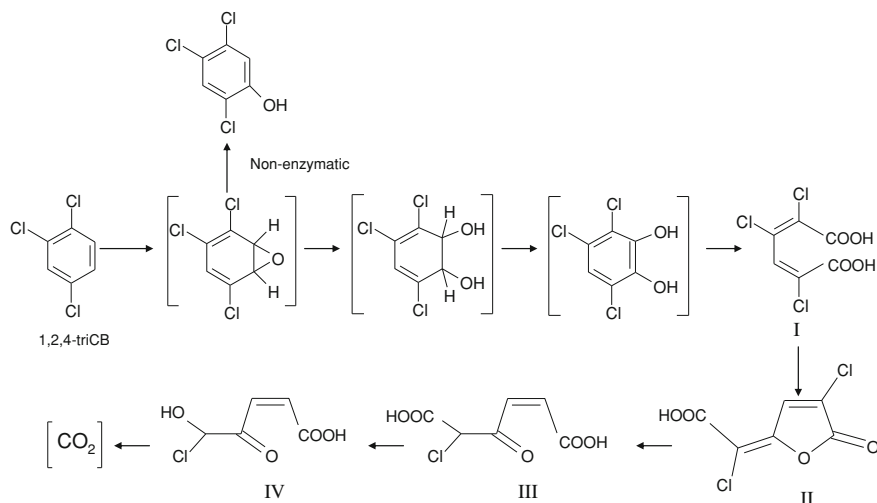


Fig. 2.8 Degradation pathway of 1,2,4-trichlorobenzene (triCB) by *T. versicolor* (Marco-Urrea et al. 2009b). Symbols: I: 2,3,5-trichloromuconate, II: (chloro-) carboxymethylenebutenolide, III: 2- or 5-chloro-4-oxo-2-hexendioic acid, IV: 2- or 5-chloro-5-hydroxy-4-oxo-2-pentenoic acid. Chemicals set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

castanella showed a consistent decrease of hexachlorobenzene concentration. The amount of ¹⁴CO₂ from [¹⁴C] 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 corn-cob-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

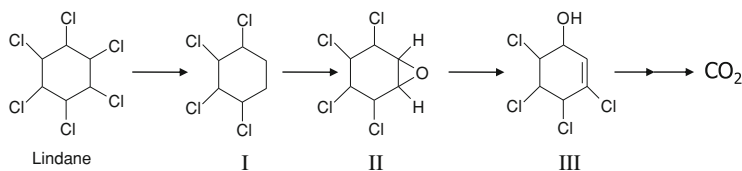


Fig. 2.9 Proposed degradation pathway for lindane by white rot fungi (Mougin et al. 1996; Singh and Kuhad 1999, 2000). Symbols: I: tetrachlorocyclohexane, II: tetrachlorocyclohexane epoxide, III: tetrachlorocyclohexenol

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, tetrachlorocyclohexene epoxide, and tetrachlorocyclohexenol, whereas only tetrachlorocyclohexenol was produced by *Cyathus bulleryi* (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-1, 1-bis(4-chlorophenyl) ethanol (dicofol), 2,2-dichloro-1,1-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.

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Chapter 3

Microbial Styrene Degradation: From Basics to Biotechnology

Dirk Tischler and Stefan R. Kaschabek

3.1 Introduction

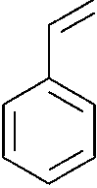
Styrene, the simplest representative of alkenylbenzenes, is one of the most important monomers produced by the chemical industry today. The compound shows a distinct toxicological behavior and is classified as a possible carcinogen due to its metabolism in human via a highly mutagenic epoxide. Considerable 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

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

Property	Value	Chemical structure
Molecular formula	C ₈ H 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

pre-narcotic 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
<i>Bacteria</i>		
Actinobacteria	<i>Corynebacterium</i>	Itoh et al. (1996)
	<i>Gordonia</i>	Alexandrino et al. (2001)
	<i>Mycobacterium</i>	Burback and Perry (1993)
	<i>Nocardia</i>	Hartmans et al. (1990)
	<i>Rhodococcus</i>	Hartmans et al. (1990), Jung and Park (2005), Patrauchan et al. (2008), Tischler et al. (2009), Warhurst et al. (1994), Zilli et al. (2003)
	<i>Streptomyces</i>	Przybulewska et al. (2006)
	<i>Tsukamurella</i>	Arnold et al. (1997)
Bacilli	<i>Bacillus</i>	Przybulewska et al. (2006)
Clostridia	<i>Clostridium</i>	Grbić-Galić et al. (1990)
α -Proteobacteria	<i>Sphingomonas</i>	Arnold et al. (1997)
	<i>Xanthobacter</i>	Hartmans et al. (1989, 1990)
γ -Proteobacteria	<i>Enterobacter</i>	Grbić-Galić et al. (1990)
	<i>Pseudomonas</i>	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)
	<i>Xanthomonas</i>	Arnold et al. (1997)
Sphingobacteria	<i>Sphingobacterium</i>	Przybulewska et al. (2006)
<i>Fungi</i>		
Agaricomycetes	<i>Bjerkandera</i>	Braun-Lüllemann et al. (1997)
	<i>Phanerochaete</i>	Braun-Lüllemann et al. (1997)
	<i>Pleurotus</i>	Braun-Lüllemann et al. (1997)
	<i>Trametes</i>	Braun-Lüllemann et al. (1997)
Eurotiomycetes	<i>Aspergillus</i>	Paca et al. (2001)
	<i>Exophiala</i>	Cox et al. (1996)
	<i>Penicillium</i>	Cox (1995), de Jong et al. (1990), Paca et al. (2001)
Sordariomycetes	<i>Gliocladium</i>	Cox (1995)
	<i>Sporothrix</i>	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 phyla 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 hydration 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

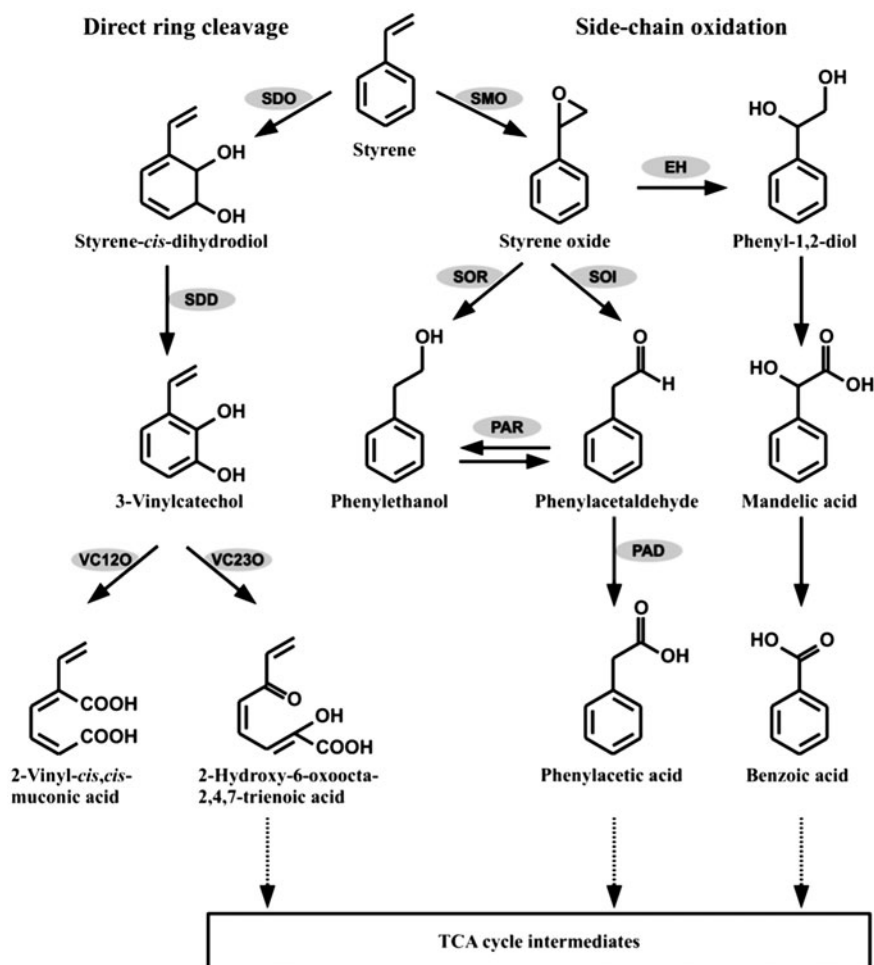


Fig. 3.1 Microbial pathways of aerobic styrene degradation. Characterized enzymes are indicated by their *abbreviation*

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

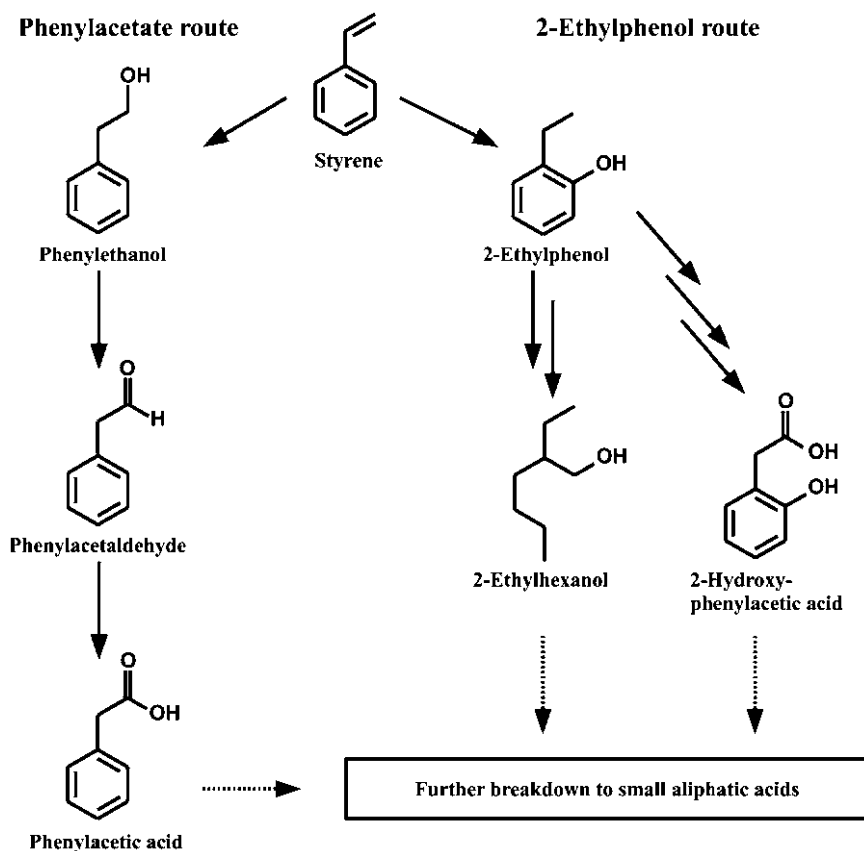


Fig. 3.2 Proposed steps of anaerobic styrene degradation pathways (adapted from Grbić-Galić et al. 1990). The herein shown routes for anaerobic styrene breakdown are based on identified metabolites from pure or mixed cultures. No data about involved enzymes are available so far

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,

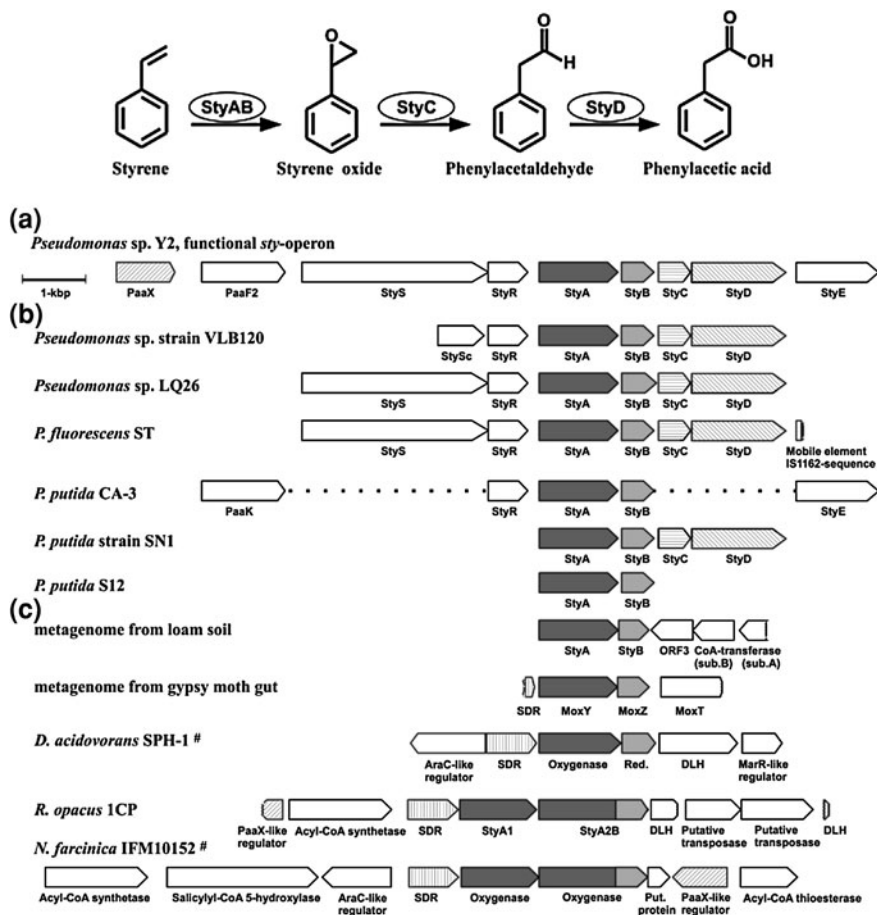


Fig. 3.3 Comparison of the organization of **a** the *styABCDE*-operon from *Pseudomonas* sp. Y2, **b** other (incomplete) styrene-catabolic gene clusters from pseudomonads, and **c** the genetic location of flavin monooxygenases with (# hypothetical) function as styrene monooxygenases. The upper pathway 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 *styC* 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 phenazine 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* ICP, 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

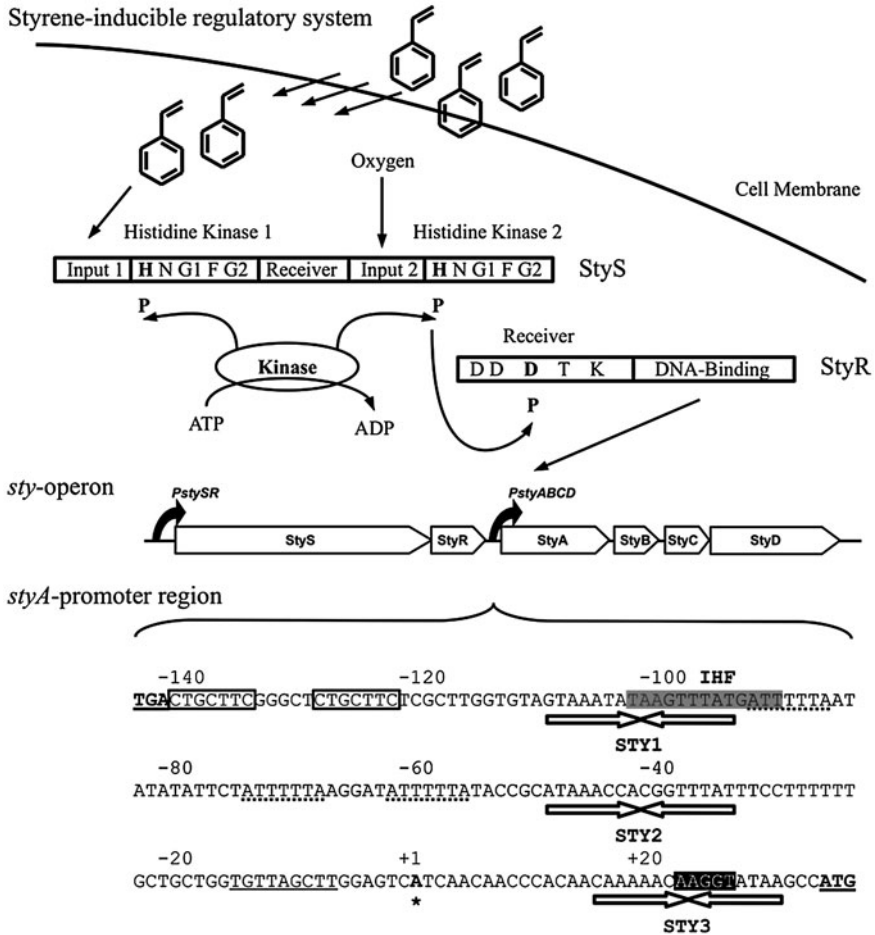


Fig. 3.4 Structure and mechanism of the styrene-inducible regulatory system of the *sty*-operon and a detailed view on the complex *styA*-promoter region (adapted from O’Leary et al. 2002b). The regulatory system (StyS/StyR with conserved motifs, *sty*-operon with indicated promoter sites *PstySR* and *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’-WAT-CAANNNTT-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’-ATTTTA-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'-WATCAANNNTTR-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 (FADH₂) 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* ICP 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* ICP 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* ICP 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 IS21-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 IS1162 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

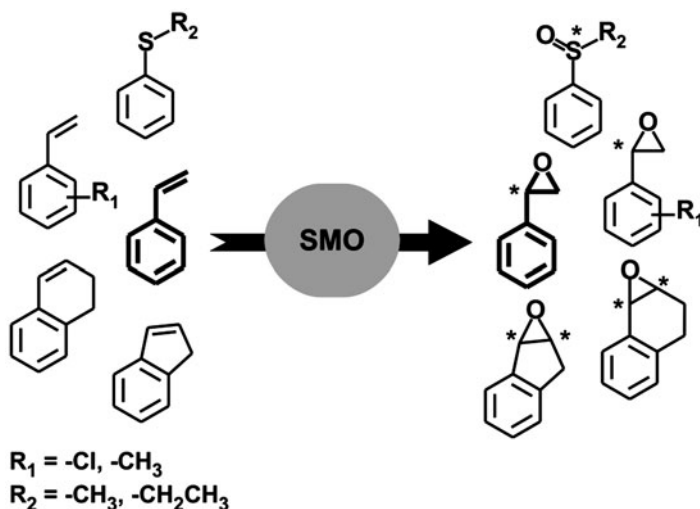


Fig. 3.5 Enantioselective oxygenations catalyzed by styrene monooxygenases (SMOs). Stars indicate optically active centers (Tischler et al. 2010)

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).

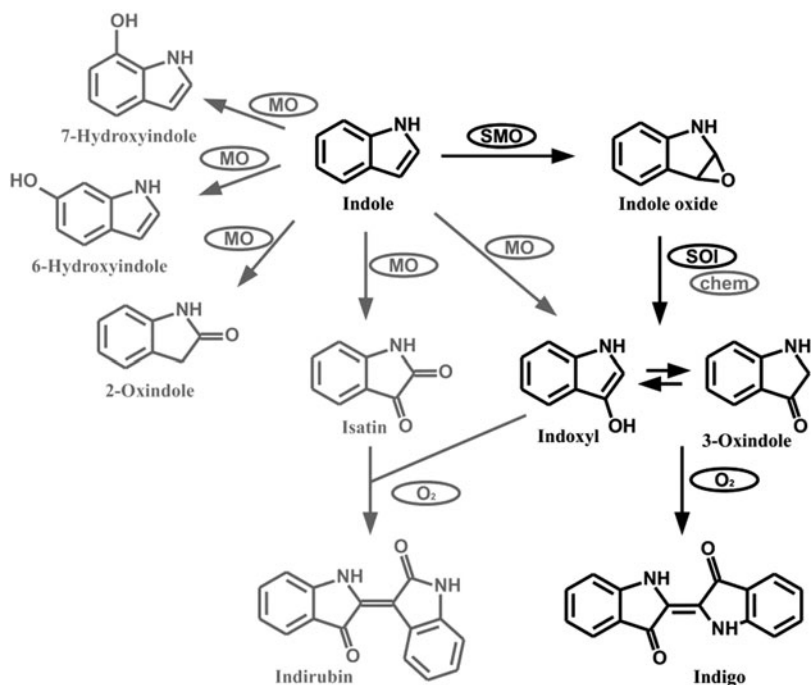


Fig. 3.6 Oxo functionalization of indole and the formation of indigoid dyes (from McClay et al. 2005). SMO/SOI-based oxygenation (*black*) and oxidation of indole (*grey*) catalyzed by various oxygenases (e.g. P450- or toluene monooxygenases; MO) can result in the formation of indigo and indirubin. Indoxyl 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-oxindole, 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 mammals. 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-coenzyme A synthetase, 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, <i>Gordonia</i> sp. strain D7, <i>Pseudomonas</i> sp. strain D26	Biofilter: crushed wood and bark compost, 74% water content	Alexandrino et al. (2001)
Inoculated with activated sludge: <i>Tsukamurella</i> sp., <i>Pseudomonas</i> sp., <i>Sphingomonas</i> sp., <i>Xanthomonas</i> 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 <i>Exophiala jeanselmei</i>	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)
<i>Pseudomonas</i> sp. IS-3	Biofilter: cubic polyurethane, minimal salt medium supplied	Kim et al. (2005)
<i>Pseudomonas</i> sp. SR-5	Biofilter: peat and/or ceramic, nutrient solution supplied	Jang et al. (2004, 2006)
<i>Pseudomonas putida</i> ST201	Packed tower biofilter: ceramic	Okamoto et al. (2003)
<i>Rhodococcus pyridinovorans</i> PYJ-1	Biofilter: compost	Jung and Park (2005)
<i>Rhodococcus rhodochrous</i> AL NCIMB 13259	Biofilter: peat and glass beads, water content and pH regulated	Zilli et al. (2003)
<i>Sporothrix variecibatus</i>	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.

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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 twentieth 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 ($\sim 350\text{--}700\text{ nm}$) 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 , $\text{--SO}_3\text{H}$, --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

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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 vicinity 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 (TiO₂, ZnO₂, 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 physicochemical 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 Veerarahavan 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 Wuhmann 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 decolorization. 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 decolorisation 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/Microaerophilic Conditions

By definition, anoxic condition refers to the absence of oxygen, where as microaerophilic 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*, *Rhodospseudomonas* and other bacterial genera decolorized azo dyes under microaerophilic 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 Mg²⁺, Ca²⁺ (1–10 mmol L⁻¹) and Pb²⁺ (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-aminobenzenesulfonate (4-ABS) degradation, however, required the additional presence of an unidentified strain, SAD4i (Coughlin et al. 2003). *Sphingomonas* 1CX 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.

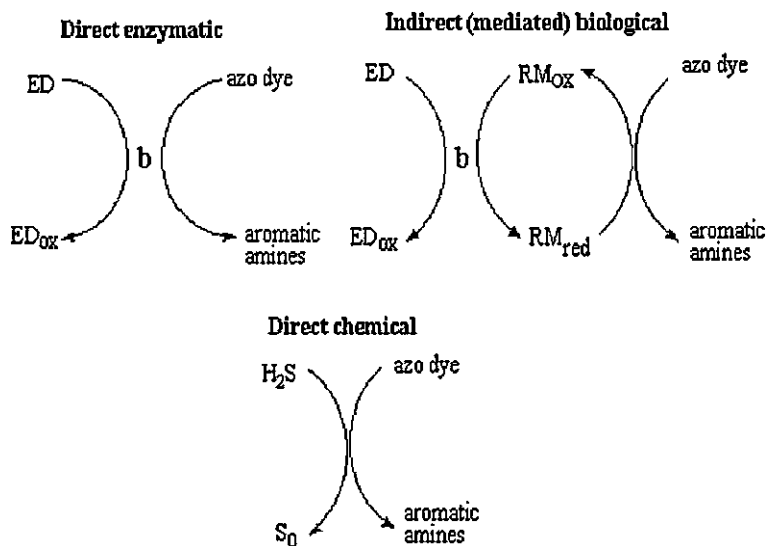


Fig. 4.1 Schematic representation of different mechanisms of anaerobic azo dye reduction. *RM* Redox mediator, *ED* Electron donor, *b* bacteria (enzymes). *Source* Pandey et al. (2007)

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 microaerophilic 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 azoreductases, 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 E_0' 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 steric 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 nanoparticles, 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 decolorization. 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 mordant 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 CH₄, CO₂, NH₄, 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 Orange 52 by methanogenic sludge has been reported by Yemashova et al. (2004). A complete bioreduction of all these azo dyes was observed in expanded granular 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-diethylphenylenediamine 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/Microaerophilic Conditions

There are a few reports on the degradation of aromatic amines under anoxic conditions. Drzyzga and Blotvogel (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 H₂S 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, *Rhodospseudomonas 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 cleavage 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 tricarboxylic 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 Lustigam 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 culture, *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 *Pseudomonas* 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

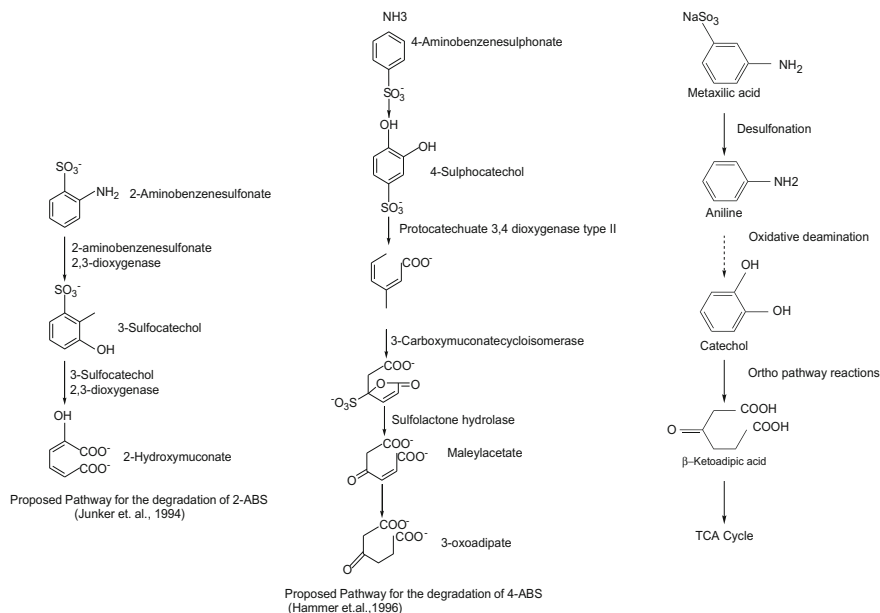


Fig. 4.2 Proposed pathways for the degradation of aminobenzene sulfonates (ABS)

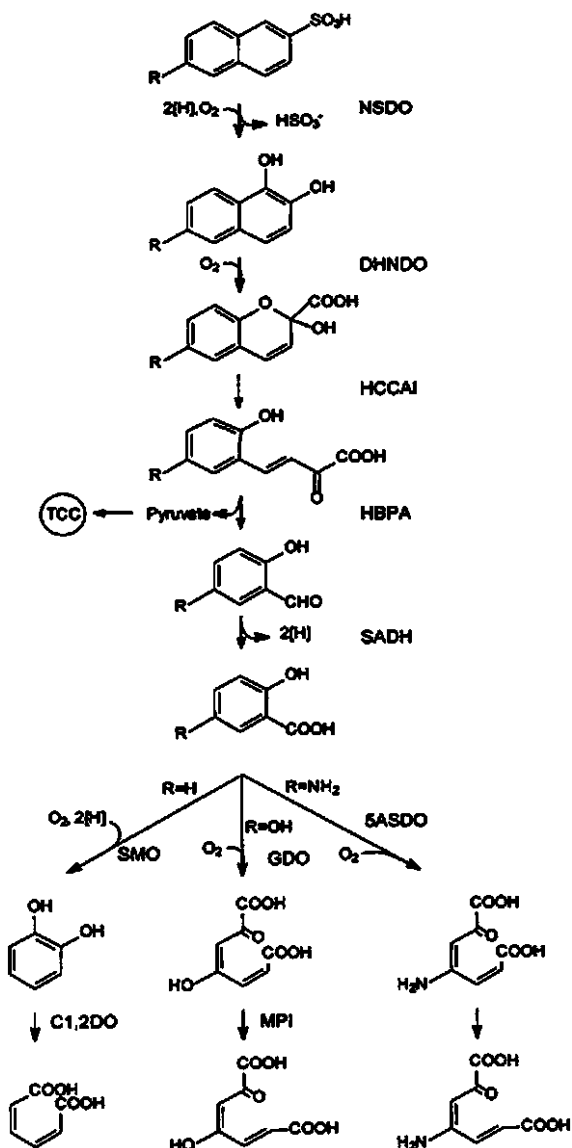
6A2NS (Kampfer et al. 1999). Available literature on the degradation of sulfonated aromatic amines (SAA) shows that cultures generally have a narrow substrate range. Mixtures of aromatic sulfonates can only be degraded by mixed bacterial consortia, which harbor diverse activities for congeneric substrates 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 stoichiometric quantities after dye reduction, indicating their degradation. However, metabolic 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 dioxygenase; *DHNDO* 1,2-dihydroxynaphthalene dioxygenase; *HCCAI* 2-hydroxychromene-2-carboxylate isomerase; *HBPA* 2'-hydroxybenzalpyruvate aldolase; *SADH* salicylaldehyde dehydrogenase; *SMO* salicylate 1-monoxygenase; *GDO* gentisate 1,2-dioxygenase; *5ASDO* 5-aminosalicylate 1,2-dioxygenase; *C1* 2DO catechol 1,2-dioxygenase; *MPI* maleylpyruvate isomerase; *TCC* tricarboxylic acid cycle. Source: Hintzer 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 bioassays, bacterial assays based on growth, enzyme inhibition etc., can be used to assess the toxicity of azo dye prior to and after decolorization and degradation. A few recent references on bacterial decolorization, 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
<i>Staphylococcus arlettae</i>	RY107, RR198, RB5, DB71	Microaerophilic-aerobic	Toxicity studies	Elisangela et al. (2009a)
<i>Klebsiella</i> sp.	RY107, RR198, DB71	Microaerophilic-aerobic		Elisangela et al. (2009b)
<i>Pseudomonas aeruginosa</i>	Remazol Orange	Microaerophilic	Degradation pathway proposed	Sarayu and Sandhya (2010)
<i>Pseudomonas, Arthrobacter, and Rhizobium</i>	AO7	Aerobic	Batch and continuously operated multistage packed-bed BAC reactor	Ruiz-Arias et al. (2010)
<i>Cardiobacterium homini</i> and <i>Pseudomonas stutzeri</i>	Direct black 38	Anoxic	Release of benzidine and its subsequent degradation to aminobiphenyl	Bafana et al. (2007)
<i>Bacillus velezensis</i>	Direct Red 28	Aerobic	Azoreductase isolation and toxicity studies	Bafana and Chakrabarti (2008)
<i>Pseudomonas putida</i> mt-2	Acid yellow 17	Aerobic	Degradation of amines	Mansour et al. (2009)
<i>Shewanella putrefaciens</i> strain AS96	Four azo dyes	Microaerophilic-aerobic	Toxicity studies	
<i>Exiguobacterium</i> sp.	X-3B	Anaerobic	Amine degradation	Khalid et al. (2008a, b)
<i>Galactomyces geotrichum</i>	Methyl red	Aerobic	Tolerant to high saline conditions	
<i>Micrococcus glutamicus</i> and <i>Proteus vulgaris</i>	Sulfonated reactive dye	Aerobic	Salt tolerant bacteria	Tan et al. (2009a, b)
<i>Halomonas</i> sp. strain GTW	Green HE4B and other dyes	Anoxic	Effect of red-ox mediator	
	Five azo dyes		Induction of various enzymes	Jadhav et al. (2008)
			Phytotoxicity studies	
			Phytotoxicity and microbial toxicity studies. Table on the performance of various microbial consortia	Saratale et al. (2010, 2011)
			Salt tolerant	Guo et al. (2007)

(continued)

Table 4.1 (continued)

Bacteria	Dye	Red-ox condition	Comments	References
<i>Pseudomonas desmolyticum</i>	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	Ayed et al. (2010)
<i>Rhodopseudomonas palustris</i> W1	Reactive Black 5	Anaerobic	Phytotoxicity studies 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 m³/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.

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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

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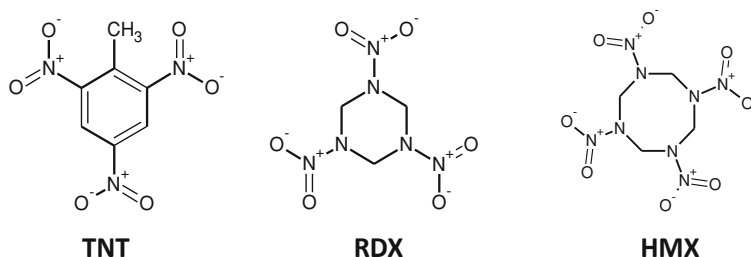


Fig. 5.1 Molecular structures of TNT, RDX and HMX

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 Spangord 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 ($\mu\text{g/l}$)
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–NO₂ 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

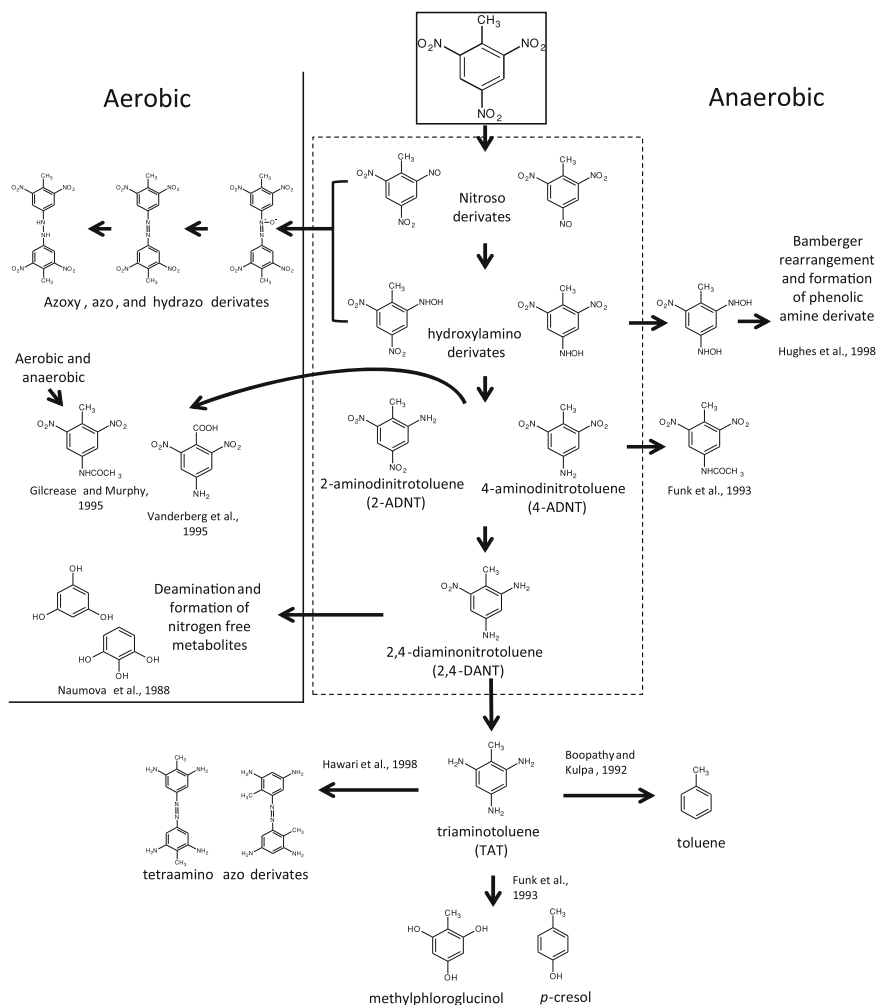


Fig. 5.2 Reduction pathway of TNT, and further transformation of the reduced derivatives under aerobic and anaerobic conditions

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
<i>Acinetobacter johnsonii</i>	Aerobic	Pathway was not determined	Fuller and Manning (1997)
<i>A. junii</i> 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)
<i>Agrobacterium</i> sp. 2PC	Aerobic	Pathway was not determined	Fuller and Manning (1997)
<i>Alcaligenes eutrophus</i>	Aerobic	Pathway was not determined	Fuller and Manning (1997)
<i>Anabaena</i> sp.		Detection of azoxy-tetranitrotoluene isomers and hydroxylaminodinitrotoluene	Pavlostathis and Jackson (1999)
<i>Arthrobacter globiformis</i>	Aerobic	Pathway was not determined	Fuller and Manning (1997)
<i>Arthrobacter</i> sp. RP17	Aerobic	Pathway was not determined	Fuller and Manning (1997)
<i>Bacillus cereus</i>	Aerobic	Pathway was not determined	Fuller and Manning (1997)
<i>Bacillus</i> sp.	Aerobic	(1) Release of nitrite, with final formation of toluene; (2) sequential reduction to the final formation of TAT	Kalafut et al. (1998)
<i>B. subtilis</i>	Aerobic	Pathway was not determined	Fuller and Manning (1997)
<i>Cellulomonas</i> sp. ES6	Anaerobic	Amino and hydroxylamino derivatives	Borch et al. (2005)
<i>Clostridium acetobutylicum</i>	Anaerobic	Degradation via Bamberger rearrangement of 2,4-diHA-6-nitrotoluene to either 2-AM-4-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)
<i>C. acetobutylicum</i>	Anaerobic	(1) Partial reduction by Bamberger rearrangement to HA intermediate; (2) TNT-TAT	Khan et al. (1997), Hughes et al. (1998)
<i>C. bifermentans</i>	Anaerobic	Detection of TAT, phenolic products of TAT hydrolysis, and TAT and pyruvic aldehyde condensation products	Lewis et al. (1996)
<i>C. bifermentans</i>	Anaerobic	Reductive TNT transformations to form TAT and phenolic products of TAT hydrolysis	Regan and Crawford (1994), Lewis et al. (1996)
<i>C. bifermentans</i> ATCC 638	Anaerobic	Reductive pathway	Ederer et al. (1997)
<i>C. bifermentans</i> KMR-1	Anaerobic	Reductive pathway	Ederer et al. (1997)

(continued)

Table 5.3 (continued)

Isolate	Conditions	Suggested pathway	Reference
<i>C. pasteurianum</i> DSM 525	Anaerobic	Reductive TNT transformations to form TAT and further transformation of TAT to undefined products	Preuss et al. (1993)
<i>C. sordellii</i>	Anaerobic	Reductive pathway	Ederer et al. (1997)
<i>C. sporogenes</i>	Anaerobic	Reductive pathway	Ederer et al. (1997)
<i>C. thermoacetium</i>	Anaerobic	Reductive pathway	Huang et al. (2000)
<i>Corynebacterium glutamicum</i>	Aerobic	Pathway was not determined	Fuller and Manning (1997)
<i>Corynebacterium</i> sp. Nap2	Aerobic	Pathway was not determined	Fuller and Manning (1997)
<i>Cytophaga pectinovora</i>	Aerobic	Pathway was not determined	Fuller and Manning (1997)
<i>Desulfotribrio gigas</i>	Anaerobic	Pathway was not determined	Boopathy and Manning (1996)
<i>D. indolicum</i>	Anaerobic	Pathway was not determined	Boopathy et al. (1997)
<i>Desulfotribrio</i> sp.	Anaerobic	Detection of TNT, DANT and TAT isomers	Drzyzga et al. (1999)
<i>Desulfotribrio</i> 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)
<i>D. vulgaris</i>	Anaerobic	Reduction to hydride-Meisenheimer complex with release of nitrite	Boopathy and Kulpa (1994)
<i>Enterobacter cloacae</i> PB2	Aerobic	Reduction to hydride-Meisenheimer complex with release of nitrite	French et al. (1998)
<i>Escherichia coli</i>	Aerobic	Pathway was not determined	Fuller and Manning (1997)
<i>E. coli</i>	Anaerobic	Reductive pathway	Ederer et al. (1997)
<i>Flavobacterium odoratum</i>	Aerobic	Pathway was not determined	Fuller and Manning (1997)
<i>Klebsiella</i> sp. IPC	Aerobic	Pathway was not determined	Fuller and Manning (1997)
<i>Klebsiella</i> sp. C1	Anaerobic	Reduced to hydroxylamino dinitrotoluenes, aminodinitrotoluenes and to nitrite via denitration	Kim et al. (2002)
<i>Lactobacillus acidophilus</i>	Anaerobic	Reductive pathway	Ederer et al. (1997)
<i>L. casei</i>	Anaerobic	Reductive pathway	Ederer et al. (1997)
<i>L. lactis</i>	Anaerobic	Reductive pathway	Ederer et al. (1997)
<i>Methanococcus deltae</i>	Anaerobic	Reductive pathway	Boopathy (1994)
<i>Methanococcus</i> sp. strain B	Anaerobic	Reductive pathway	Boopathy (1994)

(continued)

Table 5.3 (continued)

Isolate	Conditions	Suggested pathway	Reference
<i>M. thermolithotrophicus</i>			Boopathy (1994)
<i>Methylobacterium</i> sp. BJ001	Anaerobic	Reduced to ADNT	Van Aken et al. (2004)
<i>Micrococcus luteus</i>	Aerobic	Pathway was not determined	Fuller and Manning (1997)
<i>Mycobacterium</i> sp. strain HL 4-NT-1	Aerobic	Formation of a hydride-Meisenheimer complex	Vorbeck et al. (1994)
<i>M. vaccae</i> 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)
<i>Mycococcus xanthus</i>	Aerobic	Pathway was not determined	Fuller and Manning (1997)
<i>Nocardioles CB22-2</i>	Aerobic	Dinitration leads to formation of Meisenheimer complex	Behrend and Heesche-Wagner (1999)
<i>Pseudomonas</i>	Aerobic	Production of hydride-Meisenheimer complex and transformation to DNT, NT and toluene	Duque et al. (1993)
<i>P. aeruginosa</i>	Aerobic	(1) Release of nitrite, with final formation of toluene; (2) sequential reduction to the final formation of TAT	Kalafut et al. (1998)
<i>P. aeruginosa</i>	Aerobic	Detection of HA and AM derivatives	Oh et al. (2001)
<i>P. aeruginosa</i>	Aerobic	Pathway was not determined	Fuller and Manning (1997)
<i>P. aeruginosa</i> strain MA01	Aerobic	Oxidation of ADNT isomers to N(4)-acetyl-2,4-diAM-6-nitrotoluene and tetranitroazoxytoluene isomers	Alvarez et al. (1995)
<i>P. cepacia</i>	Aerobic	Pathway was not determined	Fuller and Manning (1997)
<i>P. fluorescens</i>	Aerobic	Reduction by addition of hydride to form dihydride	Pak et al. (2000)
<i>P. fluorescens</i>	Aerobic	Meisenheimer complex to catalyze reduction of nitro group	
<i>P. pseudocaligenes</i> JS52	Aerobic and anaerobic	Pathway was not determined	Fuller and Manning (1997)
<i>P. putida</i>	Aerobic	Detection of mono- and di-HADNTs, monoAM monoHA NT, and monoAM DNT	Fiorella and Spain (1997)
<i>P. putida</i> HK-6	Aerobic	Pathway was not determined	Fuller and Manning (1997)
	Aerobic	Reductive pathway	Cho et al. (2008)

(continued)

Table 5.3 (continued)

Isolate	Conditions	Suggested pathway	Reference
<i>P. putida</i> KP-T20 1	Aerobic	Amino derivatives of TNT, and denitrated products (DNT)	Park et al. (2003)
<i>Pseudomonas</i> 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	Hatidour and Ramos (1996)
<i>Pseudomonas</i> sp. DFC49	Aerobic	Pathway was not determined	Fuller and Manning (1997)
<i>Pseudomonas</i> 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)
<i>Pseudomonas</i> sp. JS150	Aerobic	Pathway was not determined	Fuller and Manning (1997)
<i>Pseudomonas</i> sp. Tol1A	Aerobic	Pathway was not determined	Fuller and Manning (1997)
<i>Rahnella aquitilis</i> BFB	Aerobic	Pathway was not determined	Fuller and Manning (1997)
<i>Raoultella terrigena</i> HB	Aerobic	AM derivatives and azoxy dimers	Claus et al. (2007)
<i>Rhodococcus erythropolis</i>	Aerobic	Dinitration leads to formation of Meisenheimer complex by F420 reductase and hydride transferase	Rieger et al. (1999)
<i>R. erythropolis</i>	Aerobic	Formation of hydride and dihydride TNT-Meisenheimer complexes	Vorbeck et al. (1998)
<i>R. erythropolis</i>	Aerobic	Pathway was not determined	Fuller and Manning (1997)
<i>R. globerulus</i>	Aerobic	Pathway was not determined	Fuller and Manning (1997)
<i>R. rhodocrous</i>	Aerobic	Pathway was not determined	Fuller and Manning (1997)
<i>Rhodococcus</i> sp. TF2	Aerobic	Pathway was not determined	Fuller and Manning (1997)
<i>Salmonella typhimurium</i>	Anaerobic	Reductive pathway	Ederer et al. (1997)
<i>Serratia marcescens</i>	Aerobic	Not defined. ADNT isomers were detected	Montpas et al. (1997)
SP1b (coryneform)	Aerobic	Pathway was not determined	Fuller and Manning (1997)
<i>Sphingomonas capsulata</i>	Aerobic	Pathway was not determined	Fuller and Manning (1997)
<i>Staphylococcus</i> 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
<i>Streptomyces albus</i>	Aerobic	Pathway was not determined	Fuller and Manning (1997)
<i>S. chromofuscus</i> A11	Aerobic		Pasti-Grigsby et al. (1996)
<i>S. griseus</i>	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)
<i>Veillonella alkalescens</i>			McCormick et al. (1976)
<i>Yarrowia lipolytica</i> 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, *DANT* aminonitrotoluene, *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 deamination (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

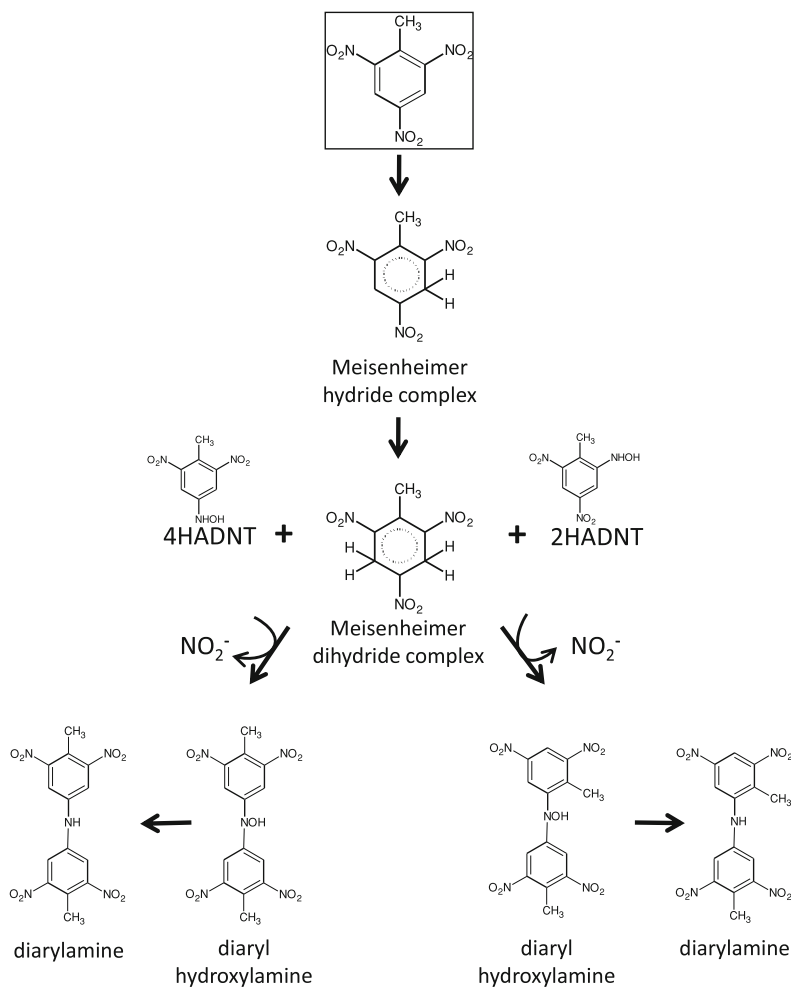


Fig. 5.3 The aerobic release of nitrite following the dimerization of dihydride complex with HADNTs

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.

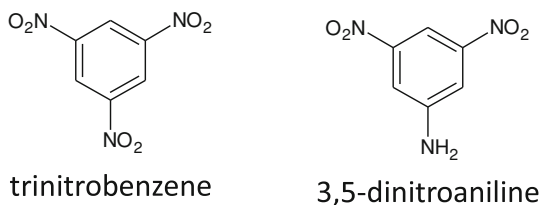
Anaerobic Denitration

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).

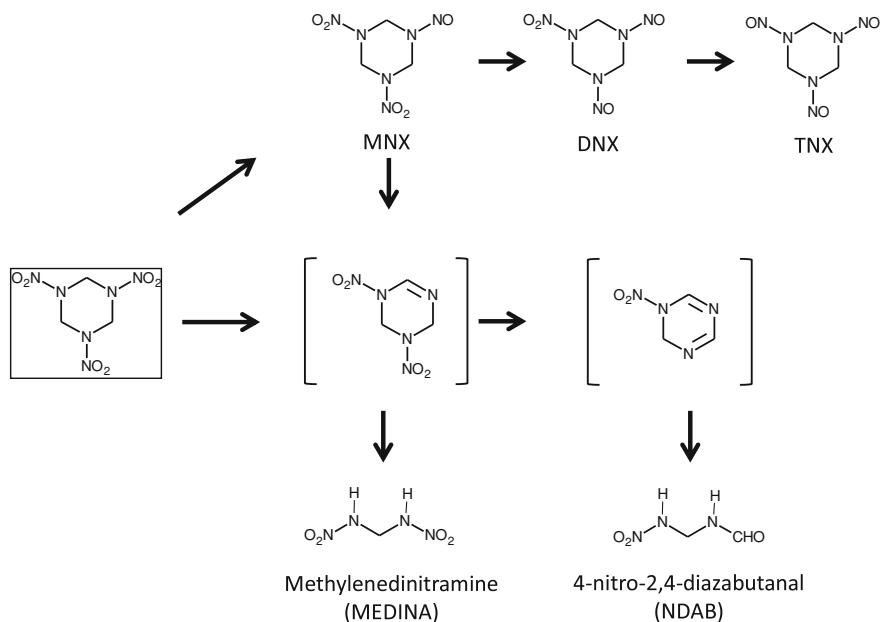


Fig. 5.5 The common degradation pathways of RDX. Compounds in *brackets* are postulated intermediates

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
<i>Bacillus</i> strains HPB2, HPB3	Denitrifying	Pathway not defined, products not identified	Singh et al. (2009)
<i>Acetobacterium malicum</i> strain HAAP-1	Anaerobic	Denitration of RDX and MNX followed by ring cleavage. Detection of MEDINA	Adrian and Arnett (2004)
<i>A. paludosum</i>	Anaerobic	Ring cleavage of RDX; nitroso derivatives were not detected. Other intermediates were not identified	Sherburne et al. (2005)
<i>Citrobacter freundii</i>	Anaerobic	Sequential reduction. Detection of MNX, DNX, TNX	Kitts et al. (1994)
<i>C. freundii</i> NS2	Anaerobic	Sequential reduction. Detection of MNX, DNX, TNX	Kitts et al. (1994)
<i>Clostridium acetobutylicum</i>	Anaerobic	Reduction and formation of amino products. Detection of mononitroso-, monohydroxylamino-, mononitrosomonohydroxylamino-, monoamino-, diamino-, and triamino-compounds	Zhang and Hughes (2003)
<i>C. bifermentans</i> HAW-1	Anaerobic	(1) Reduction to MNX; (2) MNX denitration followed by ring cleavage	Zhao et al. (2003a, b)
<i>Clostridium</i> sp. EDB2	Anaerobic	Denitration pathway. Products were not reported	Bhushan et al. (2004)
<i>Clostridium</i> 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)
<i>Clostridium</i> sp. HAW-E3	Anaerobic	(1) Reduction to MNX; (2) MNX denitration followed by ring cleavage	Zhao et al. (2003b)
<i>Clostridium</i> 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)
<i>Clostridium</i> sp. HAW-G4	Anaerobic	(1) Reduction to MNX; (2) MNX denitration followed by ring cleavage	Zhao et al. (2003b)
<i>Clostridium</i> 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
<i>Desulfotribrio</i> 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)
<i>Desulfotribrio</i> 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)
<i>Enterobacter cloacae</i>	Anaerobic	Sequential reduction. Detection of MNX, DNX, TNX	Kitts et al. (2000), Pudge et al. (2003)
<i>Fusobacteria</i> 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)
<i>Geobacter metallireducens</i> strain GS-15	Anaerobic	Reduction to MNX, RDX and MNX denitration and ring cleavage. Detection of MEDINA	Kwon and Finneran (2008)
<i>Gordonia</i> sp. KTR9	Aerobic	Pathway not defined, products not identified	Thompson et al. (2005)
<i>Gordonia</i> strain YY1	Aerobic	RDX denitration and ring cleavage. Detection of NDAB	Ronen et al. (2008)
<i>Klebsiella pneumoniae</i> SCZ1	Anaerobic	(1) Reduction to MNX; (2) MNX denitration followed by ring cleavage	Zhao et al. (2002)
<i>Methylobacterium extorquens</i>	Aerobic	Reduction to MNX followed by denitration and ring-cleavage. Detection of MNX, MEDINA	Van Aken et al. (2004)
<i>M. organophilum</i>	Aerobic	Reduction to MNX followed by denitration and ring-cleavage. Detection of MNX, MEDINA	Van Aken et al. (2004)
<i>M. rhodesianum</i>	Aerobic	Reduction to MNX followed by denitration and ring-cleavage. Detection of MNX, MEDINA	Van Aken et al. (2004)
<i>Methylobacterium</i> sp. BJ001	Aerobic	Reduction to MNX followed by denitration and ring-cleavage. Detection of MNX, MEDINA	Van Aken et al. (2004)
<i>Morganella morganii</i> B2	Anaerobic	Sequential reduction. Detection of MNX, DNX, TNX	Kitts et al. (1994)
<i>M. morganii</i>	Anaerobic	Sequential reduction. Detection of MNX, DNX, TNX	Kitts et al. (1994)

(continued)

Table 5.4 (continued)

Isolate	Conditions	Suggested pathway, indicative intermediates	Reference
<i>Providencia rettgeri</i> B1	Anaerobic	Sequential reduction. Detection of MNX, DNX, TNX	Kitts et al. (1994)
<i>P. rettgeri</i>	Anaerobic	Sequential reduction. Detection of MNX, DNX, TNX	Kitts et al. (1994)
<i>Pseudomonas putida</i> HK-6	Aerobic	Pathway not defined, products not identified	Cho et al. (2008)
<i>Pseudomonas</i> strain HPB1	Denitrifying	Pathway not defined, products not identified	Singh et al. (2009)
<i>Rhodococcus rhodochrous</i> sp. 11Y	Aerobic	Two denitration steps followed by ring cleavage. Detection of NDAB	Seth-Smith et al. (2002)
<i>Rhodococcus</i> 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)
<i>Rhodococcus</i> sp. YH1	Aerobic	Two denitration steps followed by ring cleavage. Detection of NDAB	Nejjidat et al. (2008)
<i>Serratia marcescens</i>	Anaerobic	Sequential reduction. Detection of MNX, DNX, TNX	Young et al. (1997)
<i>Shewanella halifaxensis</i> HAW-EB4	Anaerobic	Sequential reduction. Detection of MNX, DNX, TNX	Zhao et al. (2004a, 2006)
<i>S. sediminis</i> HAW-EB3	Anaerobic	Sequential reduction. Detection of MNX, DNX, TNX	Zhao et al. (2004a, 2005)
<i>Shewanella</i> 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)
<i>Stenotrophomonas maltophilia</i> PB1	Aerobic	Pathway not determined, detection of methylene-N-(hydroxymethyl)-hydroxylamine-N'-(hydroxymethyl)nitroamine	Binks et al. (1995)
<i>Williamsia</i> 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 (Nejdat 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 *Rhodococcus* strains worldwide and since it was absent in *Rhodococcus* 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 ($\text{RDX}^{\bullet-}$) 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 (Bleher 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 H₂ 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

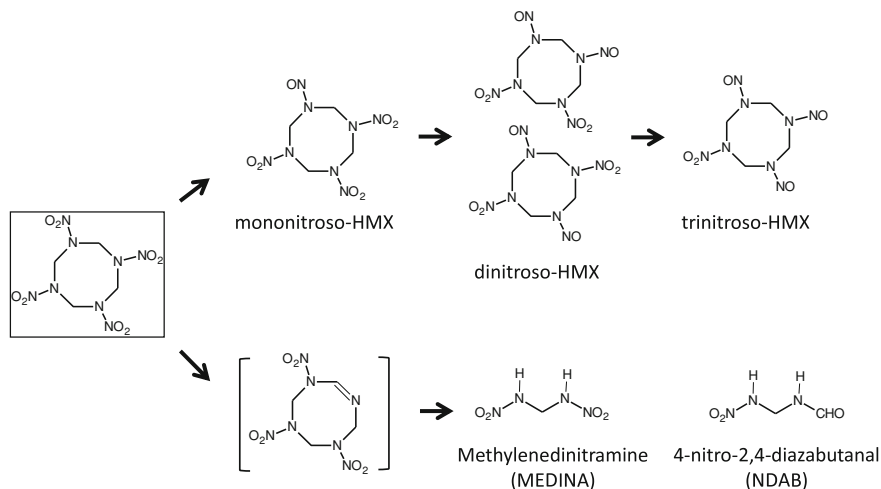


Fig. 5.6 Postulated degradation pathways of HMX. Compounds in *brackets* are postulated intermediates

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
<i>Bacillus</i> strain HPB2, strain HPB3	Denitrifying	Pathway not defined, products not identified	Singh et al. (2009)
<i>Citrobacter freundii</i> NS2	Anaerobic	Sequential reduction. Detection of mono- and dinitroso HMX	Kitts et al. (1994)
<i>Clostridiale</i> strain UXO5-19	Anaerobic	(1) Denitration followed by ring cleavage; (2) reduction of one or more nitro groups. Detection of monitroso HMX, MEDINA	Zhao et al. (2007)
<i>Clostridium bifermentans</i> HAW-1	Anaerobic	(1) Sequential reduction; (2) denitration	Zhao et al. (2004b)
<i>Clostridium</i> sp. EDB2	Anaerobic	Pathway not defined, Products not identified	Bhushan et al. (2004)
<i>Clostridium</i> sp. HAW-E3	Anaerobic	(1) Sequential reduction; (2) denitration	Zhao et al. (2004b)
<i>Clostridium</i> sp. HAW-EB17	Anaerobic	Pathway not defined	Zhao et al. (2004b)
<i>Clostridium</i> sp. HAW-G4	Anaerobic	(1) Sequential reduction; (2) denitration	Zhao et al. (2004b)
<i>Clostridium</i> sp. HAW-HC1	Anaerobic	(1) Sequential reduction; (2) denitration. Detection of mono-, di- and trinitroso HMX	Zhao et al. (2004b)
<i>Desulfovibrio</i> sp. HAW-EB18	Anaerobic	Pathway not defined. Detection of monitroso HMX	Zhao et al. (2004b)
<i>Desulfovibrio</i> strain Midref-29	Anaerobic	(1) Denitration followed by ring cleavage; (2) reduction of one or more nitro groups. Detection of monitroso HMX, MEDINA	Zhao et al. (2007)
<i>Fusobacteria</i> isolate HAW-EB21	Anaerobic	Pathway not defined. Detection of monitroso HMX	Zhao et al. (2004b)
<i>Klebsiella pneumoniae</i> SCZ1	Anaerobic	(1) Sequential reduction; (2) denitration. Detection of monitroso HMX, MEDINA	Zhao et al. (2004b)
<i>Methylobacterium extorquens</i>	Aerobic	Pathway not defined, products not identified	Van Aken et al. (2004)
<i>M. organophilum</i>	Aerobic	Pathway not defined, products not identified	Van Aken et al. (2004)
<i>M. rhodesianum</i>	Aerobic	Pathway not defined, products not identified	Van Aken et al. (2004)
<i>Methylobacterium</i> sp. BJ001	Aerobic	Reduction	Van Aken et al. (2004)
<i>Morganella morganii</i> 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
<i>Paenibacillus</i> 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)
<i>Providencia rettgeri</i> B1	Anaerobic	Sequential reduction. Detection of mono- and dinitroso HMX	Kitts et al. (1994)
<i>Pseudomonas</i> strain HPB1	Denitrifying	Pathway not defined, products not identified	Singh et al. (2009)
<i>Pseudomonas</i> 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; Nejjidat 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 $\delta^{15}\text{N}$ 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 $\delta^2\text{H}$ 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 $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ 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 $\delta^{15}\text{N}$ 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 $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ 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 $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ 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.

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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

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Table 6.1 Examples of fungal strains degrading aliphatic compounds

Fungal species	Substrate	Reference
<i>Paecilomyces variotii</i>	Methanol	Sakaguchi et al. (1975)
<i>Gliocladium deliquescens</i>	Methanol	Sakaguchi et al. (1975)
<i>Paecilomyces variotii</i>	Formaldehyde	Sakaguchi et al. (1975)
<i>Gliocladium deliquescens</i>	Formaldehyde	Sakaguchi et al. (1975)
<i>Acremonium</i> sp.	C ₂ –C ₄ Alkanes	Davies et al. (1973)
<i>Cladosporium resinae</i>	C ₆ –C ₁₉ Alkanes	Cofone et al. (1973); Lindley and Heydeman (1983)
<i>Scedosporium</i> sp.	C ₁ –C ₉ Alkanes	Onodera et al. (1989)
<i>Graphium</i> sp.	<i>n</i> -Butane	Hardison et al. (1997)
<i>Trichosporon veenshuisii</i>	<i>n</i> -Hexadecane	Middelhoven et al. (2000)
<i>Graphium</i> sp.	Di-ethyl-ether	Hardison et al. (1997)
<i>Graphium</i> sp.	Methyl- <i>tert</i> -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 CO₂ 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 peroxidase-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 ligninolytic 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

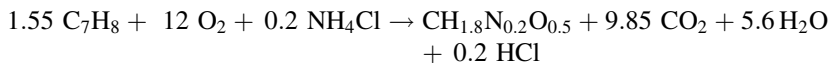
Although studies on the biodegradation of oxygenated aromatic compounds by fungi were already published more than 40 years ago (Neujahr and Varga 1970), much less research was done at that time on the non-oxygenated mono-aromatic pollutants, such as the benzene-compounds. Most of the research on oxygenated 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
<i>Trichosporon cutaneum</i>	Phenol	Neujahr and Varga (1970)
<i>Aspergillus japonicus</i>	Phenol	Milstein et al. (1983)
<i>Penicillium</i> spp	Phenol	Scow et al. (1990); Hofrichter et al. (1993)
<i>Phanerochaete chrysosporium</i>	Phenol	Kennes and Lema (1994b)
<i>Trichosporon guehoae</i>	Phenol	Middelhoven et al. (1999)
<i>Trichosporon veenhuisii</i>	Phenol	Middelhoven et al. (2000)
<i>Trichosporon cutaneum</i>	Fluorinated phenols	Peelen et al. (1995)
<i>Trichosporon cutaneum</i>	<i>o</i> -, <i>m</i> -, <i>p</i> -cresols	Hasegawa et al. (1990)
<i>Aspergillus fumigatus</i>	<i>p</i> -cresol	Jones et al. (1993)
<i>Phanerochaete chrysosporium</i>	<i>p</i> -cresol	Kennes and Lema (1994b)
<i>Penicillium frequentans</i>	<i>o</i> -cresol	Hofrichter et al. (1995)
<i>Trichosporon guehoae</i>	<i>m</i> -cresol	Middelhoven et al. (1999)
<i>Trichosporon veenhuisii</i>	<i>m</i> -cresol	Middelhoven 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 C₄ or even C₈ or C₉, 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 CO₂ per mole toluene degraded (Estévez et al. 2005a) taking biomass growth into account, and with ammonium chloride as nitrogen source:



Weber et al. (1995) reported that about 220 $\mu\text{moles CO}_2$ was generated from 60 $\mu\text{moles toluene}$ 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 $\text{nmol}/\text{min}.\text{mg}$ 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 sphaerospermum*, *Exophialalecanii-corni*, *Phanerochaete chrysosporium*, or *Paecilomyces variotii* (Qi et al. 2002; García-Peña

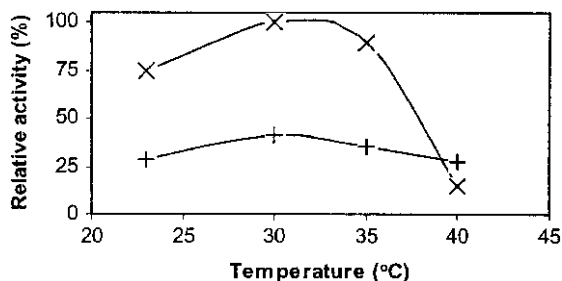
Table 6.3 Fungal strains degrading non-oxygenated mono-aromatic compounds

Fungal species	Substrate	Reference
<i>Cladosporium sphaerospermum</i>	Benzene	Qi et al. (2002)
<i>Exophiala lecanii-corni</i>	Benzene	Qi et al. (2002)
<i>Phanerochaete chrysosporium</i>	Benzene	Qi et al. (2002)
<i>Cladosporium resinae</i>	Ethylbenzene	Qi et al. (2002)
<i>Cladosporium sphaerospermum</i>	Ethylbenzene	Qi et al. (2002)
<i>Exophiala lecanii-corni</i>	Ethylbenzene	Qi et al. (2002)
<i>Mucor rouxii</i>	Ethylbenzene	Qi et al. (2002)
<i>Cladophialophora</i> sp.	Ethylbenzene	Prenafeta-Boldú et al. (2002)
<i>Ophiostoma stenoceras</i>	α -Pinene	Jin et al. (2006)
<i>Exophiala jeanselmei</i>	Styrene	Cox et al. (1993)
<i>Cladosporium sphaerospermum</i>	Styrene	Qi et al. (2002)
<i>Exophiala lecanii-corni</i>	Styrene	Qi et al. (2002)
<i>Sporothrix varicibatus</i>	Styrene	Rene et al. (2010a, b)
<i>Cladosporium sphaerospermum</i>	Toluene	Weber et al. (1995)
<i>Cladosporium sphaerospermum</i>	Toluene	Prenafeta-Boldú et al. (2001b)
<i>Cladophialophora</i> sp.	Toluene	Prenafeta-Boldú et al. (2001b)
<i>Pseudeurotium zonatum</i>	Toluene	Prenafeta-Boldú et al. (2001b)
<i>Exophiala</i> sp.	Toluene	Prenafeta-Boldú et al. (2001b)
<i>Leptodontium</i> sp.	Toluene	Prenafeta-Boldú et al. (2001b)
<i>Exophiala oligosperma</i>	Toluene	Estévez et al. (2005b)
<i>Paecilomyces variotii</i>	Toluene	Estévez et al. (2005b)
<i>Cladophialophora</i> sp.	<i>o</i> -Xylene	Nikolova and Nenov (2005)
<i>Cladophialophora</i> sp.	<i>m</i> -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 *Paecilomyces 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)

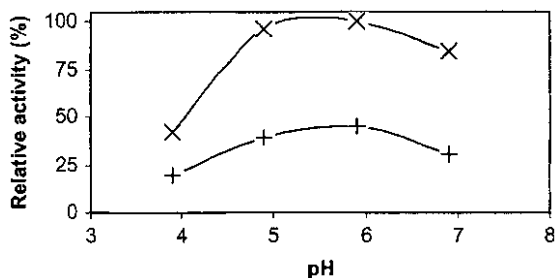
6.4.3 Influence of Environmental Conditions on the Fungal Biodegradation of Non-Oxygenated Mono-Aromatic Compounds

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 *Paecilomyces 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).

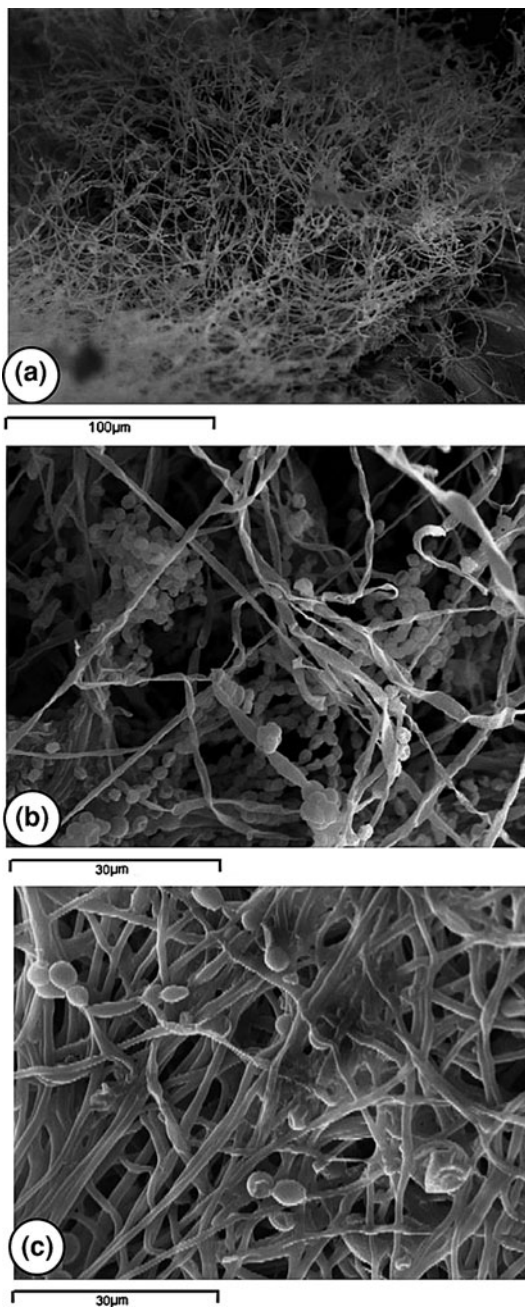
Fungi have often been inoculated in gas-phase biofilters for the treatment of air polluted with volatile benzene-related compounds (Kennes and Veiga 2004). Such packed-bed bioreactors are open systems and a mixed microbial community may often develop in the long-run. In all cases, simple microscopic 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.

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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 $\mu\text{g}/\text{kg}$. Air-borne PAHs originating from incomplete combustion of organic materials can give rise to high PAH levels in the atmosphere ($60 \mu\text{g}/\text{m}^3$ – $3 \text{mg}/\text{m}^3$ 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

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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

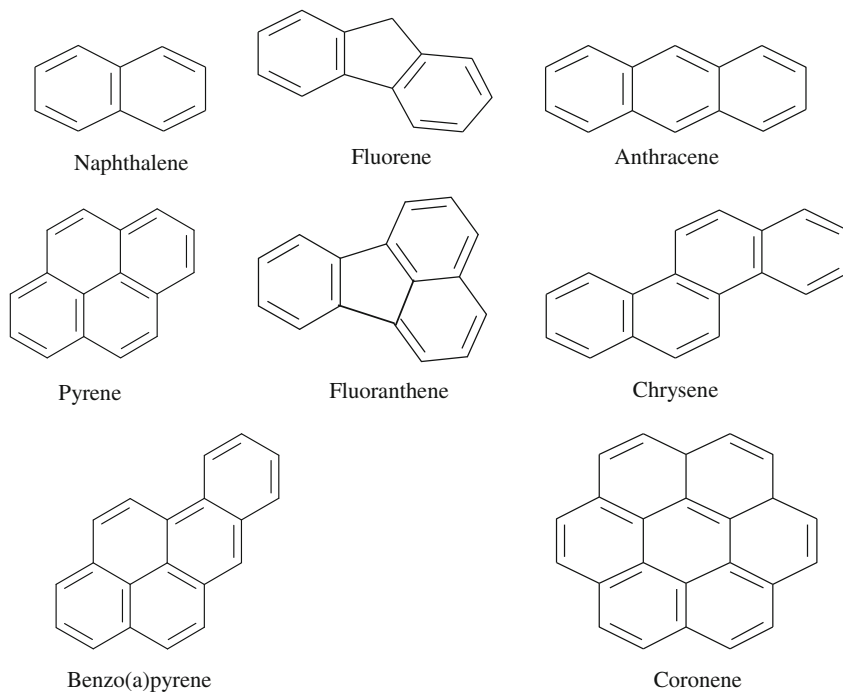
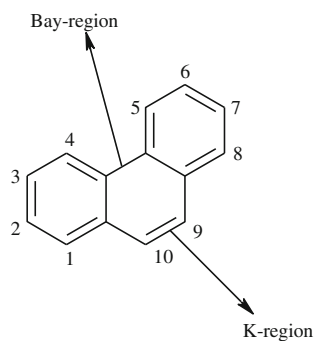


Fig. 7.1 Chemical structure of some polynuclear aromatic hydrocarbons

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 (Juhász et al. 2000). The highly hydrophobic and lipophilic

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 (LD₅₀, mg/Kg-Body weight) in animal assays. LD₅₀ 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 LD₅₀ value for anthracene, fluoranthene, pyrene and benzo(a)pyrene (BaP) are > 450, 100, 514 and 232 mg/Kg, respectively, based on mice bioassays with intraperitoneal 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 CO₂ 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 CO₂. A metabolite of molecular formula C₁₆H₁₀O₄ 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
<i>Rhodococcus</i> sp. UW1	ND	ND	ND	0.08	72% (500 mg/L)	14	Walter et al. (1991)
<i>M. flavescens</i> ATCC 700033	ND	ND	ND	0.806	38.8% (50 mg/L)	14	Dean-Ross and Cerniglia (1996)
<i>Mycobacterium</i> sp. KR2	ND	ND	ND	0.037	60% (500 mg/L)	8	Rehmann et al. (1998)
<i>Pseudomonas</i> sp. strain LP1	0.018	ND	ND	0.159	68% (100 mg/L)	30	Obayori et al. (2008)
<i>P. aeruginosa</i> LP5	0.024	ND	ND	0.118	67% (100 mg/L)	30	Obayori et al. (2008)
<i>P. aeruginosa</i> 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.

$$F_{max} = 0.003(K_{ow}C_{aq}/MW^{0.5}) \quad (7.1)$$

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. Efroymsen 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 *Acinetobacter*. 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 mucocidal (M) and non-mucocidal (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 1 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 1. 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.

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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

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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 Reki 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

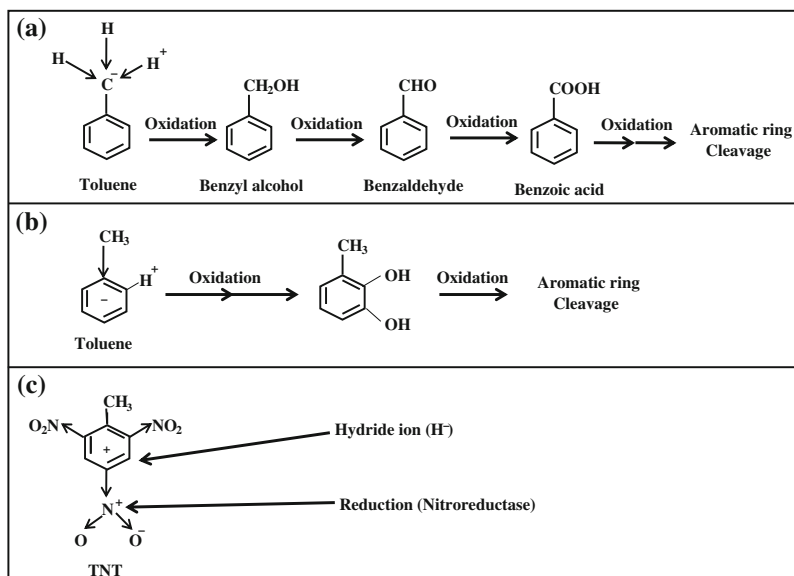


Fig. 8.1 Difference of the electron state within a molecule of toluene (a and b) and 2,4,6-trinitrotoluene (TNT) (c). By the electron-releasing effect of methyl group, the carbon atom at the methyl group of toluene is apparently with electron-rich nature. Therefore, oxidation reaction is more likely 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; Spangord 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 aminodinitrotoluenes (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 preferred 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

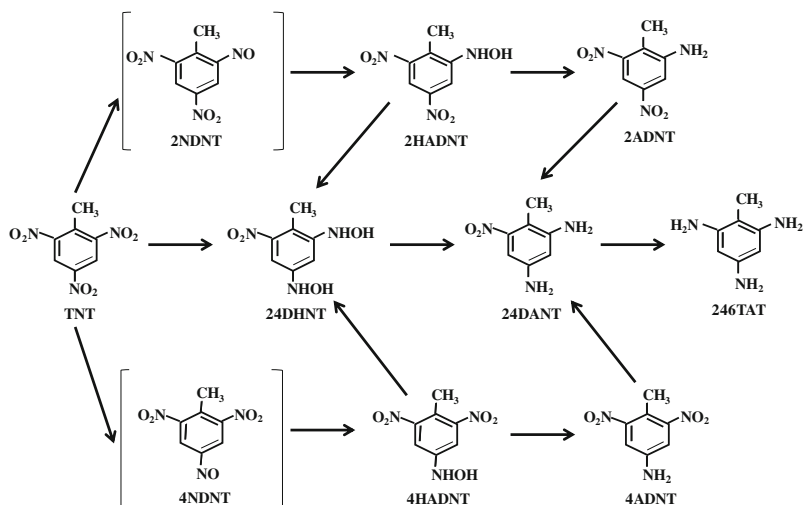


Fig. 8.2 Reductive pathway of TNT by microorganisms. TNT is commonly biotransformed by converting nitro groups into nitroso, hydroxylamino and amino groups by mostly the enzymatic reaction of nitroreductase. The major reduction metabolites from TNT are 2-hydroxylamino-4,6-dinitrotoluene (2HADNT), 4-hydroxylamino-2,6-dinitrotoluene (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 (4NDNT) 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;

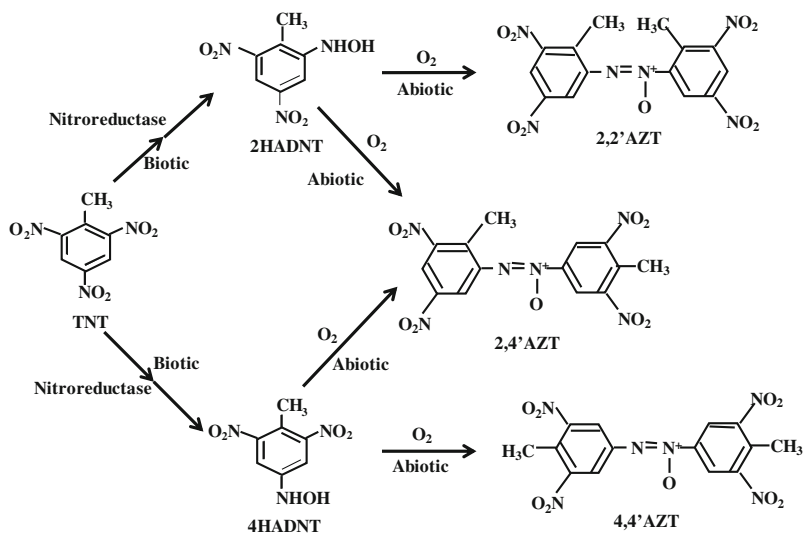


Fig. 8.3 Abiotic degradation of hydroxylamino-dinitrotoluenes (HADNTs). 2HADNT and 4HADNT which are unstable compounds are spontaneously destroyed under an oxic condition; thereby, 4,4',6,6'-Tetranitro-2,2'-azoxytoluene (2,2'AZT), 2,2',6,6'-tetranitro-4,4'-azoxytoluene, and 4,2',6,6'-tetranitro-2,4'-azoxytoluene (2,4'AZT) are produced from a mixture of 2HADNT and 4HADNT

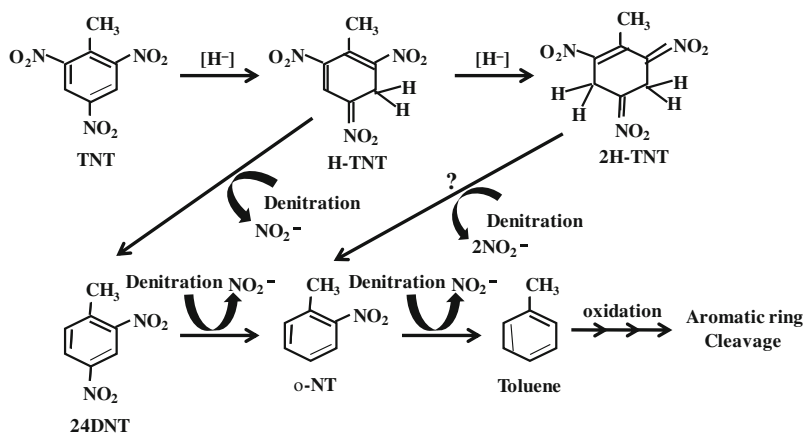


Fig. 8.4 Elimination pathway of nitro group from TNT. Denitration of TNT can occur through hydrogenation of aromatic ring via a Meisenheimer complex. The hydride-Misenheimer complex (H-TNT) is subsequently denitrated to 2,4-dinitrotoluene (24DNT); as a result, nitrite ions are released through the reaction. 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

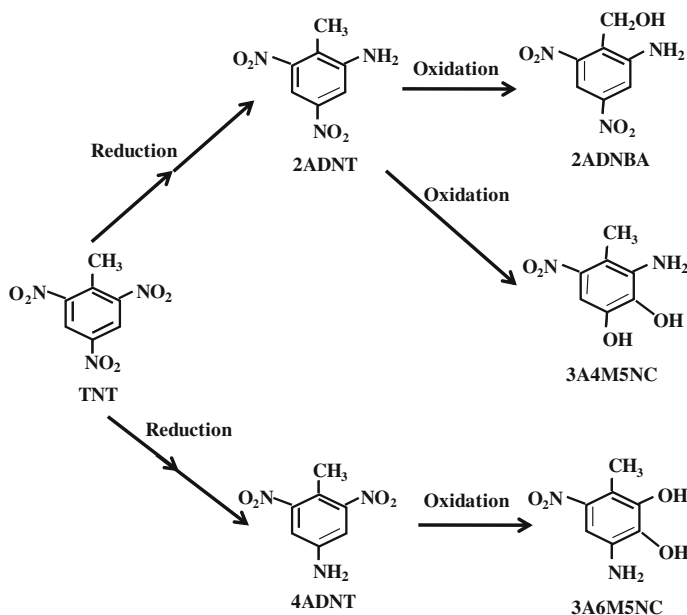


Fig. 8.5 Oxidative pathway for TNT metabolites. Unlike TNT which is relatively-resistant to oxidative reaction, the TNT metabolites such as 2ADNT and 4ADNT are possible to be oxidized by several dioxygenases. The oxidation of 2ADNT by the 24DNT dioxygenase from *Burkholderia* sp. produces 3-amino-4-methyl-5-nitrocatechol (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 fischerii* (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 H₂-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/CO₂ 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 NemaA 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 complexes 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 mutagenesis 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 Reikik 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 (Marozzi 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 without 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-1 α , 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 mutation-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 the XylR). 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.

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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

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biotransformation pathways. Biodegradation studies on these energetic chemicals under both aerobic and anaerobic conditions have been discussed.

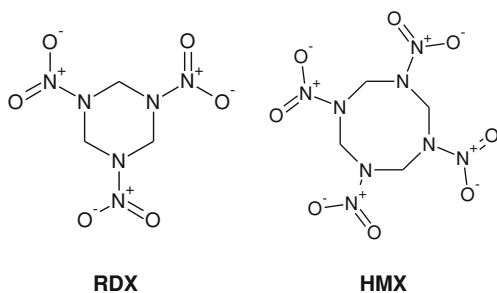
9.1.1 Structure and Properties of RDX and HMX

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, $[(\text{CH}_2\text{NNO}_2)_n]$; $n = 3$ 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 $\text{C}_3\text{H}_6\text{N}_6\text{O}_6$ 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–NO₂ 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 (N₂O, NO₂, NH₃) and small carbon-containing (HCHO, HCOOH and CO₂) 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 $\text{CH}_2 = \text{N}-\text{NO}_2$ 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 $\text{CH}_2-\text{N}-\text{NO}_2$ 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 ($-\text{NO}_2$) groups to nitroso ($-\text{NO}$) 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) [$\text{CH}_2(\text{NHNO}_2)_2$] 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 $-\text{N}-\text{C}-\text{N}-$ or outer $\text{N}-\text{NO}_2$ 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 hydroxylamino 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 $\text{C}-\text{N}$ 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) $_2\text{NNH}_2$] 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).

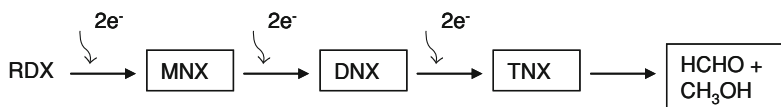
Zhao et al. (2002) investigated degradation of RDX by a facultative anaerobic bacterium which was isolated from the anaerobic sludge and identified as *Klebsiella pneumoniae* strain SCZ-1. Strain SCZ-1 degraded RDX to formaldehyde (HCHO), methanol (CH_3OH), carbon dioxide (CO_2), and nitrous oxide (N_2O) through intermediary formation of methylenedinitramine ($\text{O}_2\text{NNHCH}_2\text{NHNO}_2$). 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.

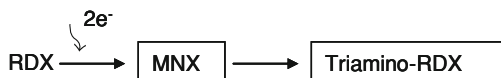
An et al. (2010) investigated the performance of anaerobic mesophilic granular sludge for the degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). Anaerobic granular sludge exhibited good performance in treating RDX as the sole substrate. Biodegradation was the main mechanism responsible for 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

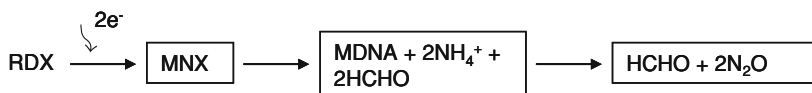
Metabolic pathways involved in the biodegradation of the cyclic nitramines RDX and HMX have been reviewed (Hawari et al. 2000a; Crocker et al. 2006). The potential degradation routes known for RDX are presented in Fig. 9.2. Three mechanisms for the transformation of RDX have been proposed: two-electron reduction, single-electron reduction/denitration, and direct enzymatic cleavage.



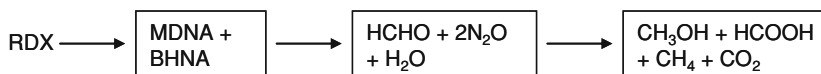
Path A: Reduction of RDX to nitroso derivatives before ring cleavage (McCormick et al, 1981)



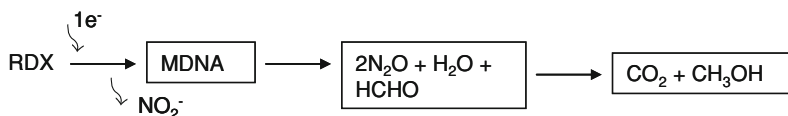
Path B: Reduction of RDX to 1,3,5-triamino-1,3,5-triazine (Zhang and Hughes, 2003)



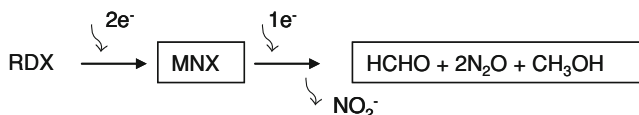
Path C: Reduction of RDX via nitrate oxidoreductase enzyme (Bhushan et al, 2002)



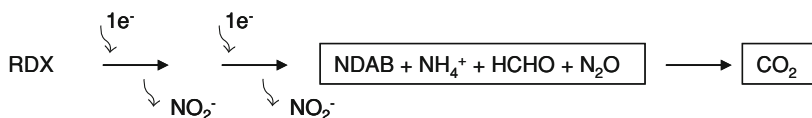
Path D: Direct enzymatic cleavage of RDX (Hawari et al, 2000b)



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-diazabutanol

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 H₂ 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–NO₂, 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

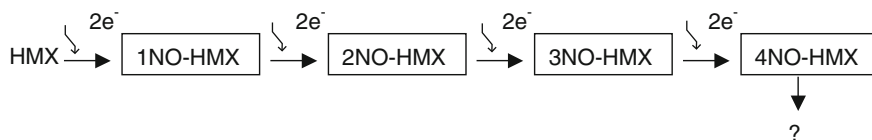
The biodegradation mechanism for HMX has been found to be similar to that of RDX. Under anaerobic conditions, nitroso derivatives are formed as intermediates from the two-electron reduction of the nitro groups on the HMX ring (Fig. 9.3, Path A). Usually, octahydro-1-nitroso-3,5,7-trinitro-1,3,5,7-tetrazocine (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.

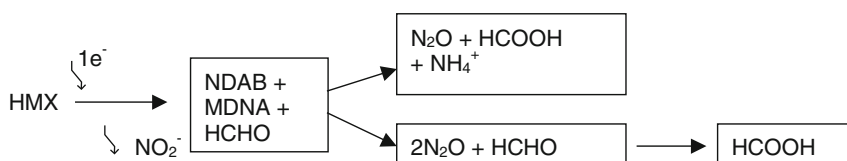
Products from biodegradation of cyclic nitramine explosives under aerobic conditions are poorly understood, particularly ring cleavage products (Binks et al. 1995; Hawari 2000). Jones et al. (1995b) isolated a *Rhodococcus* sp. strain A from explosives-contaminated soil and demonstrated its potential 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 ($\text{C}_2\text{H}_5\text{N}_3\text{O}_3$), 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 $-\text{NO}_2$ group in RDX readily accepts a free electron to form an anion radical. The elimination of nitrite anion $-\text{NO}_2^-$ 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), $66.6 \pm 4.1\%$ of the [^{14}C] RDX was converted to $^{14}\text{CO}_2$ over a 30 day period. Similarly, $76.0 \pm 3.9\%$ of the [^{14}C] RDX added (1.25 nmoles/10 g soil), was degraded to $^{14}\text{CO}_2$ 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% [^{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 $^{14}\text{CO}_2$. *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 $^{14}\text{CO}_2$ 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 [U¹⁴C]-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 NO₂⁻. 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.

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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




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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)	Melting point (°C) ^a	Vapor pressure (P ^v /Pa) ^b	Solubility in water (g/m ³) ^a	log K _{ow} ^a	LARC group ^b
Naphthalene	91-20-3	C ₁₀ H ₈		128.171	80.26	10.4	31	3.37	2B
Acenaphthylene	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)	Melting point (°C) ^a	Vapor pressure (P ^v /Pa) ^b	Solubility in water (g/m ³) ^a	log K _{ow} ^a	LARC 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 as 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.

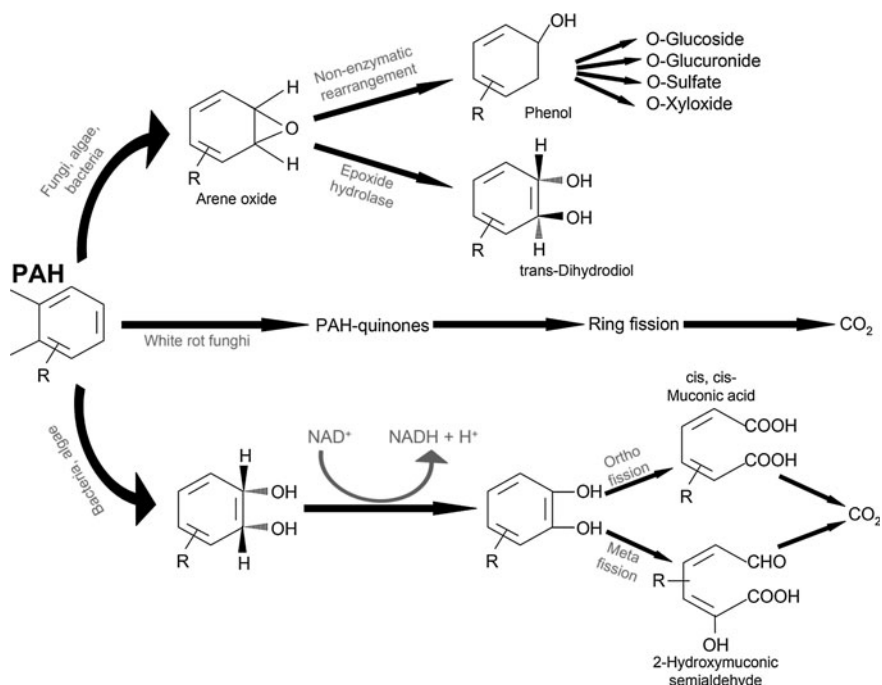


Fig. 10.1 General pathways for PAHs degradation (Redraw from Bamforth and Singleton 2005)

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, H₂O, CO₂ (aerobic) or CH₄ (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	
<i>Acinetobacter calcoaceticus</i>	<i>Absidia glauca</i>	<i>Mortierella ramanniana</i>	<i>Agmenellum quadruplicatum</i>
<i>Acinetobacter</i> sp.	<i>Acheremonium</i> sp.	<i>Mortierella verrucosa</i>	<i>Agmenellum</i> sp.
<i>Aeromonas</i> sp.	<i>Aspergillus niger</i>	<i>Mucor hiernalis</i>	<i>Anabaena</i> sp. (strain CA)
<i>Agrobacterium</i> sp.	<i>Aspergillus ochraceus</i>	<i>Mucor racemosus</i>	<i>Anabaena</i> sp. (strain 1F)
<i>Alcaligenes denitrificans</i>	<i>Aspergillus</i> sp.	<i>Neurospora crassa</i>	<i>Amphora</i> sp.
<i>Alcaligenes faecalis</i>	<i>Basidiobolus ranarum</i>	<i>Penicillium chrysogenum</i>	<i>Aphanocapsa</i> sp.
<i>Arthrobacter polychromogenes</i>	<i>Candida maltosa</i>	<i>Penicillium</i> sp.	<i>Chlorella autotrophica</i>
<i>Arthrobacter</i> sp.	<i>Candida tropicalis</i>	<i>Pestalotia</i> sp.	<i>Chlorella sorokiniana</i>
<i>Bacillus cereus</i>	<i>Candida utilis</i>	<i>Phanaerochaete chrysosporium</i>	<i>Chorella vulgaris</i>
<i>Bacillus</i> sp.	<i>Choanephora campinter</i>	<i>Phanaerochaete sordid</i>	<i>Chlamydomonas angulosa</i>
<i>Beijerinckia</i> sp.	<i>Chrysosporum pannorum</i>	<i>Phlebia radiata</i>	<i>Coccochloris elabrens</i>
<i>Burkholderia</i> sp.	<i>Circinella</i> sp.	<i>Phlyctochytrium reinboldtae</i>	<i>Cylindrotheca</i> sp.
<i>Corynebacterium renale</i>	<i>Claviceps paspali</i>	<i>Phytophthora cinnamomi</i>	<i>Dunaliella tertiolecta</i>
<i>Flavobacterium</i> sp.	<i>Cokeromices poitrassi</i>	<i>Rhizoctonia solani</i>	<i>Microcoleus chthonoplastes</i>
<i>Haemophilus</i> sp.	<i>Conidiobolus gonimodes</i>	<i>Rhizophlyctis harderi</i>	<i>Navicula</i> sp.
<i>Klebsiella</i> sp.	<i>Cunninghamella bainieri</i>	<i>Rhizopus oryzae</i>	<i>Nitzschia</i> sp.
<i>Micrococcus</i> sp.	<i>Cunninghamella blakesleeana</i>	<i>Rhizopus stolonifer</i>	<i>Nostoc</i> sp.
<i>Moraxella</i> sp.	<i>Cunninghamella echinulata</i>	<i>Saccharomices cerevisiae</i>	<i>Oscillatoria</i> sp. (strain JMC)
<i>Mycobacterium flavescens</i>	<i>Cunninghamella elegans</i>	<i>Saprolegnia parasitica</i>	<i>Oscillatoria</i> sp. (strain MEV)
<i>Mycobacterium</i> sp.	<i>Cunninghamella japonica</i>	<i>Smittium culicis</i>	<i>Phrototheca zopfii</i>
<i>Nocardia</i> sp.	<i>Emencellopsis</i> sp.	<i>Smittium culisetae</i>	<i>Porphyridium cruentum</i>
<i>Paenobacillus</i> sp.	<i>Epicoccum nigrum</i>	<i>Smittium simuli</i>	<i>Scenedesmus platydiscus</i>
<i>Pseudomonas cepacia</i>	<i>Fusarium oxyporum</i>	<i>Sordana fimicola</i>	<i>Selenastrum capricornutum</i>
<i>Pseudomonas fluorescens</i>	<i>Gilbertella persicaria</i>	<i>Syncephalastrum racemosum</i>	<i>Skeletonema costatum</i>
<i>Pseudomonas paucimobilis</i>	<i>Gliocadium</i> sp.	<i>Thamnidium anomalum</i>	<i>Synedra</i> sp.

(continued)

Table 10.2 (continued)

Some PAHs utilizing bacteria	Some PAHs utilizing fungi	Some PAHs utilizing cyanobacteria and microalgae
<i>Pseudomonas putrid</i>	<i>Helicostylum piriforme</i>	<i>Thichocladium canadense</i>
<i>Pseudomonas testosteroni</i>	<i>Hyphochytrium catenoides</i>	<i>Trichoderma viride</i>
<i>Pseudomonas vesicularis</i>	<i>Irpex lateus</i>	<i>Verticillum</i> sp.
<i>Pseudomonas</i> sp.	<i>Linderina pennispora</i>	<i>Zygorhynchus moelleri</i>
<i>Rhodococcus</i> sp.		
<i>Rhodotorula glutinis</i>		
<i>Staphylococcus auriculans</i>		
<i>Sphingomonas paucimobilis</i>		
<i>Sphingomonas</i> sp.		
<i>Streptomyces griseus</i>		
<i>Streptomyces</i> sp.		
<i>Vibrio</i> 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

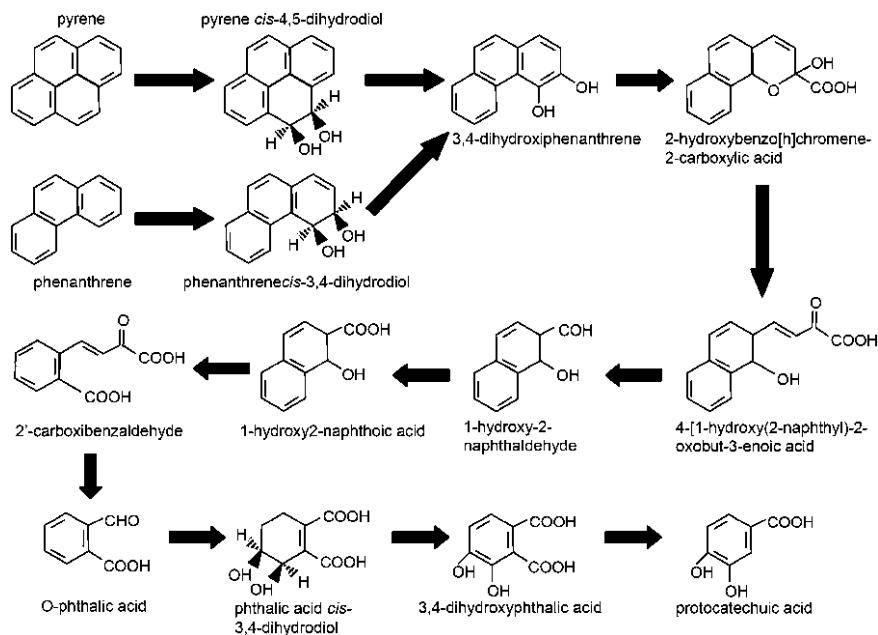


Fig. 10.2 Phenanthrene biodegradation pathway in *Mycobacterium* species (Redrawn from Pagnout et al. 2007)

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 P₄₅₀ 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. (fig), *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 2³ 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

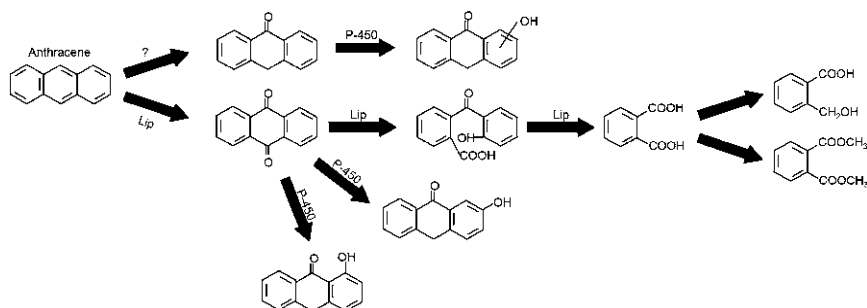


Fig. 10.3 Anthracene degradation by *I. lacteus* (Redrawn from Catjthaml et al. 2002)

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 CO₂. Non-lignolytic fungi metabolize PAHs by cytochrome P450 monooxygenase 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. *Cunninghamella 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

Lignolytic 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 CO₂ and H₂O (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

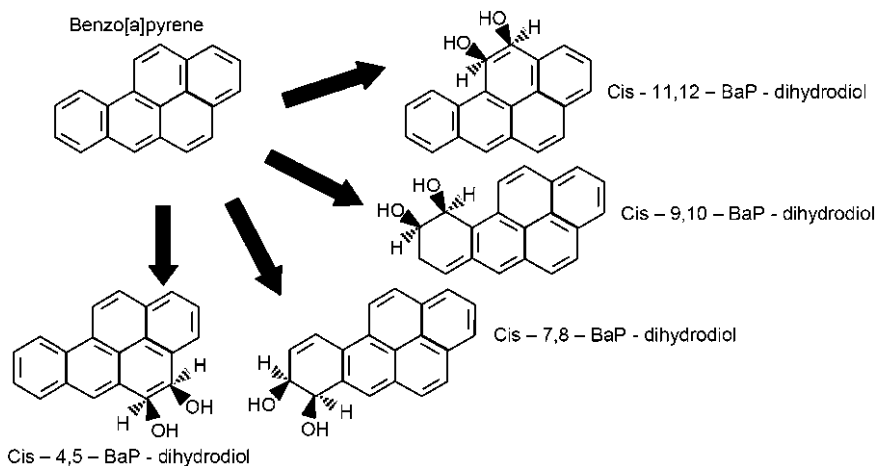


Fig. 10.4 Algal transformation products of benzo[a]pyrene (Redrawn from Juhasz and Naidu 2000)

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-normalized 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 $65.6 \pm 0.9\%$ of the initial pyrene was metabolized after three weeks, and that in basidiomycetes rich medium, *P. ostreatus* D1 metabolized up to $89.8 \pm 2.3\%$ 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

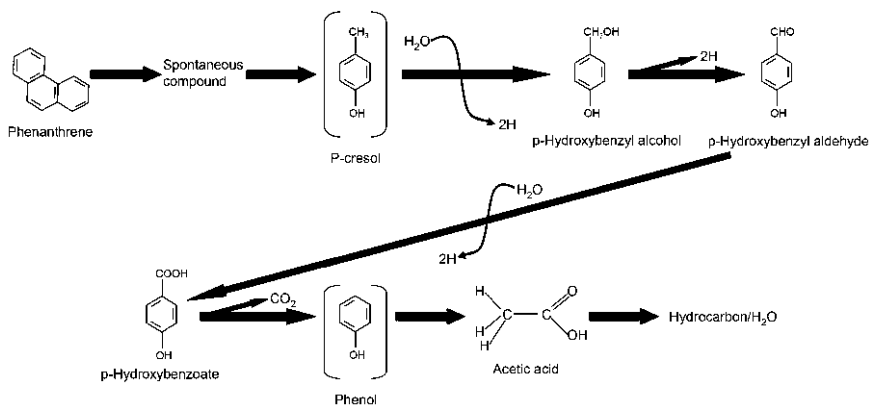


Fig. 10.5 Proposed pathways for anaerobic biotransformation of phenanthrene by sulfate-reducing bacteria (Redrawn from Tesai et al. 2009)

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 distills 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 O₂, N, and C. They concluded that the supply of O₂ 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 Majcherzyk (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 [¹⁴C]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 ¹⁴C-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 microalgae 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 pelagius* related.

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., O₂-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 N₂O, 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 phenanthrene 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 archaeobacterial 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-DCMPAHs) 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 heterogeneous 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 $\mu\text{g kg}^{-1}$ dry weight, and in Norway, ranged between 8.6 and 1050 $\mu\text{g kg}^{-1}$ 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 $\Sigma 16\text{PAHs}$ ranged from 80–7264 $\mu\text{g kg}^{-1}$.

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 CO₂ 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 ¹⁴C phenanthrene and ¹⁴C 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 Albaigüés 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 phenanthrene 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 Louisiana’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 $\mu\text{g/g}$. 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 bio-available 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 $\mu\text{g/L}$ (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 $\mu\text{g/L}$), 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.

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Chapter 11

Biodegradation of Aromatic Pollutants by Ligninolytic Fungal Strains

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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 π -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 lipophilicity, 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

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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: hemeperoxidases, 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 H₂O₂ 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 ¹⁴C-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

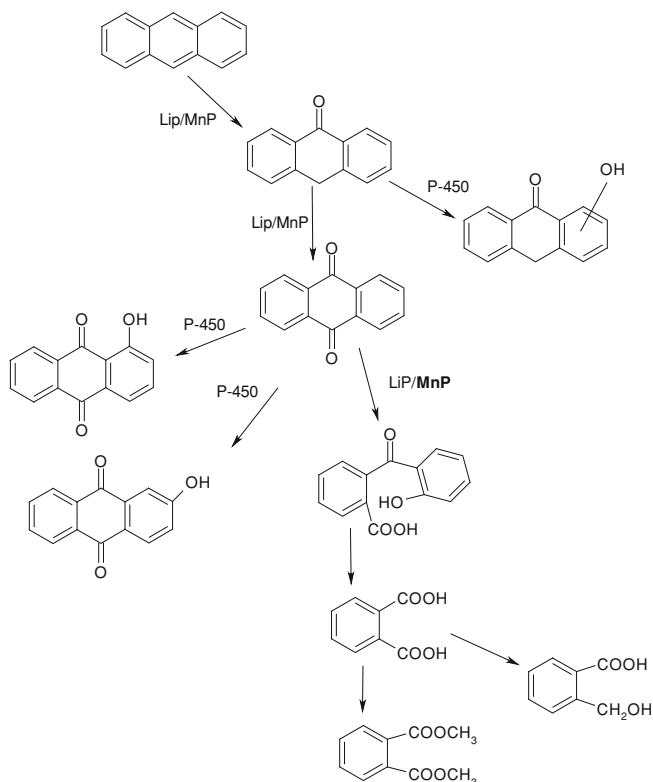


Fig. 11.1 Pathway proposed for anthracene degradation by *I. lacteus* with indication of possible enzyme involvement

(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). Schutzenhubel 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 $^{14}\text{CO}_2$ 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 $^{14}\text{CO}_2$. 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'-tetrachlorobiphenyl 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 17 α -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

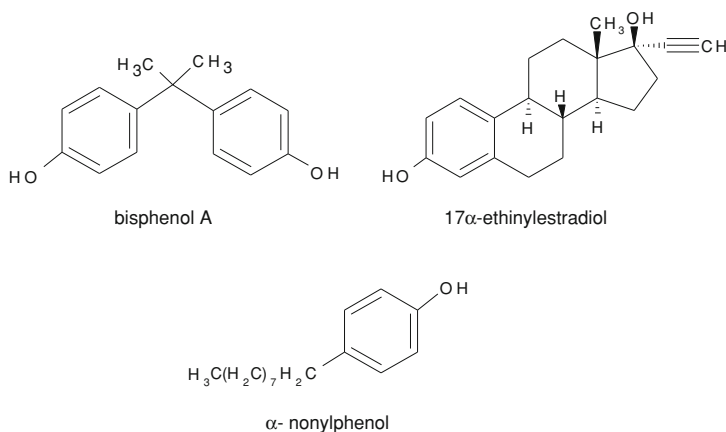


Fig. 11.2 Structures of anthropogenic EDCs

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 dibutylphthalate 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 dibutylphthalate 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).

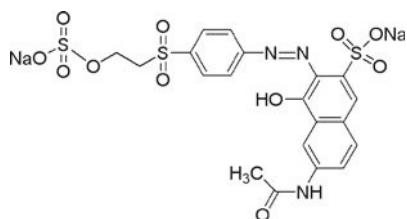
Liquid medium experiments with *Cephalosporium aphidicola* and *Cunninghamella elegans* were focused on identifying microbial transformation metabolites of oral contraceptives, EE2 and norethisterone (Choudhary et al. 2004). Transformation of norethisterone by *C. aphidicola* led to an oxidized metabolite, EE2, in this work, while the transformation of EE2 by *C. elegans* yielded several metabolites, 19-nor-17 α -pregna-1,3,5 (10)-trien-20-yne-3,4,17 β -triol, 19-nor-17 α -pregna-1,3,5 (10)-trien-20-yne-3,7 α ,17 β -triol, 19-nor-17 α -pregna-1,3,5 (10)-trien-20-yne-3,11 α ,17 β -triol, 19-nor-17 α -pregna-1,3,5 (10)-trien-20-yne-3,6 β ,17 β -triol and 19-nor-17 α -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.

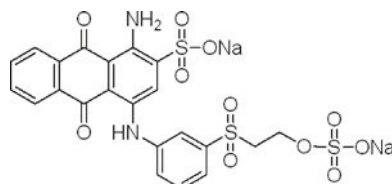
11.6 Fungal Decolorization and Degradation of Synthetic Dyes

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, triarylmethane, xanthene, anthraquinoid, acridine, cyanine, quinone-imine, phthalocyanine, and thiazole dyes. Figure 11.3 describes structures of selected group representatives.

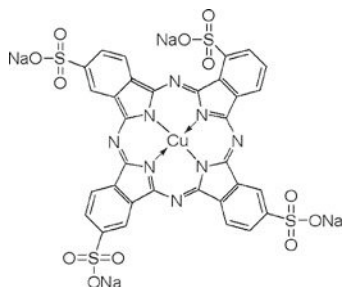
Since the first study on the fungus *P. chrysosporium* (Paszczyński et al. 1991; Pastigrigsby et al. 1992), ligninolytic fungi have been shown to degrade various synthetic dyes. Fungal decolorization and degradation of textile dyes has been



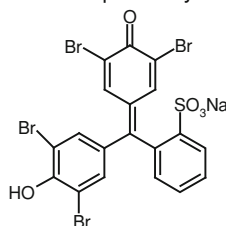
Reactive Orange 16 (RO16), azo dye



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 polymorpha* cultures (Sugimori et al. 1999). Dye molecule bio-sorption 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 *Aspergillus 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 possess 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 peroxy 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 *Corioloropsis 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).

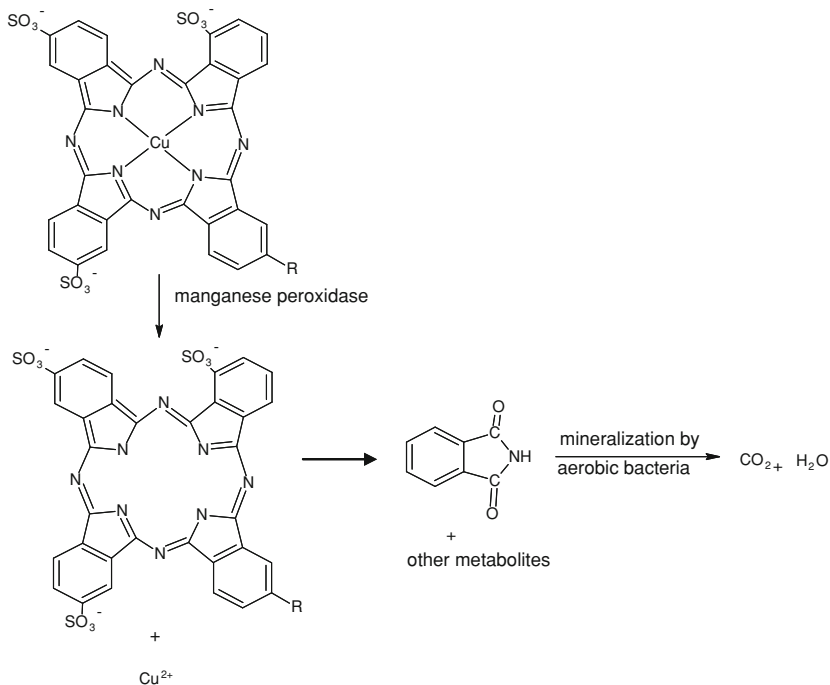


Fig. 11.4 Proposed degradation pathway for copper phthalocyanine dyes using *P. chrysosporium* and aerobic bacteria

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 Mn²⁺-independent reactions because Mn²⁺ present in the reaction mixture acted as a non-competitive 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. PCDDs 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. chrysosporium* 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 than 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.

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Chapter 12

Microbial Degradation of Dye-Containing Wastewater

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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 hydrolysed 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).

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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*-toluidine, *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*-toluidine 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 performed 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.

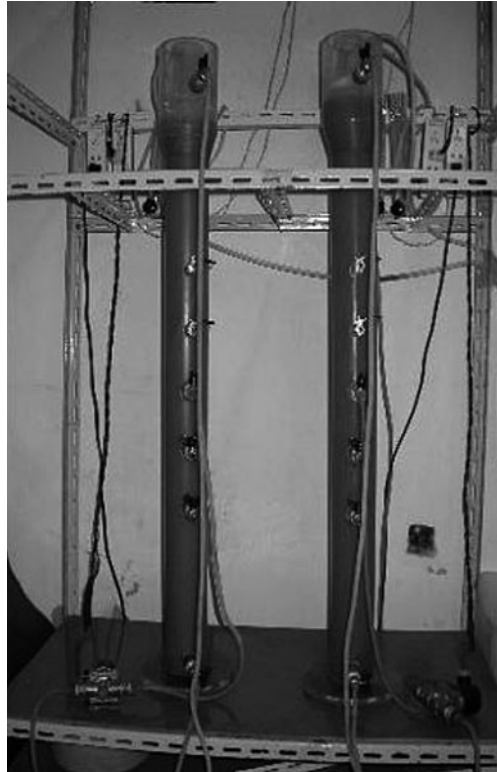
Lloyd (1992) also performed activated sludge treatment tests on two textile wastewaters. The first was a textile dyeing and finishing process water that contained reactive navy 106, and the second was a municipal wastewater consisting predominately of textile effluents. Both effluents were fed to laboratory -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

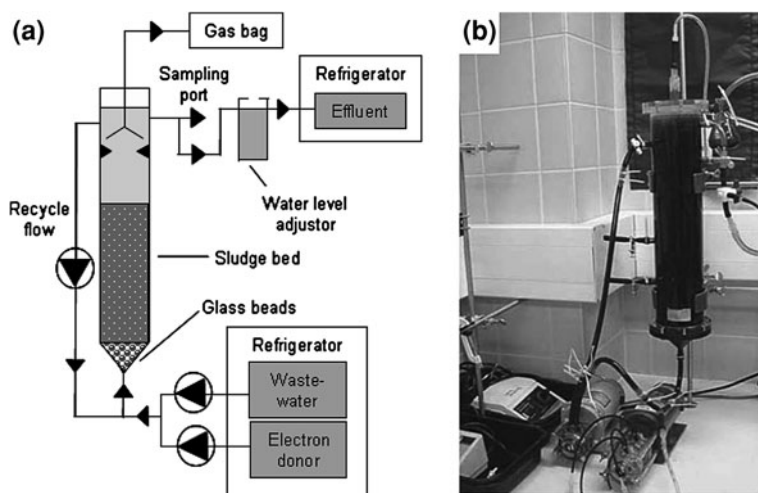


Fig. 12.2 A schematic diagram (a) and photo (b) of a laboratory scale upflow anaerobic sludge blanket reactor (UASB)

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 (Seigneur 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 (Pati and Crawford 1990; Seigneur 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). Phthalocyanine 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), *Oscillatoria* (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 bioconversion 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 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 hydrophobic 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 undoubtedly 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.

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Chapter 13

Bacterial Degradation of Petroleum Hydrocarbons

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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

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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.

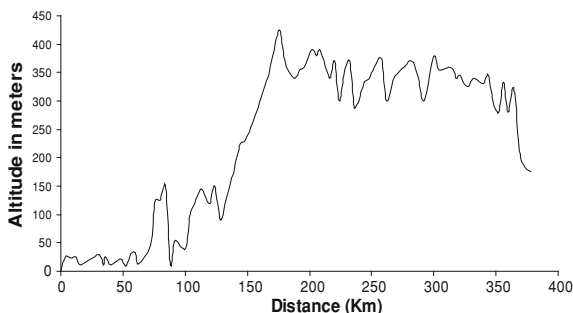
13.2 Water Contamination in Fuel Storage Tanks/Transporting Pipeline

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; Groyzman 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. Bio-degradation 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., *Aspergillus* sp., *Pseudomonas* sp., *Vibrio* sp., *Nocardia* sp. and *Rhobium* sp. Walker and Colwell (1976) isolated *Vibrio* sp., *Pseudomonas* sp. and *Acinetobacter* 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 C₁₂–C₃₀ 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* A11, *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 subtilis*, *Klebsiella* 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 Enterbacteriaceae. *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
<i>Cell morphology</i>										
Gram stain										
Shape	Negative Rod	Positive Rod	Positive Rod	Positive Rod	Positive Rod	Negative Small rod	Positive Rod	Positive Rod	Positive Rod	Positive Rod
Motility	+	+	+	+	+	+	+	+	+	+
Sporulation	-									
<i>Growth at</i>										
20°C	+	+	+	+	+	+	+	+	+	+
30°C	+	+	+	+	+	+	+	+	+	+
40°C	+	+	+	+	+	+	+	+	+	+
<i>Hydrocarbon utilization</i>										
Diesel	+		+	+	+	+	+	+	+	+
<i>Biochemical reaction</i>										
Indole production test	-	+	-	-	-	-	+	+	-	+
Methyl red test	-	-	+	+	+	-	-	-	+	+
Voges Proskauer test	+	-	-	-	-	+	-	-	-	-
Citrate utilization test	+	-	+	+	+	+	-	+	+	-
Oxidase test	+	+	+	+	+	+	+	+	+	+
Catalase test	+	+	+	+	+	+	+	+	+	+
<i>Production of acid from</i>										
Glucose	+	+	+	+	+	+	+	+	+	+
Galactose	-	-	+	+	-	+	-	-	-	-
Fructose	+	+	+	+	+	+	+	+	+	+
Sucrose	-	+	-	-	-	-	+	-	-	-
Mannitol	-	-	-	-	-	+	-	-	+	-
Lactose	-	-	+	+	+	-	-	+	+	+
Cellobiose	-	-	+	+	+	-	-	+	+	+
Adonitol	-	-	-	-	-	+	-	-	-	-

(continued)

Table 13.2 (continued)

Characteristics	ACE2	ACE4	ARI2	AR4	AI1	ACP	AP2	ANI	AN4	AN5
Arabinose	-	+	-	-	+	-	+	-	-	+
Raffinose	-	-	-	-	-	+	-	-	+	-
Inositol	-	-	-	-	-	+	-	+	-	-
<i>Hydrolysis of</i>										
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
<i>Cell morphology</i>					
Gram stain	Negative	Positive	Positive	Positive	Positive
Shape	Rod	Rod	Rod	Rod	Rod
Motility	+	+	+	+	+
Sporulation	—	+	+	+	+
<i>Growth at</i>					
20°C	+	+	+	+	+
30°C	+	+	+	+	+
40°C	+	+	+	+	+
<i>Hydrocarbon utilization</i>					
Diesel	+	+	+	+	+
<i>Biochemical reaction</i>					
Indole production test	—	+	—	—	—
Methyl red test	—	—	+	—	+
Voges Proskauer test	+	—	—	+	—
Citrate utilization test	+	—	+	—	—
Oxidase test	+	+	+	+	+
Catalase test	+	+	+	+	+
<i>Production of acid from</i>					
Glucose	+	+	+	—	+
Fructose	+	+	+	+	+
Sucrose	+	+	—	+	—
Mannitol	—	—	—	—	—
Lactose	—	—	+	+	—
Cellobiose	—	—	+	+	—
Adonitol	+	+	+	+	+
Arabinose	+	+	+	+	+
Raffinose	+	+	+	+	+
Inositol	—	—	—	—	—
<i>Hydrolysis of</i>					
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).

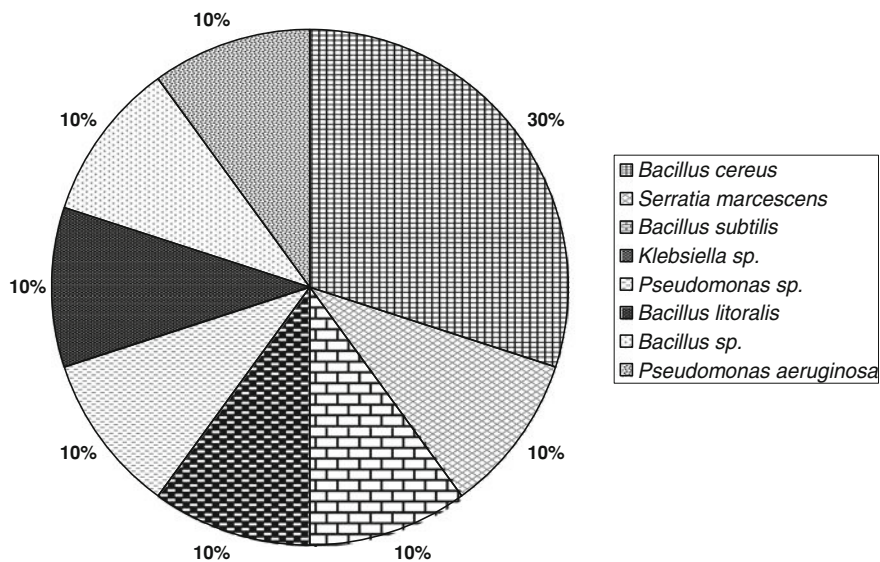


Fig. 13.2 Distribution of diesel degrading bacteria isolated from diesel pipeline

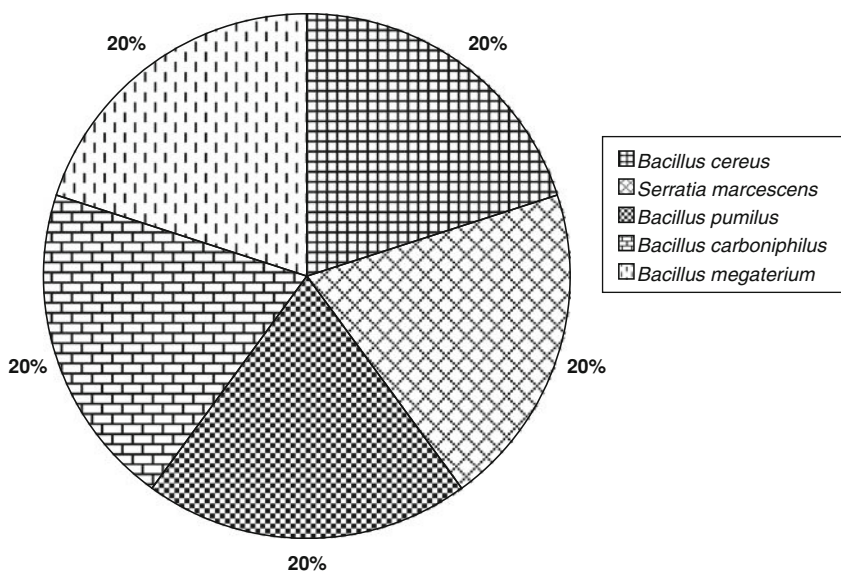


Fig. 13.3 Distribution of naphtha degrading bacteria isolated from naphtha pipeline

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

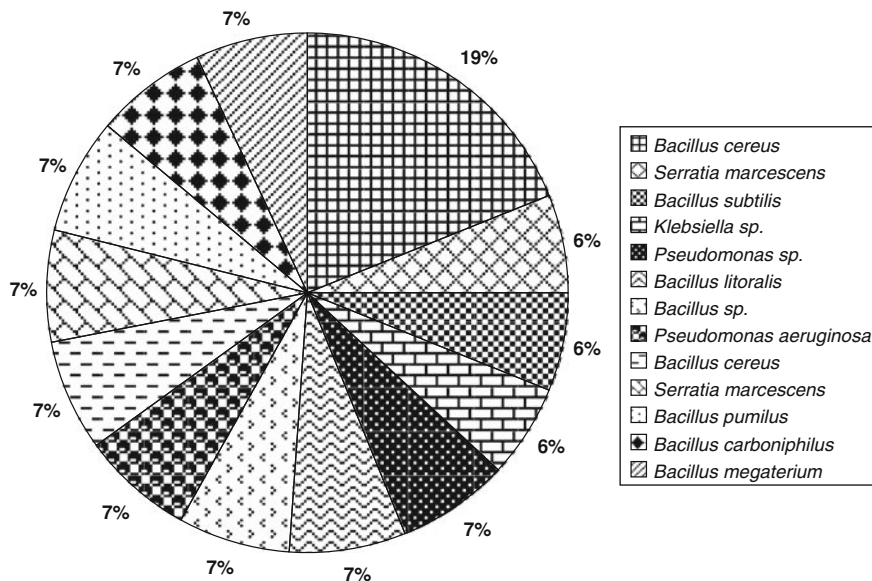


Fig. 13.4 Distribution of diesel/naphtha degrading bacteria isolated from diesel and naphtha pipeline

(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

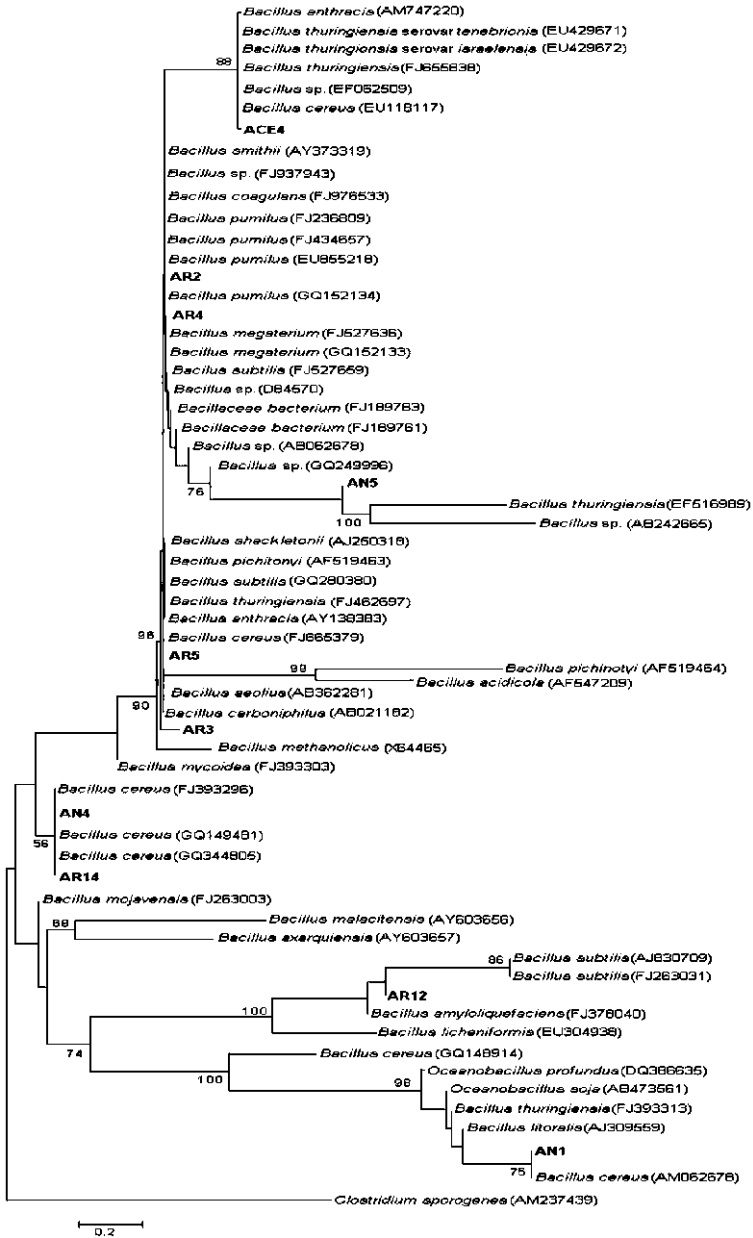


Fig. 13.5 Neighbor-joining tree based on 16S rRNA gene sequences, showing phylogenetic relationships between sequences of the bacterial phylum Firmicutes (*Bacillus* related species). *Clostridium sporogenes* was used as a bacterial outgroup. Numbers at nodes indicate bootstrap values >50% from 1000 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*

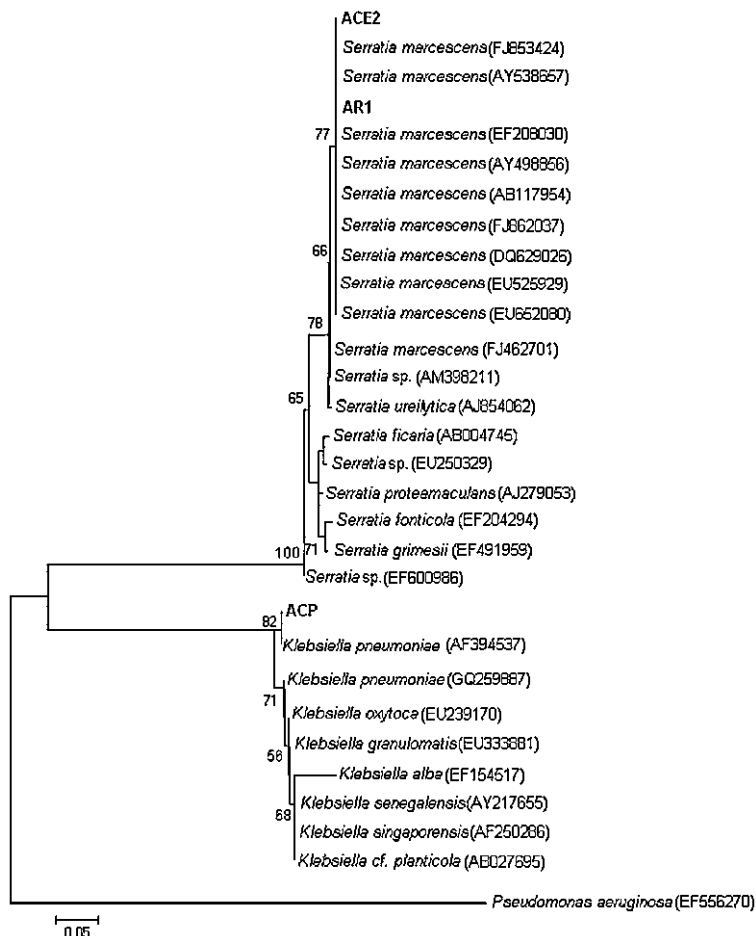


Fig. 13.6 Neighbor-joining tree based on 16S rRNA gene sequences, showing phylogenetic relationships between sequences of the Order Enterobacteriales (phylum Proteobacteria). *Pseudomonas aeruginosa* was used as the out group sequence. Numbers at nodes indicate bootstrap values >50% from 1000 replicates. 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.

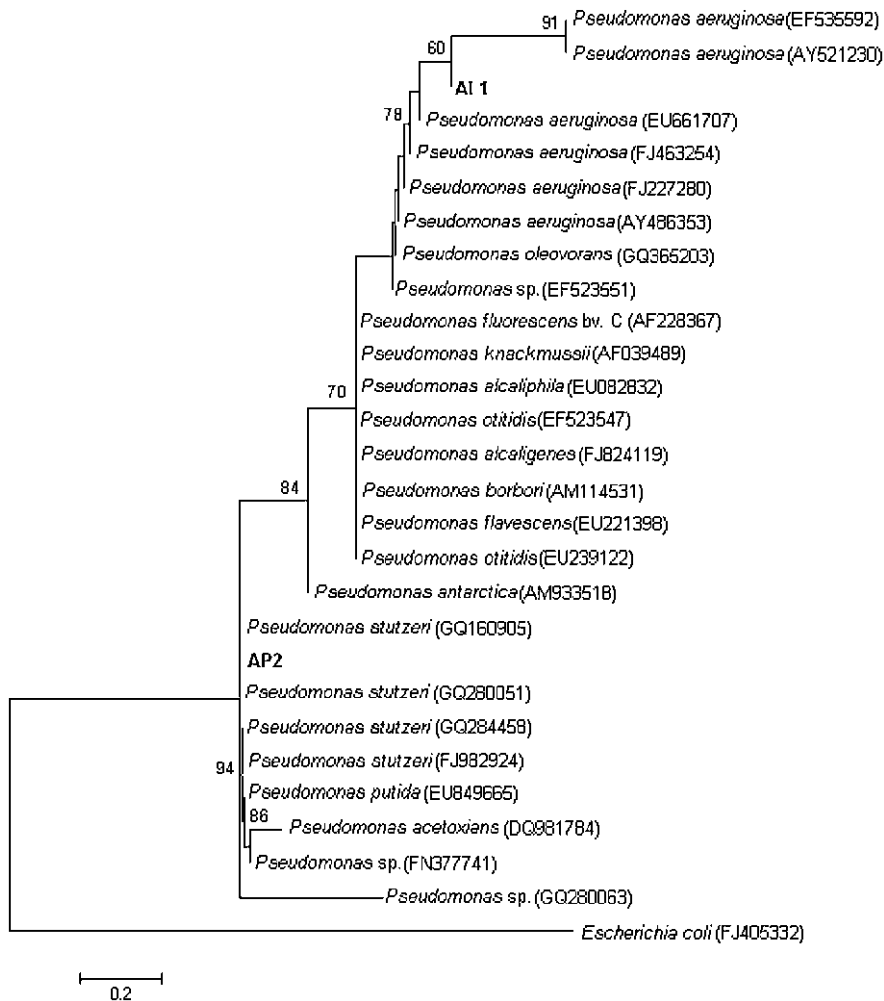


Fig. 13.7 Neighbor-joining tree based on 16S rRNA gene sequences, showing phylogenetic relationships between sequences of the Order Pseudomonadales (phylum Proteobacteria). *Escherichia coli* was used as the out group sequence. Numbers at nodes indicate bootstrap values >50% from 1000 replicates. GenBank 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
<i>Diesel degrading bacteria</i>			
1.	ACE2	DQ092416	<i>Serratia marcescens</i>
2.	ACE4	AY912105	<i>Bacillus cereus</i>
3.	AR12	EF535590	<i>Bacillus subtilis</i>
4.	AR14	EF535591	<i>Bacillus cereus</i>
5.	AI1	EF535592	<i>Pseudomonas aeruginosa</i>
6.	ACP	EF535593	<i>Klebsiella</i> sp.
7.	AP2	EF535594	<i>Pseudomonas</i> sp.
8.	AN1	EF535595	<i>Bacillus litoralis</i>
9.	AN4	EF535596	<i>Bacillus cereus</i>
10.	AN5	EF535597	<i>Bacillus</i> sp.
<i>Naphtha degrading bacteria</i>			
11.	AR1	DQ207558	<i>Serratia marcescens</i>
12.	AR2	DQ207559	<i>Bacillus pumilus</i>
13.	AR3	DQ207560	<i>Bacillus carboniphilus</i>
14.	AR4	DQ207561	<i>Bacillus megaterium</i>
15.	AR5	DQ207562	<i>Bacillus cereus</i>

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 (C₁₀–C₂₀), branched alkanes, naphthalene derivatives, substituted naphthalenes and iso prenioid alkanes (pristane, phytane) (Fig. 13.8a). The inoculated *S. marcescens* ACE2 degraded almost all the *n*-alkanes (C₁₀–C₂₀) and many of the branched alkanes found in diesel (Fig. 13.8b). Inoculation of *B. cereus* ACE4 degraded more *n*-alkanes (C₁₀–C₂₀) (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 (C₁₀ and C₂₂). Moderately degraded branched alkanes and naphthalene derivatives could be found in the degraded products, which is a

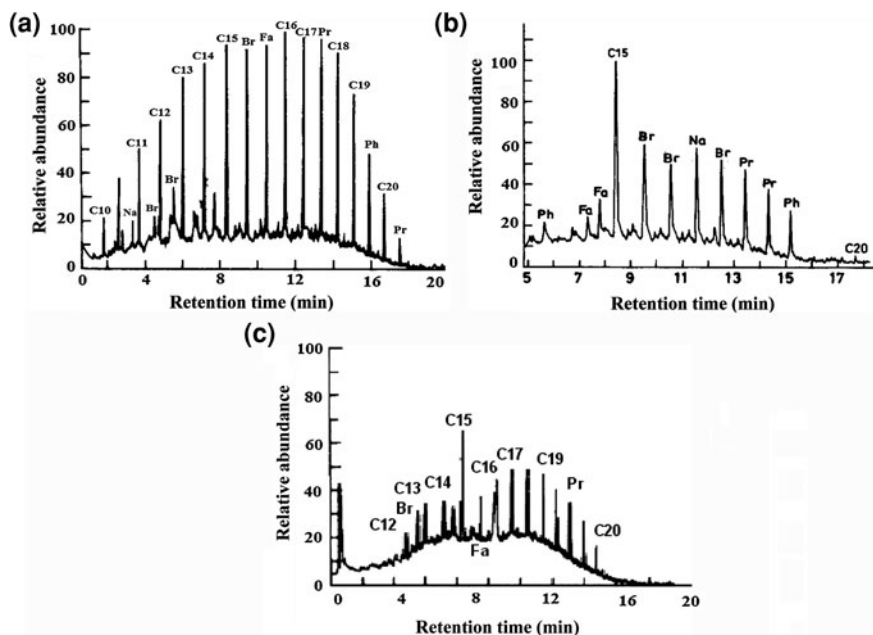


Fig. 13.8 a GC-MS profiles of abiotic control (uninoculated) C10 to C21, *n*-alkanes (numbers designate the number of C atoms); Br, branched alkanes; Na, substituted naphthalenes; Pr, pristane; Ph, phytane. The alkane, naphthalene, phytane, and pristane peaks were identified by comparison of their retention 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

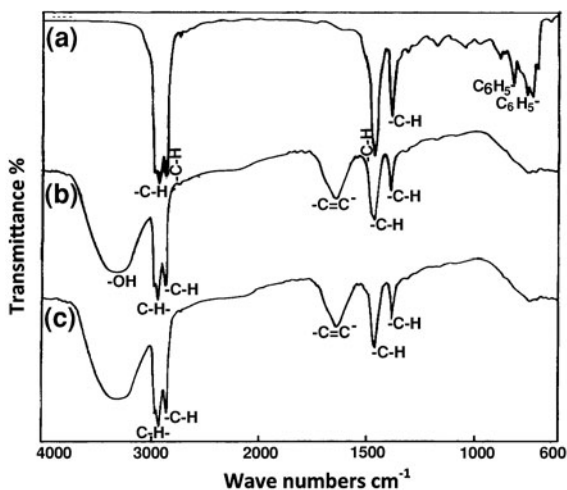
common feature of many other alkane-degrading microorganisms (Van Beilen et al. 1994; Whyte et al. 1998; Wongsu et al. 2004).

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=O) 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 (%)		Relative abundance (RA)		Biodegradation efficiency BE (%)	
		Strain ACE2		Strain ACE4		Strain ACE4		Strain ACE4	
		Control	Strain ACE2	Control	Strain ACE4	Relative abundance (RA)	Biodegradation efficiency BE (%)	Relative abundance (RA)	Biodegradation efficiency BE (%)
2	C 10	21	0	100	100	0	100	0	100
3	C 11	38	0	100	100	0	100	0	100
5.1	C 12	61	20	67.21	67.21	0	100	0	100
6	C 13	80	36	55	55	0	100	0	100
7.1	C 14	83	38	54	54	0	100	0	100
8.2	C 15	97	64	34	34	100	100	100	100
11.8	C 16	100	45	55	55	0	100	0	100
12.6	C 17	99	46	53	53	0	100	0	100
14.5	C 18	95	0	100	100	0	100	0	100
15.1	C 19	78	38	51	51	0	100	0	100
16.5	C 20	38	24	27	27	0	100	0	100
3.7	Naphthalene derivatives Compound—I	18	0	100	100	0	100	0	100
4	Naphthalene derivatives Compound—II	50	48	4	4	0	100	0	100
5.8	Branched alkanes Compound—I	39	0	100	100	37	5	37	5
9.5	Branched alkanes Compound—II	100	39	61	61	45	65	45	65
16	Phytane Compound—I	45	0	100	100	35	22	35	22
13.1	Pristine Compound—I	98	43	56	56	40	59	40	59
18.1	Pristine Compound—II	14	0	100	100	12	14	12	14
11.5	Farnithane Compound—I	98	38	61	61	35	64	35	64
7.6	Farnithane Compound—I	30	0	100	100	28	7	28	7
BE (Total percentage)				69	69		77		77

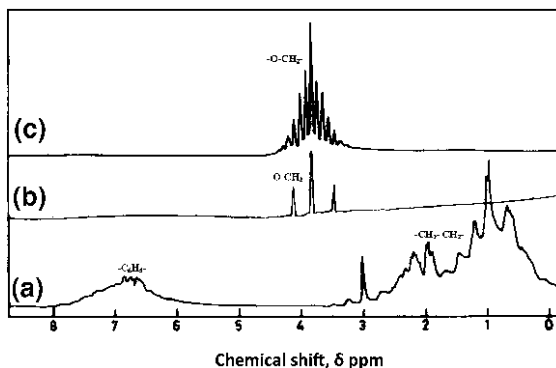
Fig. 13.9 FTIR spectrum of diesel and diesel inoculated with ACE and ACE4. **a** FTIR spectrum of diesel, **b** FTIR spectrum of diesel inoculated with ACE2, **c** FTIR spectrum of diesel inoculated with ACE4



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 ($-\text{O}-\text{CH}_2-$) 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 ($-\text{CH}_2-\text{CH}_2-$) was consumed by degrading bacteria and that the carbon–hydrogen bond was cleaved into carbon–oxygen bond ($-\text{O}-\text{CH}_2-$). From this, it is evident that aliphatic protons (CH_2-CH_2) are completely degraded by bacterial species, and converted as ($-\text{O}-\text{CH}_2$) 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 ($-\text{CH}_2-\text{CH}_2-$) 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 Falnery 1968). During degradation, n-alkanes were converted into ($-\text{O}-\text{CH}_2-$) 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

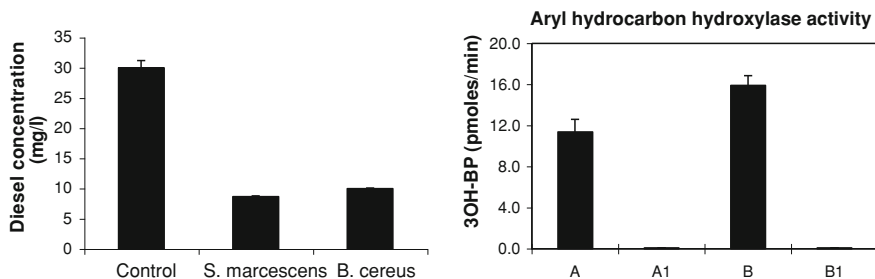


Fig. 13.11 **a** Total petroleum hydrocarbon in presence/absence of bacteria *S. marcescens* ACE4 and *B. cereus* ACE4. **b** Aryl hydrocarbon hydroxylase activity of bacteria ACE2 and ACE4, Enzyme Unit = 3OH-B(a)P pmoles/min. (A) Initial period of biodegradation (ACE2), (B) initial period of biodegradation (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 C₁₈ 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, C₅–C₉ is less likely to be degraded, but kerosene with a hydrocarbon range of C₁₀–C₁₈ is more susceptible to degradation (Hill 1984). The preferential removal of C₁₅–C₃₅ 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 H₂S, SO₂ and CO₂, are often generated in such quantities that they can dissolve in

the fuel, increasing the silver strip corrosivity index. The H₂S generation can also be a severe health hazard to the operating staff. Other metabolites, such as bio-surfactants, 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, organo-boramine, 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 bithiocyanate
- 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 scraping, 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 biocorrosion 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 marcescens* 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*).

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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.

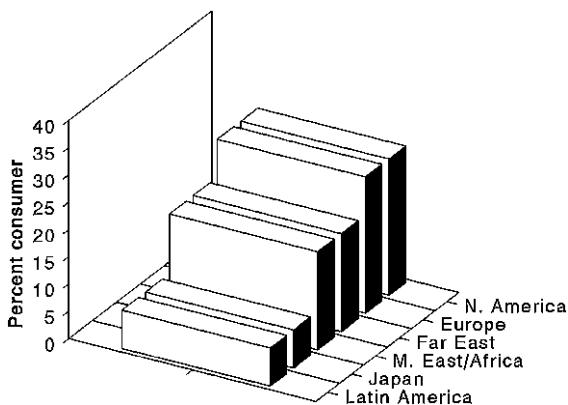
Advantages of polyurethanes are that they increase the tensile strength and melting points making them more durable (Bayer 1947). Their resistance to degradation by water, oils, and solvents makes them excellent materials for the replacement of plastics (Saunders and Frisch 1964). As coatings, they exhibit 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.

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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 (Uhlrig 1999)

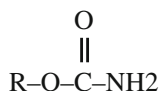


Isocyanate precursors react with hydroxyl groups of polyols, compounds with two or more hydroxyl groups, to form PU. When the polyol is a polyester resin, the product is polyester PU. By varying the isocyanate and polyol composition, chemists can synthesize an enormous diversity of PU materials, including 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 (Uhlrig 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:



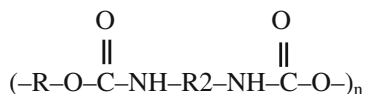
Variations in the R group and substitutions of the amide hydrogen produce multiple urethanes. Although PU may contain urethane groups, other moieties such as urea, ester, ether or an aromatic may also be included (Saunders and Frisch 1964). The addition of these functional groups may result in fewer 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	<p>Polyester-type</p> <p>Poly(butylene adipate) Poly(ethylene butylene adipate) Poly(ethylene adipate) Polycaprolactone Poly(propylene adipate) Poly(ethylene propylene adipate)</p> <p>Polyether-type</p> <p>Poly(oxytetramethylene) glycol Poly(oxypropylene) glycol Poly(oxypropylene)-poly(oxyethylene) glycol</p>
Chain extension/ crosslinking agent	1,4-Butanediol Ethylene glycol 1,3-Butanediol 2,2-Dimethyl-1,3-propanediol Trimethylpropane 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:



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 radiolabeled 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 polycaprolactonedioles 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 DLNTM) 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 phenylmethylsulphonyl fluoride (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 charged polyurethane (Fig. 14.2). This evidence was corroborated with electrical impedance measurements that showed there was an increase in the total volume of the

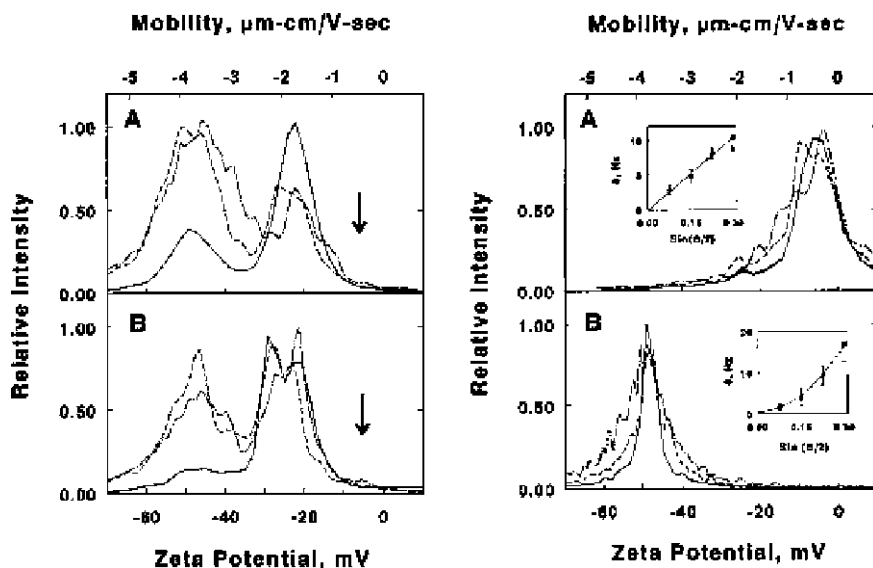
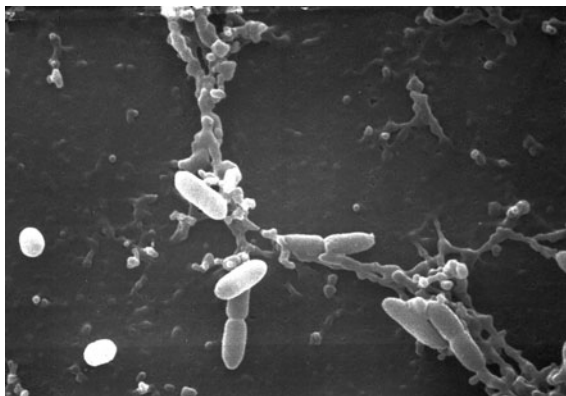


Fig. 14.2 (Left) Characterization of *Bacillus* and polyurethane by methods commonly employed in particle analysis *A* and *B*, electrokinetic measurements of *Bacillus* and polyurethane, respectively, in defined YES medium. Mobility (*top*) and zeta potential (*bottom*) spectra were calculated from 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 $\delta = \alpha \sin^2(\theta/2)$, where the value of α was determined by a linear regression analysis of δ versus $\sin^2(\theta/2)$ (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



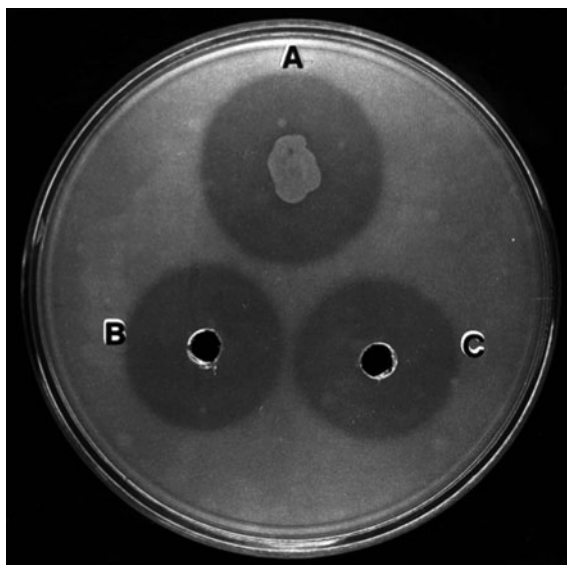
This evidence indicates that two populations exist in polyurethane cultures: one that is coated with polyurethane and one that is not. At lower concentrations of polyurethane, it may be that the two populations of bacteria are dependent on different sources of nutrition. The first population is coated 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 DLNTM, 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 DLNTM 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 $0.466 \text{ doublings h}^{-1}$ from $0.721 \text{ doublings h}^{-1}$. The μ continued to drop at higher concentrations from $0.466 \text{ doublings h}^{-1}$ at 3.0 mg ml^{-1} to $0.369 \text{ doublings h}^{-1}$ 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 Pseudomonads 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 $0.7 \text{ doublings h}^{-1}$, 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

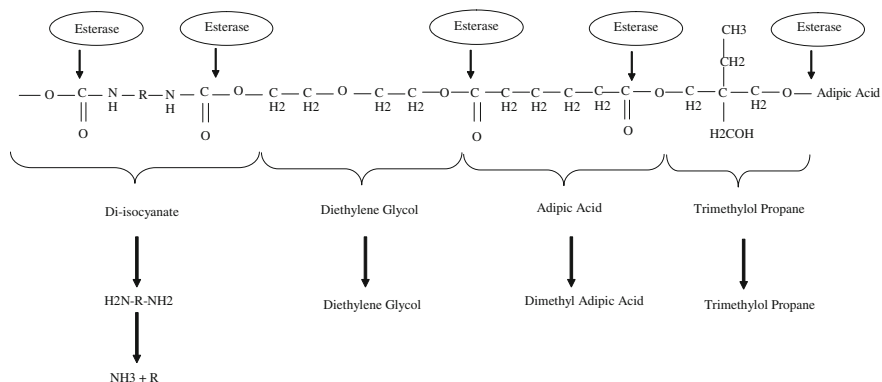


Fig. 14.5 Theoretical degradative pathway of polyester-polyurethane by esterase activity of *Pseudomonas*

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 Ser¹⁹⁹-His⁴³³-Glu³²⁴ 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 $1.3 \text{ doublings h}^{-1}$ 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, respectively (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.

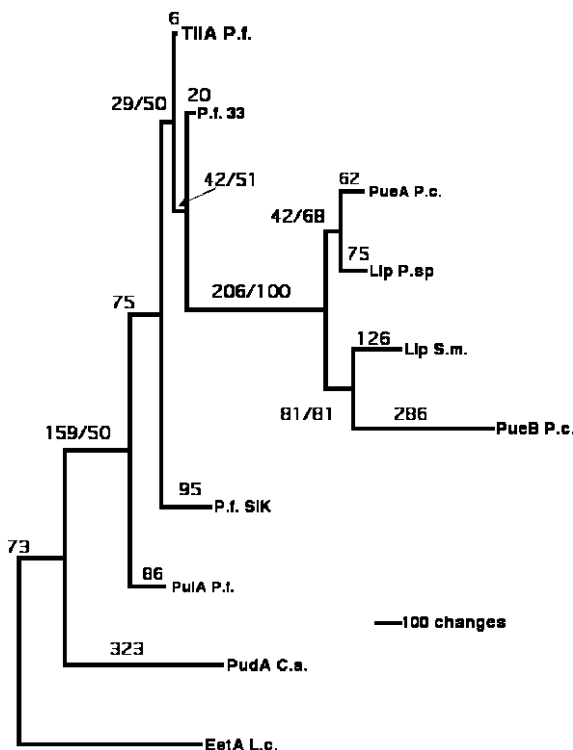
Howard et al. (2007) identified a gene cluster resembling a binding-protein-dependent ABC transport system in *Pseudomonas chlororaphis* in connection with PueA and PueB (Fig. 14.7). The identified ABC transport system

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	<i>Pseudomonas chlororaphis</i>	EF175556
PueA	617/1801	42/59	<i>Pseudomonas chlororaphis</i>	EF175556
PuLA	451/1353	24/41	<i>Pseudomonas fluorescens</i>	AF144089
PudA	548/1644	11/31	<i>Comamonas acidovorans</i>	AB009606
TliA	476/1428	26/40	<i>Pseudomonas fluorescens</i> B52	AF083061
LipA	613/1789	36/53	<i>Serratia marcescens</i> SM6	BAA02519
Lipase	617/1801	39/55	<i>Pseudomonas</i> sp. MIS38	BAA84997
LipApf33	476/1428	27/41	<i>Pseudomonas fluorescens</i> 33	BAA36468
Lipase	449/1338	25/39	<i>Pseudomonas fluorescens</i> 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*

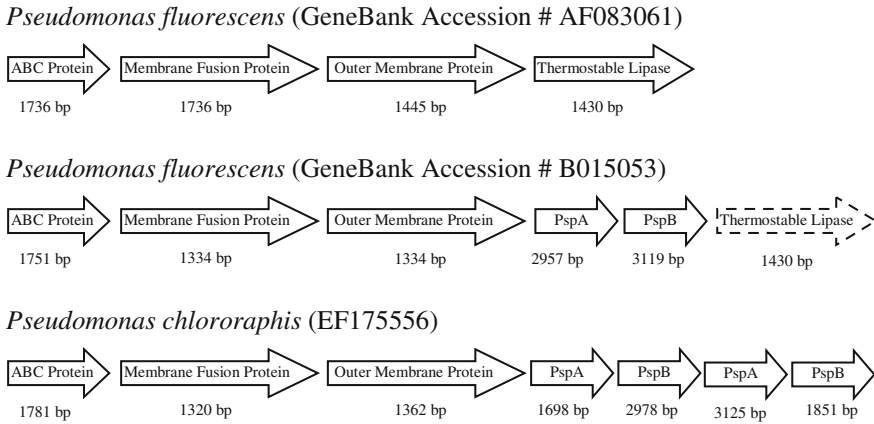


Fig. 14.7 Comparison of the gene clusters from two strains of *Pseudomonas fluorescens* and the PUase gene cluster from *Pseudomonas chlororaphis*. The ABC Reporter Protein, Membrane Fusion Protein and Outer Membrane Protein are involved in Type I translocation of the extracellular protein. The PspA 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 insertional 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 DLNTM 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 pueA::Kan^r*, had an 80% decrease in cell number ($4.66 \times 10^7 \pm 0.13$), whereas both the complements, *P. chlororaphis pueA::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)
<i>P. chlororaphis</i> (wild type)	1.32	31.5	0.800	$2.31 \times 10^8 \pm 0.87$
<i>P. chlororaphis</i> pueA::Kan ^r	1.09	38.2	0.917	$4.66 \times 10^7 \pm 0.13$
<i>P. chlororaphis</i> pueA::Kan ^r (pPueA-1)	1.41	29.5	0.710	$2.86 \times 10^8 \pm 0.09$
<i>P. chlororaphis</i> (pPueA-1)	1.54	27.0	0.649	$3.85 \times 10^8 \pm 0.98$
<i>P. chlororaphis</i> pueB::Kan ^r	1.19	34.9	0.893	$2.35 \times 10^8 \pm 0.148$
<i>P. chlororaphis</i> pueB::Kan ^r (pPueB-1)	1.37	30.4	0.735	$3.59 \times 10^8 \pm 0.187$
<i>P. chlororaphis</i> (pPueB-1)	1.41	29.5	0.781	$3.99 \times 10^8 \pm 0.813$

The concentrations of Impranal DLNTM 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 insertional mutant, *P. chlororaphis* pueA::Kan^r, exhibited a μ_{\max} of 1.09. It would be hypothesized that a deletion of the *pueA* gene would result in a decrease in growth rate. However, a large decrease in growth obtained from the insertional mutant may indicate that PueA plays a major role as compared to PueB in polyurethane degradation by *P. chlororaphis*. When multiple copies of *pueA* gene were introduced into either the wild type, *P. chlororaphis* pPueA-1, a μ_{\max} value of 1.54, or the mutant, *P. chlororaphis* pueB::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* pueB::Kan^r, had a 18% decrease in cell number ($2.35 \times 10^8 \pm 0.148$) whereas, both the complement, *P. chlororaphis* pueB::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 Impranal 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 *pueB* 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* pueB::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* pueB::Kan^r, displayed a μ_{\max} value of 1.19. Again, it would be hypothesized that the deletion of the *pueB* 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 contact with insoluble substrates (e.g. PU). In order to overcome this obstacle, enzymes that degrade insoluble substrates possess 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

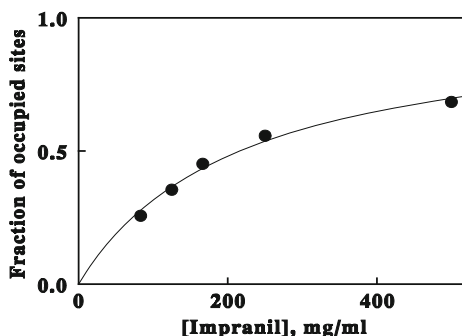


Fig. 14.8 Equilibrium binding of Impranil DLN to PueA. The concentration of occupied polyurethane binding sites present in different reaction mixtures of PueA and soluble Impranil DLN were determined by kinetic exclusion assays on a flow fluorimeter as described in the text. Each determination was expressed 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 $6.6 \mu\text{g ml}^{-1}$ 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 $220 \pm 30 \text{ mg ml}^{-1}$ 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

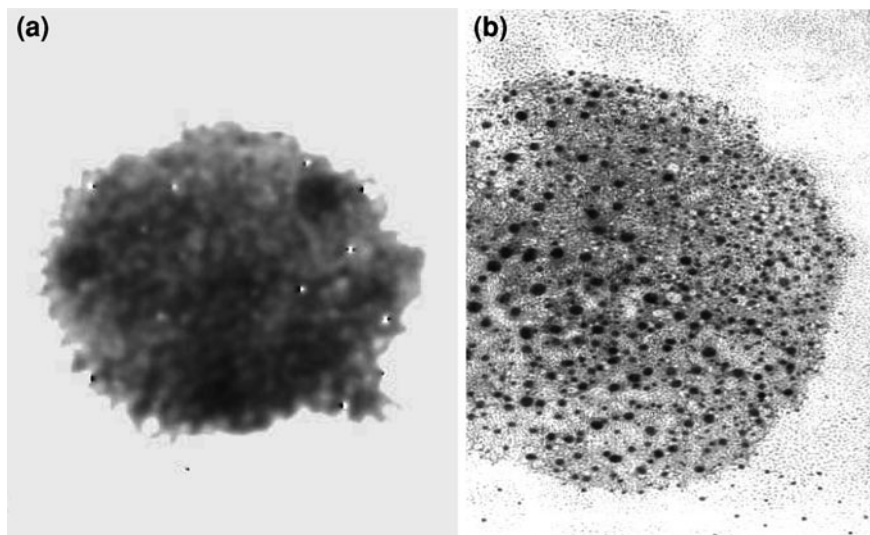


Fig. 14.9 Electron micrographs of embedded Bayhydrol 110TM polyurethane particles. **a** Electron micrograph of polyurethane particles taken at a magnification of $\times 15,000$. **b** Electron micrographs of Immunogold-labeled PueA (1:5,000,000,000 dilution of 0.83 mg ml^{-1} PueA) bound to embedded polyurethane particle at $\times 15,000$ 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, PuaA, and Pua 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.

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Chapter 15

Microbial Degradation of Recalcitrant PAHs-Microbial Diversity Involving Remediation Process

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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

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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 (C₁₈H₁₂), benzo[β]fluoranthene,

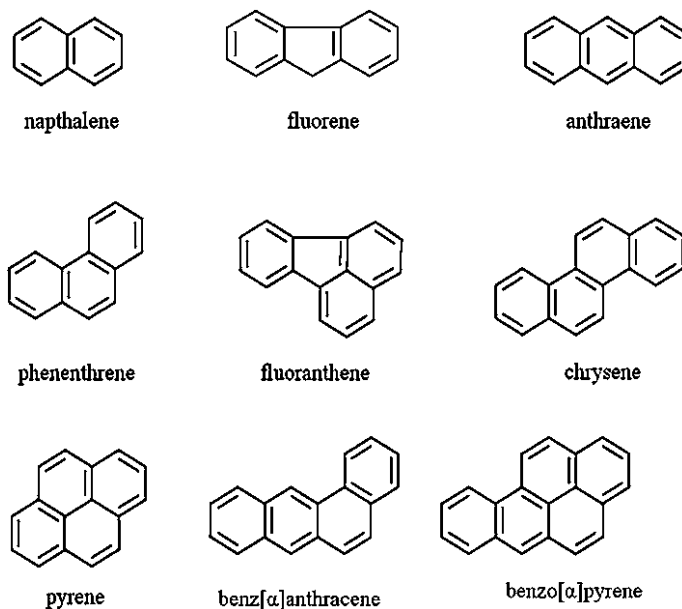


Fig. 15.1 Schematic diagrams of different PAHs

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 led 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, *Alcaligenes 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 ($E^{\circ'}$) values, and convert into less standard Gibbs free energy change ($\Delta G^{\circ'}$) 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 channelled into the intermediates of Krebs's cycle. Aerobic bioremediation of HMWPAHs, including BAP, must utilize co-metabolic degradation that requires a carbon as an energy source and oxygen (Juhász 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. H₂). 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 O₂, alternative electron acceptors, such as NO₃³⁻, Fe²⁺ and SO₄³⁻ 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, trans-hydroxylation, 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 biotransformation, 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

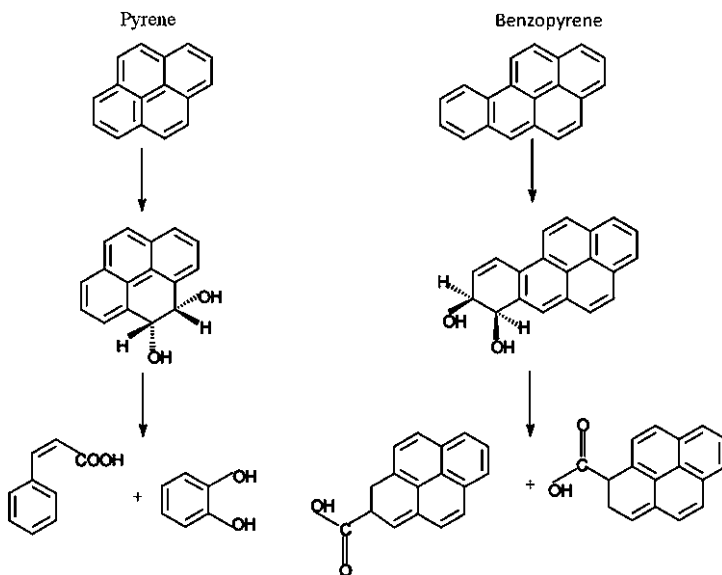


Fig. 15.2 Pathway of degradation of two significant PAH compounds

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-dihydroxypyrene 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 occurred 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-degrading 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 *nahR* 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 pKA1, 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 *nahG* (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-Ezike 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 *nahG*, 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-Ezike 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 fluoresces 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 *nahAc* genes from a miscellaneous bacterial population may be of related size when amplified with a particular set of *nahAc* 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.

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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

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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 monomer units that are metabolized by central metabolic

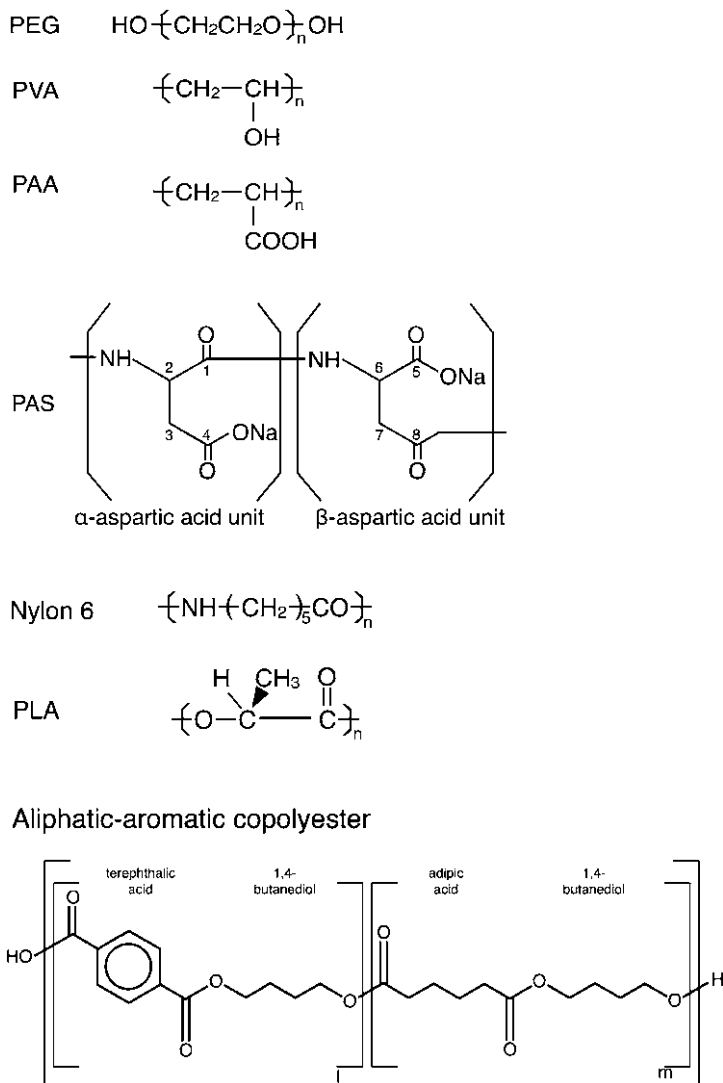


Fig. 16.1 Chemical structures of xenobiotic polymers discussed in this article

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
<i>A. Polyethers</i>			
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
Polybutylene oxide (PBO)	MW: 2,000	Exogenous	Dehydrogenase; bacteria
<i>B. Vinyl polymers</i>			
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	Exogenous	Bacteria
Butadiene	Tetramer–decamer		Bacteria
Styrene	Dimer		Bacteria
Acrylonitril	Trimer		Fungus
<i>C. Polyamides</i>			
Polyaspartate (PAS)	MW: ca. 20,000	Endogenous/exogenous	Hydrolases; bacteria
Nylon	Dimer–hexamer	Endogenous/exogenous	Hydrolases; bacteria
<i>D. Polyesters</i>			
Polycaprolactone (PCL)		Endogenous	Lipases and cutinases: bacteria and fungi
Polyactic acid (PLA)	Up to a few hundreds of thousands	Endogenous	Proteases and lipases (cutinases): actinomycetes and <i>Bacillus</i>

(continued)

Table 16.1 (continued)

Chemical structure	Biodegradable molecular size	Degradation mechanism	Relevant enzymes/ microorganism
Aliphatic		Endogenous	Lipases and cutinases; bacteria and fungi
Aromatic		Endogenous	Cutinases
Aliphatic-co- aromatic		Endogenous	Hydrolase; actinomycetes and <i>Bacillus</i> -related species
<i>E. Polyurethane</i>		?	?
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: $\text{HO}[\text{R}-\text{O}]_n\text{H}$ [$\text{R}=\text{CH}_2\text{CH}_2$ for PEG, $\text{CH}_2\text{CH}(\text{CH}_3)\text{CH}_2$ for polypropylene glycol (PPG), a polymer of 1,2-propylene oxide, $(\text{CH}_2)_4$ for polytetramethylene glycol (PTMG), and $\text{C}_2\text{H}_5(\text{CH}_2)$ 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 biore-resistant, 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

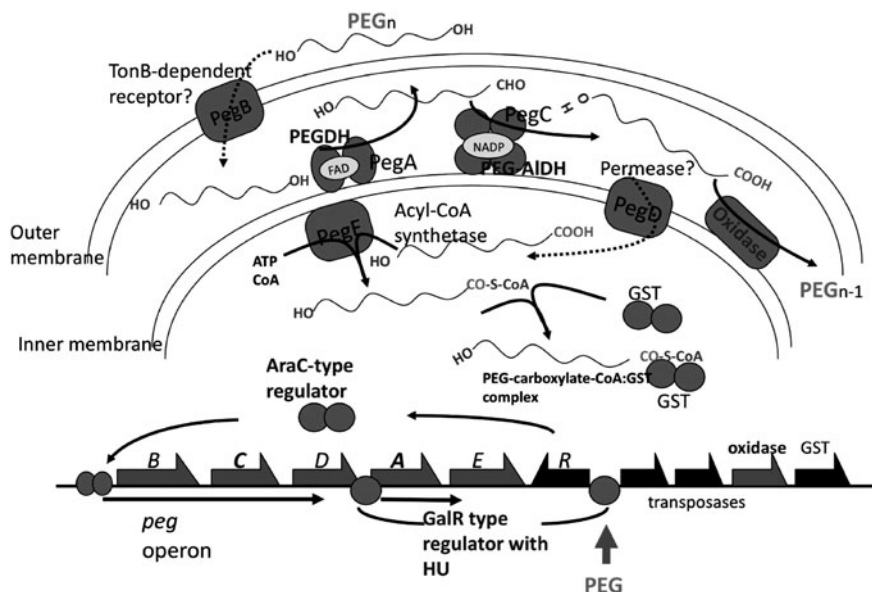


Fig. 16.2 The operonic structure of genes involved in PEG degradation and its regulation by PEG

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 *Stenotrophomonas 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 acetaldehyde. 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 quinoxinoprotein 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-quinoxinoprotein 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 maltophilia*, *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,

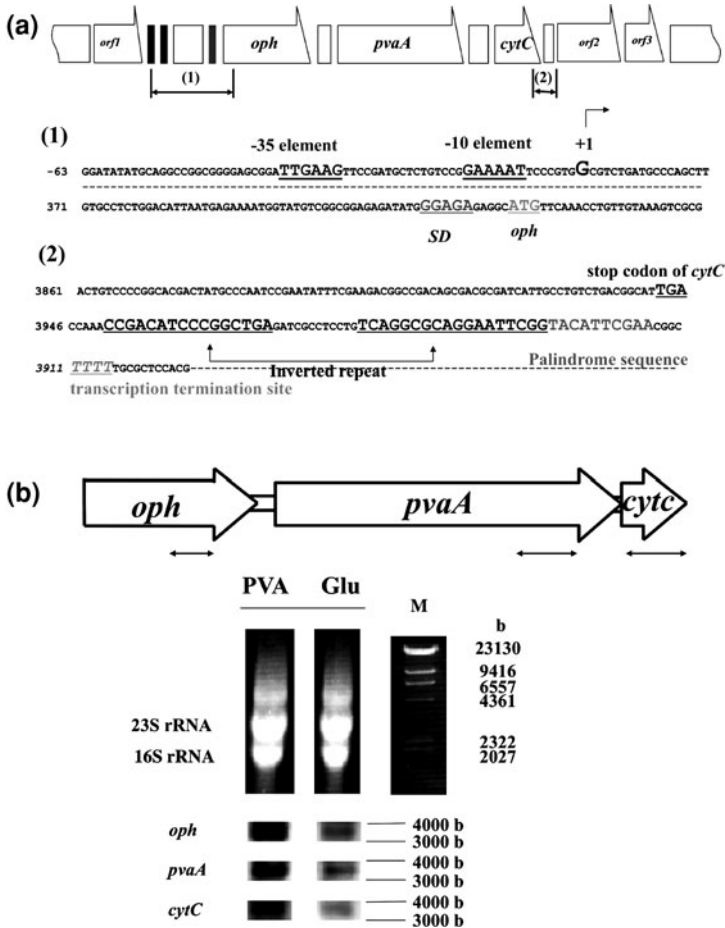


Fig. 16.3 The operonic structure of genes involved in PVA degradation and its expression. **a** The operonic structure of three genes; **b** the expression of three genes in PVA and glucose media. Symbols: *oph* oxiPVA hydrolase gene; *pvaA* PVA-DH gene; *cytC* cytochrome *c* gene

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 CO₂ 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 (Priyambada 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 ¹⁴CO₂ 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 *Escherichia 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 *Pseudonocardaceae* 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 (T_g: 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 enantioselectivity 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 chrysosporium*, 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 quinoxinoprotein 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, polystyrene, 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.

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Chapter 17

Microbial Degradation of Alkanes

S. N. Singh, B. Kumari and Shweta Mishra

17.1 Introduction

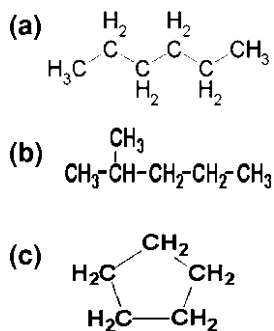
Petroleum hydrocarbons are introduced into the environment due to their extensive use as fuels and chemicals. Besides, leaks and accidental spills occur often during exploration, production, refining, transport and storage of petroleum and petroleum products which used to add an additional burden of 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 CO₂ 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

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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 erythropolis* 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, *Geobacillus 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 sparingly 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 O₂ 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

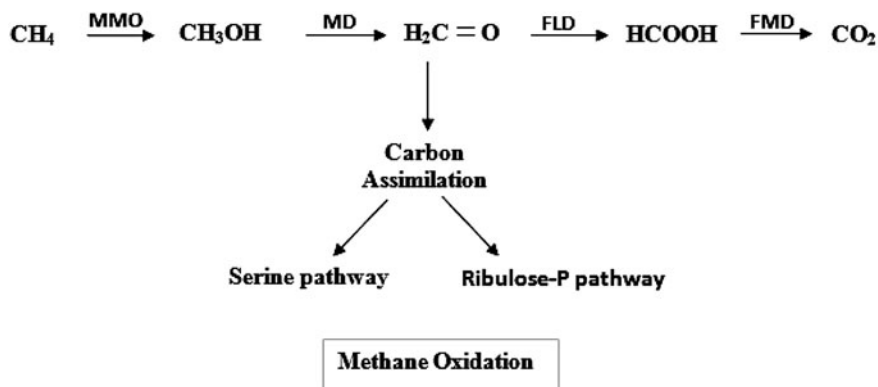


Fig. 17.2 Aerobic pathways of methane oxidation (after Rojo 2009)

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.

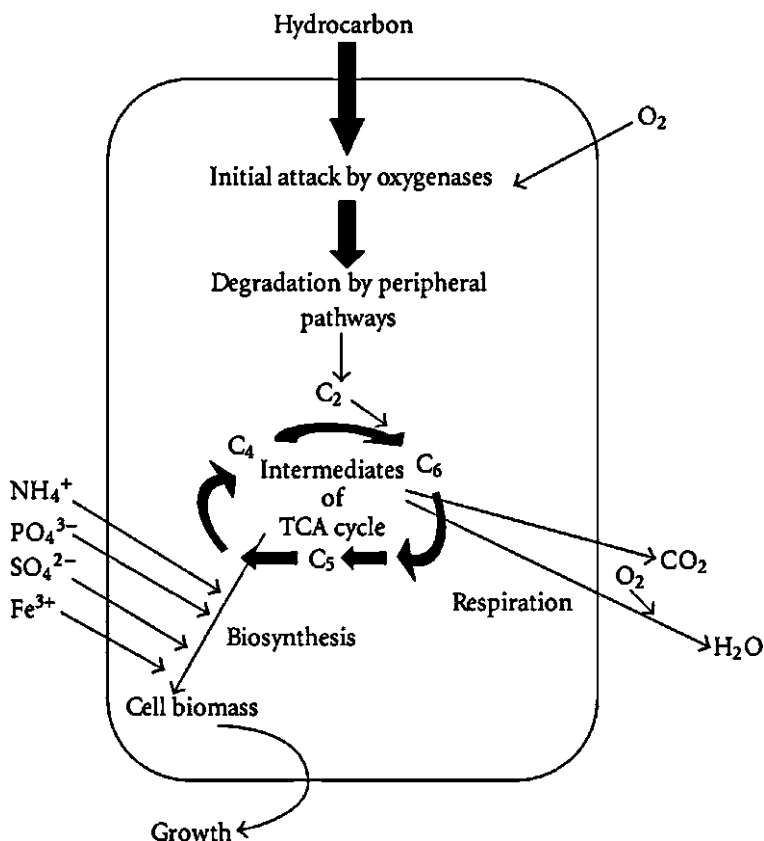
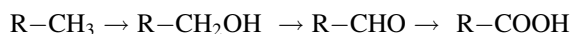


Fig. 17.3 Process of microbial aerobic degradation of hydrocarbons associated with growth process (after Fritsche and Hofrichter 2000)

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:



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.

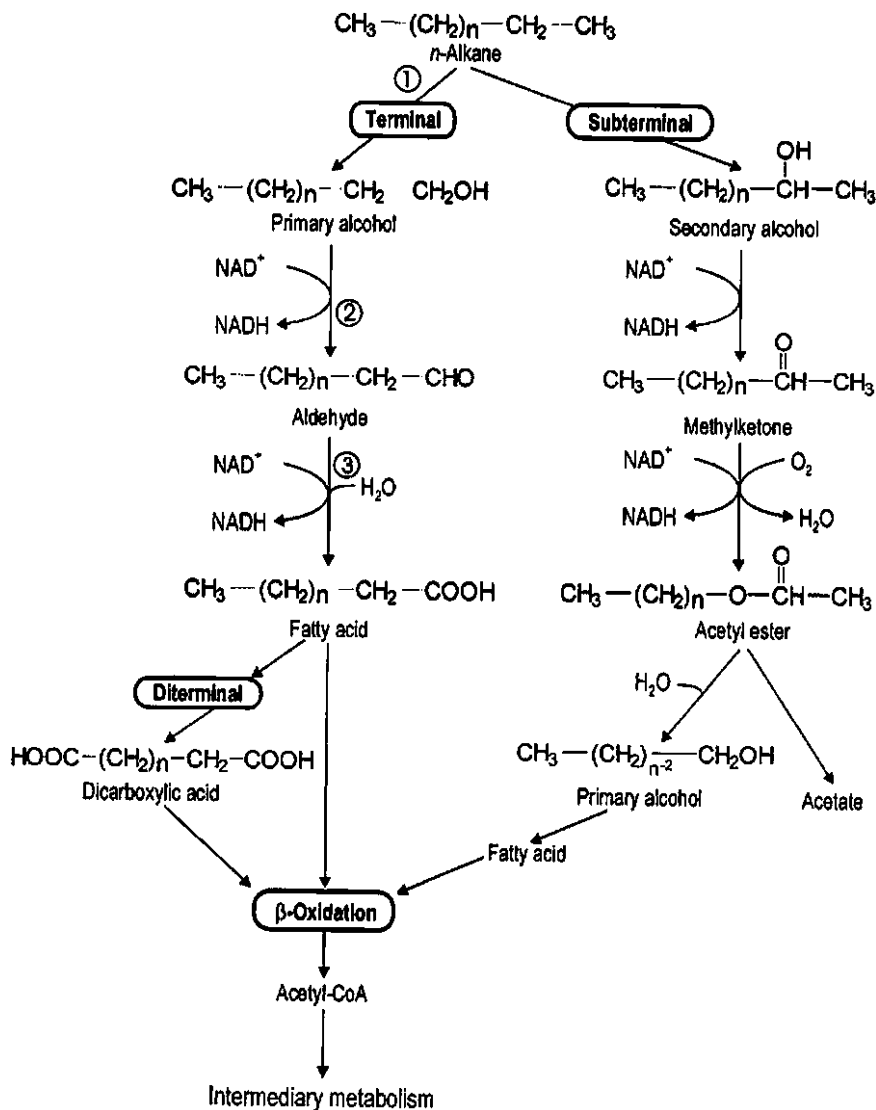


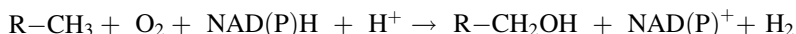
Fig. 17.4 Aerobic pathways of *n*-alkane degradation (after Fritsche and Hofrichter 2000)

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

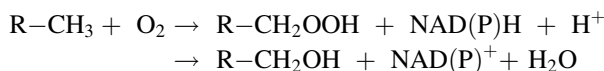
bypass. Similarly, a number of propane and butane utilizers have been reported that are also capable of growth on long chain alkanes, such as *n*-dodecane and *n*-hexadecane.

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:



2. Dioxygenase attack produces hydroperoxides, which are reduced to yield also alkan-1-ol:



3. Rarely, subterminal oxidation at C₂ 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).

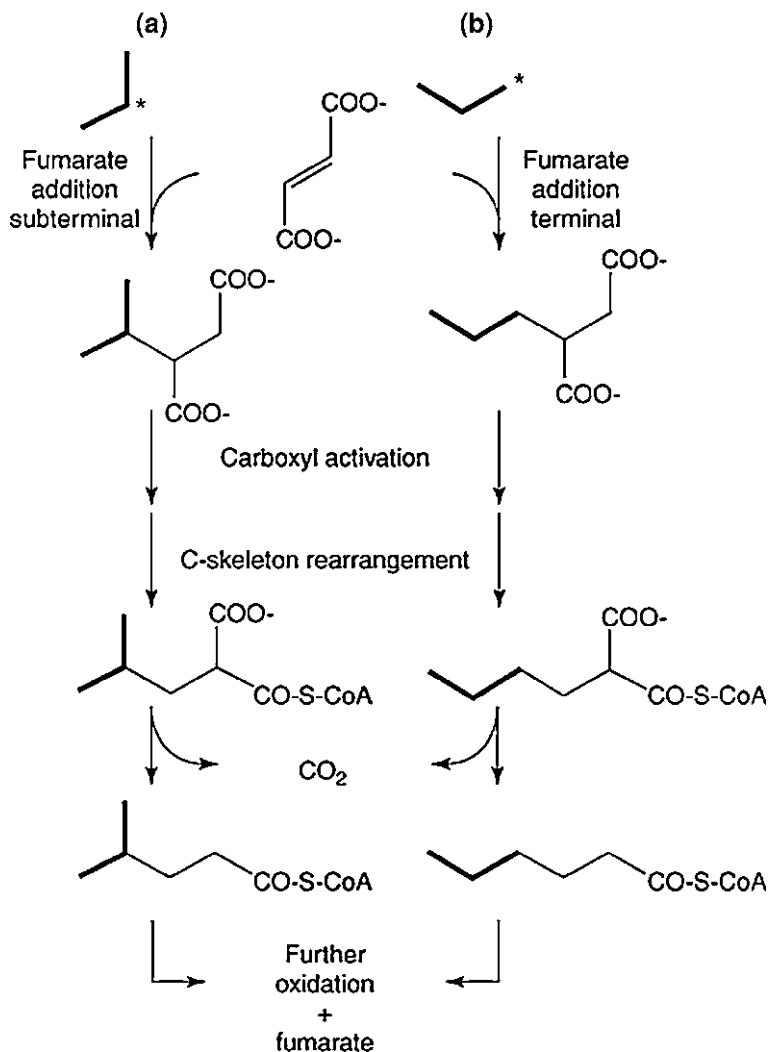


Fig. 17.5 Anaerobic activation of short chain alkanes by fumarate addition. The formed methylalkylsuccinates are activated by binding with acetyl-coenzyme A (CoA), which yields a thioester that undergoes C-skeleton rearrangement, followed by decarboxylation and β -oxidation. **a** Activation of the secondary 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 CO₂ with NO₃⁻ reduction to N₂, were compared for alkane metabolism (Fig. 17.6). Out of which,

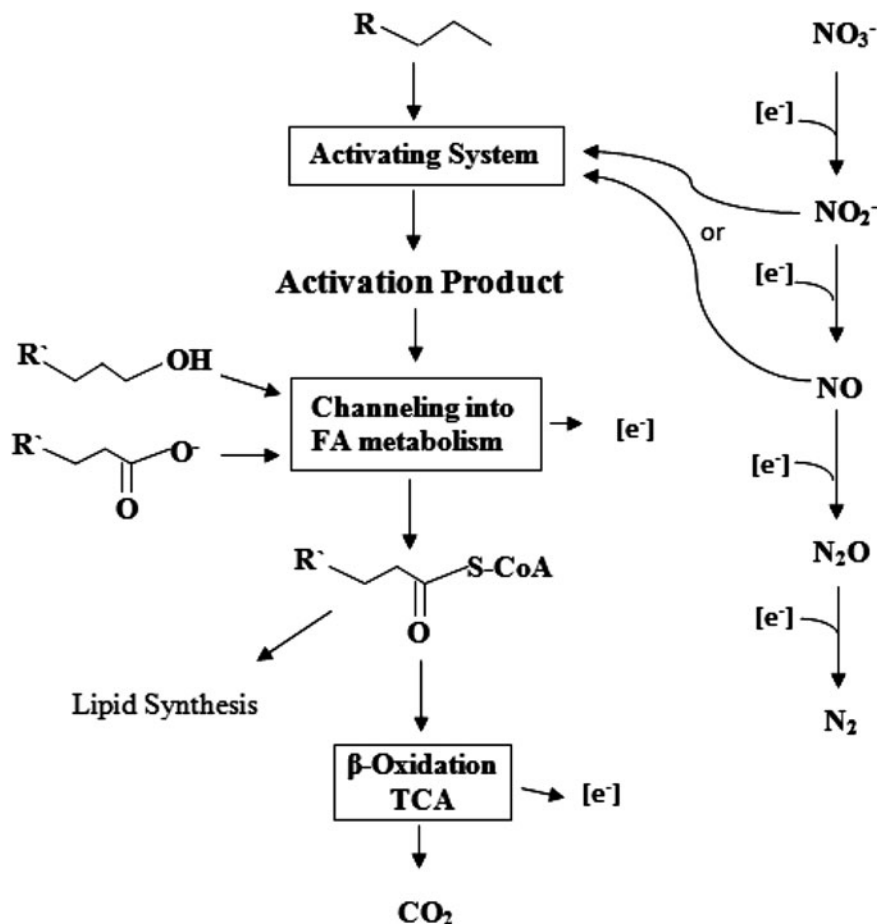


Fig. 17.6 Hypothetical involvement of denitrification intermediates in alkane activation. A small proportion of NO_2^- or NO is deviated from the respiratory chain for alkane activation. They may be used for activation indirectly (by yielding O_2 that is used by alkane monooxygenase; or by giving rise 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)

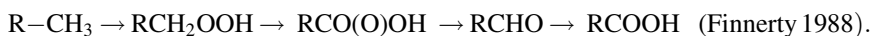
two strains HXN1 and OcN1 (both Betaproteobacteria) metabolized C6–C8 and C8–C12 alkanes, respectively. Both of them activated alkanes anaerobically in a fumarate-dependent reaction yielding alkylsuccinates as evidenced by metabolite and gene analyses. However, strain HdN1 was unique. It belonged to Gamma-proteobacteria 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 *Actinomyces*, 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-trimethyl dodecandioic 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:



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).

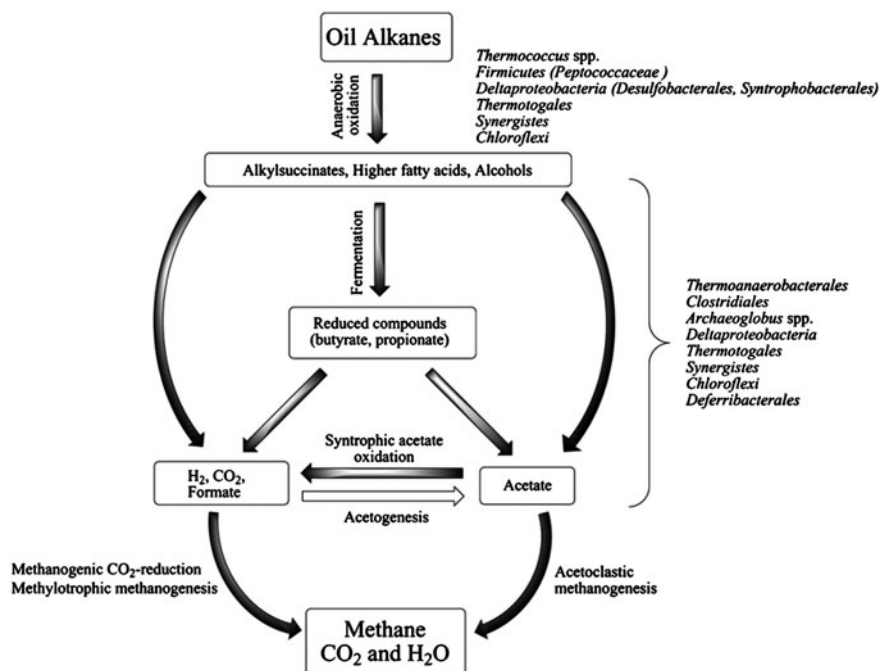


Fig. 17.7 Presumptive methanogenic degradation of oil alkanes (after Mbadinda et al. 2011)

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 CH₄ 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 CH₄ 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 CH₄ as energy source and found in methanotrophs in two forms pMMO (particulate Methane

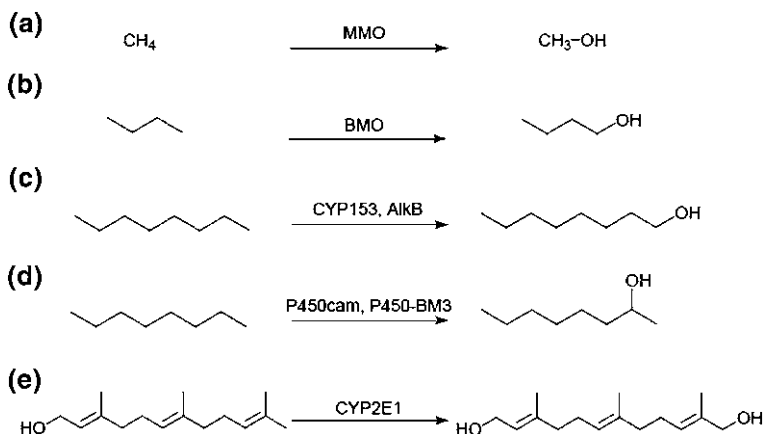


Fig. 17.8 **a** Methane to methanol by Methane Monooxygenase (MMO), **b** Butane to 1-butanol by Butane Monooxygenase (BMO), **c** Octane to 1-Octanol, **d** Octane to 2-Octanol, **e** Farnesol to 1-hydroxyfarnesol (after van Beilen and Funhoff 2005)

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, stearyl-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 heterocyclic compounds where as pMMO mediates the oxidation of a small group of alkanes (Murrell 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).

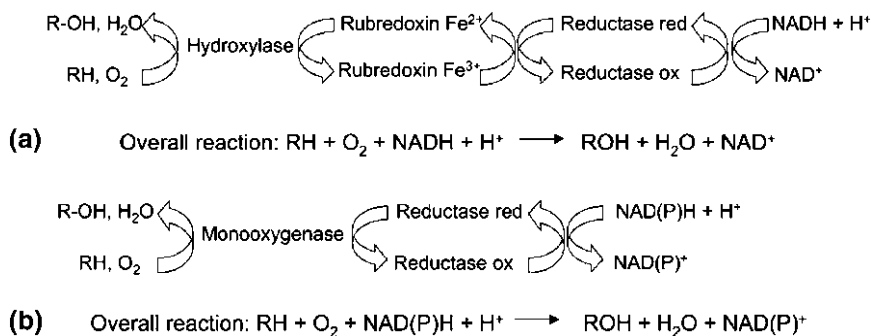


Fig. 17.9 Steps involved in the oxidation reaction catalysed by alkane hydroxylase (a) and methane monooxygenase and cytochrome P450 monooxygenase (b) (after Ayala and Torres 2004)

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 O₂-dependent activation of the alkane through a substrate radical intermediate (Shanklin et al. 1997; Bertrand et al. 2005). One of the O₂ atoms is transferred to the terminal methyl group of the alkane, rendering an alcohol, while the other one is reduced to H₂O 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, Cu²⁺-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 FADH₂, could serve as co-factor of the enzyme. Size of isolated enzyme was closer to that reported for *Fusarium 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	<i>Methylococcus capsulatus</i> <i>Methylisinus trichosporum</i> OB3b	C1–C10	Baik et al. (2003)
pMMO	All methanotrophs	C1–C5	Leieberman and Rosenzweig (2004)
Propane monooxygenase	<i>Pseudomonas butanovora</i> (ATCC 43655)	C2–C8	Kotani et al. (2003)
Butane monooxygenase	<i>Gordonia</i> sp. TYP	C3 and C13–C22	Sluis et al. (2002)
AlkB	<i>Acinetobacter</i> , <i>Alcanivorax</i> , <i>Burkholderia</i> , <i>Mycobacterium</i> , <i>Pseudomonas</i> , <i>Rhodococcus</i> etc.	C5–C16	Smits et al. (2002)
Cytochrome P450 (CYP153) monooxygenase	<i>Sphingomonas</i> sp. HXN-200, <i>Mycobacterium</i> sp. HXN1500 <i>Acinetobacter</i> sp. EB104	C4–C16	Maier et al. (2001)
Cytochrome P450 (CYP52) monooxygenase	<i>Candida maltosa</i> , <i>Candida tropicalis</i> , <i>Yarrowia lipolytica</i>	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. (Efroymsen 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 oleovorans* ATCC 29347 to test the ability to assimilate short/medium chain of 54 bacterial 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. Östeberg 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 soybeans, 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

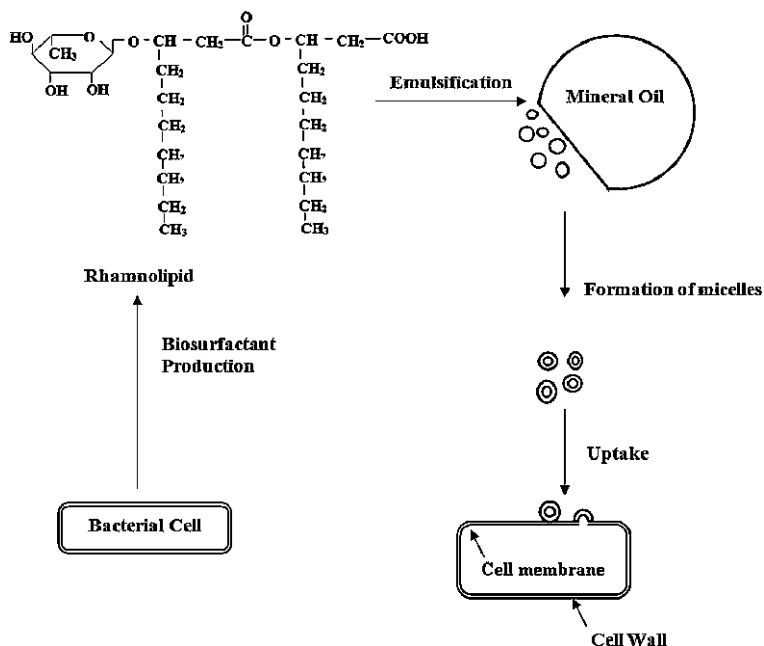


Fig. 17.10 Involvement of biosurfactants in the uptake of hydrocarbons and the emulsifying effect of a rhamnolipid produced by *Pseudomonas* spp. within the oil–water interphase and the formation of micelles (after Fritsche and Hofrichter 2000)

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 RRL 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.

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