Garima Kaushik *Editor*

Applied Environmental Biotechnology: Present Scenario and Future Trends

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Preface

Applied environmental biotechnology is the field of environmental science and biology that involves the use of living organisms and their by-products in solving environmental problems like waste and wastewaters. It includes not only the pure biological sciences such as genetics, microbiology, biochemistry, and chemistry but also subjects from outside the sphere of biology, such as chemical engineering, bioprocess engineering, information technology, and biophysics.

Cleaning up the contamination and dealing rationally with wastes is, of course, in everybody's best interests. Considering the number of problems in the field of environmental biotechnology and microbiology, the role of bioprocesses and biosystems for environmental cleanup and control based on the utilization of microbes and their products is highlighted in this work. Environmental remediation, pollution control, detection, and monitoring are evaluated considering the achievement as well as the perspectives in the development of environmental biotechnology. Various relevant articles are chosen up to illustrate the main areas of environmental biotechnology: industrial waste water treatment, soil treatment, oil remediation, phytoremediation, microbial electroremediation, and development of biofuels dealing with microbial and process engineering aspects. The distinct role of environmental biotechnology in future is emphasized considering the opportunities to contribute new approaches and directions in remediation of a contaminated environment, minimizing waste releases, and developing pollution prevention alternatives using the end-of-pipe technology. To take advantage of these opportunities, new strategies are also analyzed and produced. These methods would improve the understanding of existing biological processes in order to increase their efficiency, productivity, flexibility, and repeatability.

The responsible use of biotechnology to get economic, social, and environmental benefits is highly attractive since the past, such as fermentation products (beer, bread) to modern technologies like genetic engineering, rDNA technology, and recombinant enzymes. All these techniques are facilitating new trends of environment monitoring. The twenty-first century has found microbiology and biotechnology as an emerging area in sustainable environmental protection. The requirement of alternative chemicals, feedstocks for fuel, and a variety of commercial products has grown dramatically in the past few decades. To reduce the dependence on foreign exchange, much research

has been focussed on environmental biotechnology to develop a sustainable society with our own ways of recovery and reusing the available resources.

An enormous amount of natural and xenobiotic compounds are added to the environment every day. By exploring and employing the untapped potential of microbes and their products, there are possibilities of not only removing toxic compounds from the environment but also the conversion and production of useful end products. Basic methodologies and processes are highlighted in this book which will help in satisfying the expectations of different level of users/readers.

This work focuses on the alarming human and environmental problems created by the modern world, and thus provides some suitable solutions to combat them by applying different forms of environmental studies. With the application of environmental biotechnology, it enhances and optimizes the conditions of existing biological systems to make their course of action much faster and efficient in order to bring about the desired outcome. Various studies (genetics, microbiology, biochemistry, chemistry) are clubbed together to find solutions to environmental problems in all phases of the environment like, air, water, and soil. The 3R philosophy of waste reduction, reuse, and recycling is a universally accepted solution for waste management. As these are end-of-pipe treatments, the best approach is developing the approach of waste prevention through cleaner production. However, even after creation of waste the best solution to deal with is through biological means, and today by applying various interdisciplines we can create various by-products from this waste and utilize them best. Treatment of the various engineering systems presented in this book will show how an engineering formulation of the subject flows naturally from the fundamental principles and theories of chemistry, microbiology, physics, and mathematics and develop a sustainable solution.

The book introduces various environmental applications, such as bioremediation, phytoremediation, microbial diversity in conservation and exploration, in-silico approach to study the regulatory mechanisms and pathways of industrially important microorganisms, biological phosphorous removal, ameliorative approaches for management of chromium phytotoxicity, sustainable production of biofuels from microalgae using a biorefinary approach, bioelectrochemical systems (BES) for microbial electroremediation, and oil spill remediation.

This book has been designed to serve as a comprehensive environmental biotechnology textbook as well as a wide-ranging reference book. The authors thank all those who have contributed significantly in understanding the different aspects of the book and submitted their reviews, and at the same time hope that it will prove of equally high value to advanced undergraduate and graduate students, research scholars, and designers of water, wastewater, and other waste treatment systems. Thanks are also due to Springer for publishing the book.

Kishangarh, Rajasthan, India Garima Kaushik

Acknowledgments

Foremost, I must acknowledge the invaluable guidance I have received from all my teachers in my academic life. I also thank all my coauthors for their support, without which this book would have been impossible.

I thank my family for having the patience and taking yet another challenge which decreased the amount of time I spent with them. Especially, my daughter Ananya, who took a big part in that sacrifice, and also my husband Dr. Manish, who encouraged me in his particular way and assisted me in completing this project.

Speaking of encouragement, I must mention about my head of department and dean of Earth Sciences School, Central University of Rajasthan, Prof. K. C. Sharma, whose continuous encouragement and trust helped me in a number of ways in achieving endeavors like this.

I also thank my colleagues, Dr. Devesh, Dr. Sharmila, Dr. Ritu, and Dr. Dharampal for their support and invaluable assistance.

No one is a bigger source of inspiration in life than our parents. I have come across success and failures in my academic life but my parents have been a continuous source of encouragement during all ups and downs in my life. I really appreciate my in-laws for always supporting me throughout my career.

It will be unworthy on my part if I do not mention Prof. I. S. Thakur, my Ph.D. supervisor who gave me an opportunity to work, learn, and explore the subject knowledge under his guidance and leadership.

Thank you all for your insights, guidance, and support!

Garima Kaushik

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About the Editor

Dr. Garima Kaushik is currently working as Assistant Professor in Department of Environmental Science, School of Earth Science, Central University of Rajasthan. A gold medallist in B. Sc. and M.Sc. from University of Rajasthan, she obtained Ph.D. in the field of Environmental Biotechnology, from Jawaharlal Nehru University, New Delhi. She has also served as an Environmental Consultant to World Bank funded projects with government of Rajasthan, namely; Health Care Waste Management (HCWM) and Rajasthan Rural Livelihood Project (RRLP). Her areas of research interest are environmental microbiology, chiefly bioremediation of industrial effluents, biomedical waste management, enzyme kinetics, applications and bioprocess engineering. Another area of her research includes climate change and rural livelihoods and promotion of environmentally friendly activities in rural areas for adaptation to climate change. She is also pursuing her future research in the area on education for sustainable development.

Dr. Kaushik has published several research papers in the field of bioremediation, climate change adaptation in international and national journals and has contributed in organizing various conferences and seminars. She has also participated in various academic events at national and international level and is also the life member of many academic societies.

Abbreviations

µM	Micromolar
AAS	Atomic absorption spectrophotometer
ABTS	2,2′-azinodi-3-ethyl-benzothiazoline-6-sulfuric acid
ANOVA	Analysis of variance
APHA	American Public Health Association
ARDRA	Amplified ribosomal DNA restriction analysis
ATP	Adenosine triphosphate
BHC	Benzene hexachloride
BLAST	Basic local alignment search tool
BOD	Biological oxygen demand
CBD	Convention on Biological Diversity
CLPP	Community level physiological profiling
COD	Chemical oxygen demand
CPCB	Central Pollution Control Board
CU	Color unit
DAPI	Diamidino-2-phenylindole
DDT	Dichloro diphenyl trichloroethane
DEAE cellulose	Diethylaminoethyl cellulose
DGGE	Denaturing gradient gel electrophoresis
EEA	European Environment Agency
EPA	Environmental Protection Agency
FISH	Fluorescence in situ hybridization
FT-IR	Fourier transformation infrared spectroscopy
GC-MS	Gas chromatography and mass spectrometry
GMOs	Genetically modified organisms
HRT	Hydraulic retention time
IAS	In situ air sparging
IC	Ion chromatography
IR	Infra-red band
LMWOA	Low molecular weight organic acids
LNAPL	Light nonaqueous phase liquid
MEGAN	MEta Genome Analyzer
MOCB	Miniature oil containment boom
NADH	Nucleotide adenosine dihydride
NCBI	National Center for Biotechnology Information

PAH	Poly aromatic hydrocarbon
PCB	Polychlorinated biphenyl
PCDD	Polychlorinated dibenzodioxin
PCDF	Polychlorinated dibenzofuran
PCP	Pentachlorophenol
PGDB	Pathways/Genome Databases
RF	Radio frequency
RFLP	Restriction fragment length polymorphism
SSCP	Single strand conformation polymorphism
TCE	Trichloroethylene
UNCED	United Nations Conference on Environment and Development
UNESCO	The United Nations Organization for Education, Science and Culture
UVF	Ultraviolet Fluorescence Spectrometry
WF	Water footprint
WFCC	World Federation for Culture Collection
WNO	World Nature Organization

Bioremediation Technology: A Greener and Sustainable Approach for Restoration of Environmental Pollution

1

Shaili Srivastava

Abstract

Bioremediation has the potential technique to restore the polluted environment including water and soil by the use of living plants and microorganisms. The bioremediation technology is greener clean and safe technology for the cleanup of contaminated site. This chapter will focus on the biological treatment processes by microorganisms that currently play a major role in preventing and reducing the extent of organic and inorganic environmental contamination from the industrial, agricultural, and municipal waste. Bioremediation is concerned with the biological restoration of contaminated sites and content of the chapter also reflects the current trends of bioremediation technology and the limitations of bioremediation. Environmental genomics technique is the useful for the advanced treatment of waste site as well as genome-enabled studies of microbial physiology and ecology which are being applied to the field of bioremediation, and to anticipate additional applications of genomics that are likely in the near future.

Keywords

Bioremediation · Environment · Genomics · Microbes

1.1 Introduction

The organic and inorganic compounds are released during the production, storage, transport, and use of organic and inorganic chemicals into the environment every year as a result of various developmental activities. In some cases these releases are deliberate and well regulated (e.g., industrial emissions) while in other cases they are accidental (e.g., chemical or oil spills). Detoxification of the contaminated sites is expensive and time consuming by conventional chemical or physical methods. Bioremediation is a combination of two words, "bio," means living and "remediate" means to solve a problem or to bring the sites and affairs into the original state, and "bioremediate" means to use biological organisms to solve an environmental problem such as contaminated soil or ground water, through the technological innovations. The technique of bioremediation uses living microorganisms usually bacteria and fungi to remove pollutants from soil and water. This approach is potentially more

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cost-effective than traditional techniques like incineration of waste and carbon filtration of water.

Bioremediation technologies can be generally classified as in situ or ex situ. In situ bioremediation involves treating the contaminated material at the site while ex-situ involves removal of the contaminated material to be treated elsewhere. Some examples of bioremediation technologies are bioventing, landfarming, bioreactor, composting, bioaugmentation, rhizofiltration, and biostimulation.

However, not all contaminants are easily treated by bioremediation using microorganisms. For example, heavy metals such as cadmium and lead are not readily absorbed or captured by organisms. The assimilation of metals such as mercury into the food chain may worsen matters. Phytoremediation is useful in these circumstances, because natural plants or transgenic plants are able to bioaccumulate these toxins in their above-ground parts, which are harvested for removal. The heavy metals in the harvested biomass may be further concentrated by incineration or even recycled for industrial use. A wide range of bioremediation strategies is being developed to treat contaminated soils. In bioremediation, microorganism transform hazardous chemical compounds to nonhazardous end products, however, in phytoremediation plants are used for this purpose (Brar et al. 2006). Two basic methods are available for obtaining the microorganism to initiate the bioremediation: bioaugmentation—in which adapted and genetically coded toxicants degrading microorganism are added; biostimulation—which involves the injection of necessary nutrients to stimulate the growth of the indigenous microorganism.

The bioremediation systems in operation today rely on microorganisms native to the contaminated sites, encouraging them to work by supplying them with the optimum levels of nutrients and other chemicals essential for their metabolism. Thus, today's bioremediation systems are limited by the capabilities of the native microbes. However, researchers are currently investigating ways to augment contaminated sites with nonnative microbes, including genetically engineered microorganisms—especially suited to degrading the contaminants of concern at particular sites. It is possible that this process, known as bioaugmentation, could expand the range of possibilities for future bioremediation systems.

The effectiveness of bioremediation is mainly influenced by degradability and toxicity of the chemical compounds. Based on this the chemical may be divided into degradable and nontoxic, degradable and toxic, nondegradable and toxic, and nondegradable and nontoxic chemical compounds. The main goal of bioremediation can be fulfilled by enhancing the rate and extent of biodegradation of the pollutants, utilizing or developing microorganisms.

1.2 Current Practice of Bioremediation

The key players in bioremediation are bacteria—microscopic organisms that live virtually everywhere. Microorganisms are ideally suited to the task of contaminant destruction because they possess enzymes that allow them to use environmental contaminants as food and because they are so small that they are able to contact contaminants easily. In situ bioremediation can be regarded as an extension of the purpose that microorganisms have served in nature for billions of years: the breakdown of complex human, animal, and plant wastes so that life can continue from one generation to the next. Without the activity of microorganisms, the earth would literally be buried in wastes, and the nutrients necessary for the continuation of life would be locked up in detritus.

The goal in bioremediation is to stimulate microorganisms with nutrients and other chemicals that will enable them to destroy the contaminants. The bioremediation systems in operation today rely on microorganisms native to the contaminated sites, encouraging them to work by supplying them with the optimum levels of nutrients and other chemicals essential for their metabolism. Researchers are currently investigating ways to augment contained sites with nonnative microbes including genetically engineered microorganisms specially suited to degrading the contaminants of concern at particular sites. It is

possible that this process, known as bioaugmentation, could expand the range of possibilities for future bioremediation systems (USEPA 1987).

Regardless of whether the microbes are native or newly introduced to the site, an understanding of how they destroy contaminants is critical to understanding bioremediation. The types of microbial processes that will be employed in the cleanup dictate what nutritional supplements the bioremediation system must supply. Furthermore, the byproducts of microbial processes can provide an indication that the bioremediation is successful. Whether microorganisms will be successful in destroying man made contaminants in the subsurface depends on three factors: the type of organisms, the type of contaminant, and the geological and chemical conditions at the contaminated site. Biological and nonbiological measures to remedy environmental pollution are used the same way. All remediation techniques seek first to prevent contaminants from spreading. In the subsurface, contaminants spread primarily as a result of partitioning into ground water. As the groundwater advances, soluble components from a concentrated contaminant pool dissolve, moving forward with the groundwater to form a contaminant plume. Because the plume is mobile, it could be a financial, health, or legal liability if allowed to migrate off-site. The concentrated source of contamination, on the other hand, often has settled into a fixed position and in this regard is stable. However, until the source can be removed by whatever cleanup technology, the plume will always threaten to advance off-site.

Selection and application of a bioremediation process for the source or the plume require the consideration of several factors. The first factor is the goal for managing the site, which may vary from simple containment to meeting specific regulatory standards for contaminant concentrations in the groundwater and soil. The second factor is the extent of contamination. Understanding the types of contaminants, their concentrations, and their locations, is critical in designing in-situ bioremediation procedures. The third factor are the types of biological processes that are effective for transforming the contaminant. By matching established metabolic capabilities with the contaminants found, a strategy for encouraging growth of the proper organisms can be developed. The final consideration is the site's transport dynamics, which control contaminant from spreading and influence the selection of appropriate methods for stimulating microbial growth.

1.3 Microorganisms in Bioremediation

In microbial bioremediation, living microorganisms are used to convert complex toxic compounds into harmless by-products of cellular metabolism such as CO_2 and H_2O. However, in phytoremediation plants are used to remove contamination from the soil and water. In a nonpolluted environment, microorganisms are constantly at work, utilizing toxic compounds; however, most of the organisms die in contaminated sites. A few of them due to their inherent genetic material, grow, survive, and degrade the chemicals. The successful use of microorganisms in bioremediation depends on the development of a basic understanding of the genetics of a broad spectrum of microorganisms and biotechnological innovations. Pure, mixed, enriched, and genetically engineered microorganisms have been used for degradation of these compounds. Routes of degradation of the major natural compounds have been well established. The entire spectrum of microbial degradation is related to the breakdown of xenobiotic chemicals, which are nondegradable and is recalcitrant. A large number of microorganisms have been isolated in recent years that are able to degrade compounds that were previously considered to be nondegradable. This suggests that, under the selective pressure of environmental pollution, a microbial capacity for the degradation of recalcitrant xenobiotics is developing that might be harnessed for pollutant removal by biotechnological processes. Nevertheless, the fact that many pollutants persist in the environment emphasizes the current inadequacy of this catabolic capacity to deal with such pollutants.

1.3.1 Degradation by Fungi

The process of natural bioremediation of persistent compounds involves a range of microorganism. Most fungi are robust organisms and are generally more tolerant to a high concentration of polluting chemicals than bacteria. A variety of fungi have been used for degradation of pollutants in the environment. The contaminants present in water and soil from industrial and agriculture activities are degraded and utilized by fungi. But use of fungi for degradation of industrial pollutants such as chlorophenols, nitrophenols, and polyaromatic hydrocarbons are limited. In spite of the toxicity of the effluent and presence of chlorophenols, the microbial flora of tannery liquid wastes is relatively rich, with the *Aspergillus niger* group predominant. The extracellular enzymes and cell mass from the pregrown *Phanerochaete chrysosporium* cultures were used by researchers for the degradation of pentachlorophenol (PCP). The lignin degrading fungi *P. chrysosporium, Phanerochaete sordida, Trametes hirusta,* and *Ceriporiopsis subvermispora* were evaluated for their ability to decrease the concentration of pentachlorophenol.

Fungi are especially well suited to polycyclic aromatic hydrocarbon (PAH) degradation relative to other bacterial decomposers for a few reasons. They can degrade high molecular weight PAHs, whereas bacteria are best at degrading smaller molecules. They also function well in nonaqueous environments where hydrophobic PAHs accumulate; a majority of other microbial degradation occurs in aqueous phase. Also, they can function in the very low oxygen conditions that occur in heavily PAH-contaminated zones. Fungi possess these decomposing abilities to deal with an array of naturally-occurring compounds that serve as potential carbon sources. Hydrocarbon pollutants have similar or analogous molecular structures which enable the fungi to act on them as well. When an area is contaminated, the ability to deal with the contamination and turn it into an energy source is selected for the fungal population and leads to a population that is better able to metabolize the contaminant.

1.3.2 Degradation by Bacteria

Bacteria can be separated into aerobic types, which require oxygen to live, and anaerobic, which can live without oxygen. Aerobic bioremediation is usually preferred because it degrades pollutants 10–100 times faster than anaerobic bioremediation. Facultative types can thrive under both aerobic and anaerobic conditions. Certain bacteria belonging to *Bacillus* and *Pseudomonas* species have these desirable characteristics. They consume organic waste thousands of times faster than the types of bacteria that are naturally present in the waste. Bacteria, *Arthobacteria*, *Flavobacterium*, *Pseudomonas*, and *Sphingomonas*, have been isolated and applied for the degradation of chlorinated phenol and other toxic organic compounds. A number of bacteria viz., *Pseudomonas*, *Flavobacterium*, *Xanthomonas*, *Nocardia*, *Aeromonas*, and *Arthrobarterium* are known to utilize lignocellulosic components of the bleached plant effluent containing lignosulphonics and chlorinated phenols. One particularly promising mechanism for the detoxification of polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) is microbial reductive dechlorination. In current scenario research data suggested that, only a limited number of phylogenetically diverse anaerobic bacteria have been found that couple the reductive dehalogenation of chlorinated compounds the substitution of chlorine for a hydrogen atom to energy conservation and growth in a process called dehalorespiration. Microbial dechlorination of PCDDs occurs in sediments and anaerobic mixed cultures from sediments, but the responsible organisms have not yet been identified or isolated. Various microbial cultures capable of aerobic polychlorinated biphenyl (PCB) biodegradation have been isolated by researchers (Fetzner and Lingens 1994). Up to 85% degradation of Arochlors 1248 and 1242 has been shown. The more highly chlorinated 1254 and 1260 Arochlors have not shown significant aerobic biodegradation in the laboratory or in the field. Anaerobic degradation by dechlorination reactions is widespread even for the 1254 and 1260 Arochlors.

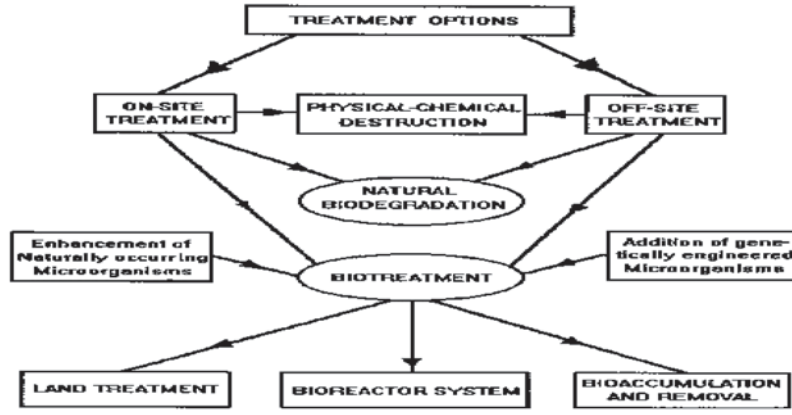

Fig. 1.1 In vivo and in vitro design strategies. (Source: Biotechnology in Medicine and Agriculture Principles and Practices)

1.4 Bioremediation Processes and Technologies

Bioremediation techniques are divided into three categories; in situ, ex situ solid, and ex situ slurry (Fig. 1.1). With in situ techniques, the soil and associated groundwater is treated in place without excavation, while it is excavated prior to treatment with ex-situ applications. The potential applications of biotechnology can be applied in terms of the contaminated matrix, degrading organisms of the contaminants, the type of reactor technology used, and the types of compounds present. The anaerobic and aerobic treatment methods applied for reducing the pollution load have been proved successful up to some extent. Pump-and-treat systems, which are applied to saturated-zone remediation, involve the removal, treatment, and return of associated water from a contaminated soil zone. The returned water is supplemented with nutrients and saturated with oxygen. Percolation consists of applying water, containing nutrients and possibly a microbial inoculum, to the surface of a contaminated area and allowing it to filter into the soil and mix with the groundwater, if present. Bioventing supplies air to an unsaturated soil zone through the installation of a well(s) connected to associated pumps and blowers, which draw a vacuum on the soil. Air sparging involves the injection of air into the saturated zone of a contaminated soil.

Ex situ solid-phase techniques consist of soil treatment units, compost piles, and engineered biopiles. Soil treatment units consist of soil contained and tilled (to supply oxygen) with application of water, nutrients, and possibly microbial inocula to soil. Compost piles consist of soil supplemented with composting material (i.e., wood chips, straw, manure, rice hulls, etc.) to improve its physical handling properties and its water- and air-holding capacities. Compost piles require periodic mixing to provide oxygen to the soil. Biopiles are piles of contaminated soil that contain piping to provide air and water. Ex situ solid applications involve the addition of water, nutrients, and sometimes addition of cultured indigenous microbes or inocula. They are often conducted on lined pads to ensure that there is no contamination of the underlying soil. Ex situ slurry techniques involve the creation and maintenance of soil–water slurry as the bioremediation medium. The slurry can be maintained in either a bioreactor or in a pond or lagoon. Adequate mixing and aeration are key design requirements for slurry systems. Nutrients and, perhaps, inoculum may be added to the slurry.

1.5 Monitoring the Efficacy of Bioremediation

The general acceptance of bioremediation technology as an environmentally sound and economic treatment for hazardous waste requires the demonstration of its efficacy, reliability and predictability, as well as its advantages over conventional treatments. An effective monitoring

design includes protocols for treatment-specific, representative sampling, control, and monitoring: these should take into account abiotic and biotic pollutant fate processes in all relevant process compartments. A number of well-established and novel chemical and molecular biological monitoring techniques and parameters are available (Schneegurt and Kulp 1998).

Bioremediation research is generally conducted at one of the three scales: laboratory, pilot scale, or field trial. To help ensure that results achieved at the first two scales can be translated to the field, the research program should be conceived as a continuum, with investigators working at each scale involved throughout the research conceptualization and planning process. The aim is to translate research findings from the laboratory into viable technologies for remediation in the field mechanisms of bioremediation that include bioaugmentation in which microbes and nutrients are added to the contaminated site or biostimulation in which nutrients and enzymes are added to supplement the intrinsic microbes. In the injection method, bacteria and nutrients are injected directly into the contaminated aquifer, or nutrients and enzymes, often referred to as "fertilizer," that stimulate the activity of the bacteria that are added. In soil remediation, usually nutrients and enzymes are added to stimulate the natural soil bacteria, though sometimes both nutrients and bacteria are added. When the treatment is stopped, the bacteria die. This technique works best on petroleum contamination.

1.6 Types of Bioremediation

1.6.1 Ex situ Bioremediation Bioreactors—Place of Action of Microbes

The most promising areas for technology development efforts as well as the critical issues have been identified, which must be addressed in moving from laboratory scale testing to the development of commercially viable technologies. Experiments are conducted by operating a laboratory scale completely mixed continuous flow activated sludge system to treat settled chrome tannery wastewater and to develop biokinetic parameters for the same. Occasionally, a large amount of phenol gets into the wastewater treatment plant in the phenol discharging industries, creating shock loading conditions on activated sludge systems. The immobilization of microbial cells on solid supports, is an important biotechnological approach introduced only recently in bioremediation studies. Treatment of industrial cells has also been attempted successfully. Bioreactors using immobilized cells have several advantages over conventional effluent treatment technologies. Various bioreactors have been designed for the application of microbial consortium for the treatment of tannery effluent. Upflow anaerobic sludge blanket (UASB) reactors were used to treat tannery waste water containing high sulfate concentration, competition between sulfate-reducing (SRB) and methane-producing (MPB) bacteria. Bench scale continuous flow activated sludge reactors were used to study the removal of PCP mixed with municipal wastewater.

Ex situ solid phase techniques consist of soil treatment units, compost piles, and engineered biopiles. Soil treatment units consist of soil contained and tilled (to supply oxygen) with application of water, nutrients, and possibly microbial inoculate to the soil. Compost piles consist of soil supplemented with composting material (i.e., wood chips, straw, manure, rice hulls, etc.) to improve its physical handling properties and its water- and air-holding capacities.

Flavobacterium cells are immobilized on polyurethane and the degradation activity of cells in semicontinuous batch reactor is studied. The ability of *Arthrobacter* cells to degrade PCP in mineral salt medium was evaluated for immobilized, nonimmobilized and coimmobilized cells. The immobilized cells were encapsulated in alginate. A microbial consortium able to degrade PCP in contaminated soil was used in a fed batch bioreactor. The microorganism in the biofilm employs natural biological processes to efficiently degrade complex chemical process and can remediate high volume of waste more cheaply than other available cleanup procedures (Figs. 1.2 and 1.3).

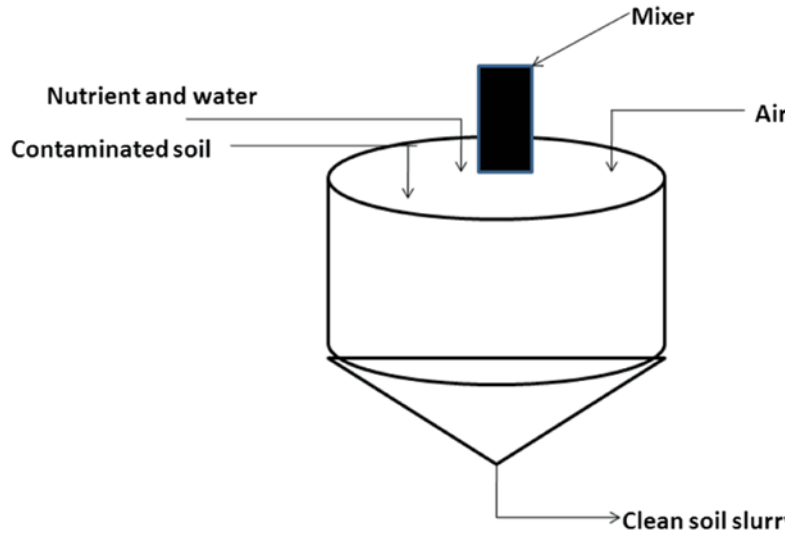

Fig. 1.2 Ex-situ bioremediation technique

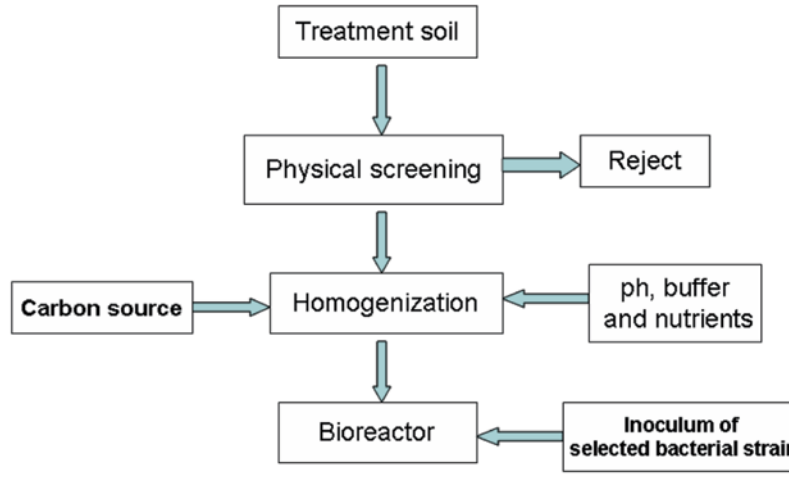

Fig. 1.3 Bioremediation treatment strategies in bioreactor. (Source: Biotechnology in Medicine and Agriculture Principles and Practices)

1.6.2 In situ Bioremediation

With in situ techniques, the soil and associated ground water is treated in place without excavation, while it is excavated prior to treatment with ex situ applications. Pump-and-treat systems, which are applied to saturated-zone remediation, involve the removal, treatment, and return of associated water from a contaminated soil zone. The returned water is supplemented with nutrients and saturated with oxygen. Percolation consists of applying water, containing nutrients and possibly a microbial inoculum, to the surface of a contaminated area and allowing it to filter into the soil and mix with the groundwater, if present. Bioventing supplies air to an unsaturated soil zone through the installation of a well(s) connected to associated pumps and blowers that draw a vacuum on the soil. Air sparging involves the injection of air into the saturated zone of a contaminated soil.

It has long been recognized that microorganisms have distinct and unique roles in the detoxification of polluted soil environments and, in recent years, this process has been termed as bioremediation or bioreclamation. The role of microorganisms and their limitations for bioremediation must be better understood so that they can be more efficiently utilized. Application of the principles of microbial ecology will improve

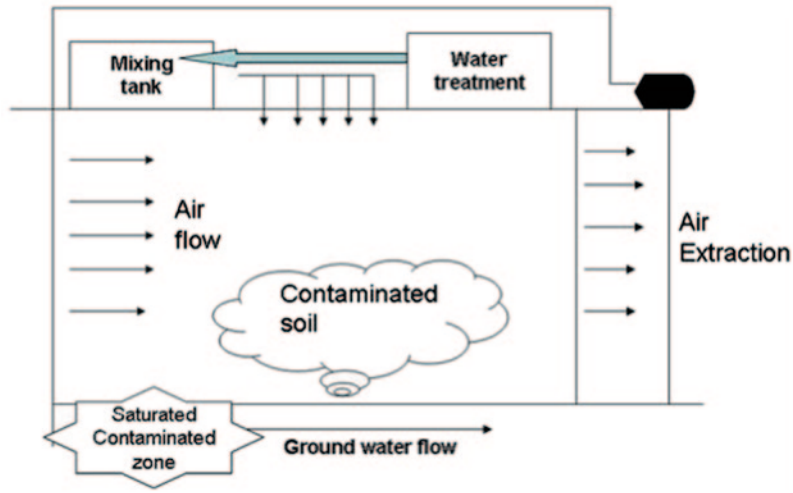

Fig. 1.4 In situ bioremediation of contaminated site. (Source: Biotechnology in Medicine and Agriculture Principles and Practices, Kumar et al. 2013)

methodology. The enhancement of microbial degradation as a means of bringing about the in-situ clean-up of contaminated soils has spurred much research. The rhizosphere, in particular, is an area of increased microbial activity that may enhance transformation and degradation of pollutants. The most common methods to stimulate degradation rates include supplying inorganic nutrients and oxygen, but the addition of degradative microbial inocula or enzymes as well as the use of plants should also be considered. Approximately 750 tons of soil, which had been contaminated by a wood preservative, was bioremediated in North Carolina using white rot fungi. Primary contaminants of concern at the site included pentachlorophenol and lindane. The field degradation of PCDDs and PCDFs in soil at a former wood treatment facility in North Carolina has been demonstrated. Toxaphene-contaminated soils present at a crop dusting facility in northern California were bioremediated using white rot fungi. The soils were mixed with a suitable substrate that had been inoculated with the fungi and placed in biotreatment cells. During operation of the project, toxaphene concentrations and environmental conditions (e.g., oxygen levels, moisture content, carbon dioxide levels, and temperature) within the treatment cells were monitored to track progress of fungal bioremediation. Chlorophenols are recalcitrant compounds that have been used for decades to impregnate wood, and many residues can be found in the environment long after the uses of chlorophenols have been discontinued. Chlorophenols are soluble in water and may leach from contaminated soil to groundwater. Therefore, the contaminated sites must be cleaned up to prevent further contamination into ground water. There have been only very limited field trials of PCB bioremediation. General Electric Corporation has carried out most in efforts to clean up their own contaminated sites. One in 1987 basically "land farmed" the PCB contaminated soils. They tilled the soils and added bacteria that degraded PCBs together with appropriate nutrients. The treatment result was less than laboratory results had shown and may have been due to bioavailability problems with the PCBs in the field (Fig. 1.4).

In situ Physical/Chemical Treatment

In situ Air Sparging (IAS)

IAS was first implemented in Germany in 1985 as a saturated zone remedial strategy. It involves the injection of pressurized air into the saturated zone. IAS induces a transient, air-filled porosity in which air temporarily displaces water as air bubbles migrate laterally from the sparge point and also vertically toward the water table. IAS induces a separate phase flux in which air travels in continuous, discrete air channels of relatively smaller diameter from the sparge point to the water table. Air movement through the saturated

zone typically does not occur as migrating air bubbles, with the exception of within homogeneous, highly permeable formations of unconsolidated course sand and gravel deposits. IAS enhances physical or biological attenuation processes and physical attenuation by volatilizing polycyclic hydrocarbons (PHCs) adsorbed to the formation matrix and stripping those dissolved in groundwater. IAS stimulates aerobic biodegradation of absorbed and dissolved-phase PHCs amenable to metabolism. Physical processes are a more significant attenuation mechanism for volatile PHCs, whereas biological processes are a more significant attenuation mechanism for PHCs of low volatility and varying aqueous solubilities.

Blast-Enhanced Fracturing

A technique used at sites with fractured bedrock formations to improve the rate and predictability of recovery of contaminated groundwater by creating "fracture trenches" or highly fractured areas through detonation of explosives in boreholes (shotholes). Blast-enhanced fracturing is distinguished from hydraulic or pneumatic fracturing in that the latter technologies do not involve explosives, are generally conducted in the overburden, and are performed within individual boreholes.

Directional Wells

Encompasses horizontal wells, trenched or directly drilled wells are installed at any nonvertical inclination for purposes of groundwater monitoring or remediation. This technology can be used in the application of various remediation techniques such as groundwater and/or nonaqueous phase liquid extraction, air sparging, soil vapor extraction, in situ bioremediation, in situ flushing, permeable reactive barriers, hydraulic and pneumatic fracturing, etc.

Groundwater Recirculation Well

This technique encompasses in situ vacuum, vapor, or air stripping, in-well vapor stripping, in-well aeration, and vertical circulation wells. Creation of groundwater circulation "cell" through injection of air or inert gas into a zone of contaminated groundwater through center of double-cased stripping well which is designed with upper and lower double-screened intervals.

Hydraulic and Pneumatic Fracturing

Techniques to create enhanced fracture networks to increase soil permeability to liquids and vapors and accelerate contaminant removal. The technique is especially useful for vapor extraction, biodegradation, and thermal treatments. Hydraulic fracturing involves injection of high pressure water into the bottom of a borehole to cut a notch; a slurry of water, sand and thick gel is pumped at high pressure into the borehole to propagate the fracture from the initial notch.

In situ Flushing

The technique is also known as injection/recirculation or in situ soil washing. General injection or infiltration of a solution into a zone of contaminated soil/groundwater, followed by down gradient extraction of groundwater and elutriate (flushing solution mixed with the contaminants) and above-ground treatment and/or reinjection. Solutions may consist of surfactants, cosolvents, acids, bases, solvents, or plain water.

In situ Stabilization/Solidification

The technique is also known as in situ fixation, or immobilization. The process of alteration of organic or inorganic contaminants to innocuous and/or immobile state by injection or infiltration of stabilizing agents into a zone of contaminated soil/groundwater. Contaminants are physically bound or enclosed within a stabilized mass (solidification), or their mobility is reduced through chemical reaction (stabilization).

Permeable Reactive Barrier

Encompasses passive barriers, passive treatment walls, treatment walls, or trenches. An in-ground trench is backfilled with reactive media to provide passive treatment of contaminated groundwater passing through the trench. Treatment wall is placed at strategic location to intercept the contaminant plume and backfilled with media such as zero-valent iron, microorganisms, zeolite,

activated carbon, peat, bentonite, limestone, saw dust, or other.

Thermal Enhancements

Use of steam, heated water, or radio frequency (RF) or electrical resistance (alternating current or AC) heating to alter temperature-dependent properties of contaminants In-situ to facilitate their mobilization, solubilization, and removal. Volatile and semivolatile organic contaminants may be vaporized; vaporized components then rise to the vadose zone where they are removed by vacuum extraction and treated.

Electrokinetics

An in situ process involving application of low intensity direct electrical current across electrode pairs implanted in the ground on each side of a contaminated area of soil, causing electroosmosis and ion migration. Contaminants migrate toward respective electrodes depending upon their charge. Process may be enhanced through use of surfactants or reagents to increase contaminant removal rates at the electrodes. Process separates and extracts heavy metals, radionuclides, and organic contaminants from saturated or unsaturated soils, sludges, and sediments.

Biological Treatment

Bioslurping

Use of vacuum-enhanced pumping to recover light nonaqueous phase liquid (LNAPL) and initiate vadose zone remediation through bioventing. In bioventing, air is drawn through the impacted vadose zone via extraction wells equipped with low vacuums to promote biodegradation of organic compounds.

Intrinsic Bioremediation

Natural, nonenhanced microbial degradation of organic constituents by which complex organic compounds are broken down to simpler, usually less toxic compounds through aerobic or anaerobic processes.

Monitored Natural Attenuation

Encompass intrinsic bioremediation process. Reliance on a variety of physical, chemical, or biological processes (within the context of a carefully controlled and monitored site cleanup approach) that, under favorable conditions, act without human intervention to reduce the mass, toxicity, mobility, volume, or concentration of contaminants in soil or groundwater.

Biocolloid Formation

Solid materials containing the basic elements produced by bacterial transformation assume a discrete particle which may be referred as biocolloids. Biological colloid is the negative charge that is usually present on the particle surface and forms the electric double layer surrounding the colloid particles. The biocolloid system may be appropriate in remediation of groundwaters and flowing surface water. The basic requirements would be the addition of bacteria and metabolism in the presence of the metal followed by recovery of the biocolloids. Biocolloid methods can be used for treatment of contaminated ground water in-situ in recovery of metals (Lovley 1995).

1.7 Limiting Factors of Intrinsic Biodegradation

Physical, chemical, and biological factors have complex effects on hydrocarbon biodegradation in soil. For this reason, experts frequently recommend that soil bioremediation projects begin with treatability studies to empirically test the biodegradability of the (Spormann and Widdel 2000) contaminants and to optimize treatment conditions. On the other hand, it is possible that the expense of such treatability studies could be avoided or minimized, if certain soil characteristics could be measured and used to predict the potential for bioremediation of a site, the kinetics of hydrocarbon removal or the optimal values for certain controllable treatment conditions. For example, certain cocontaminants such as heavy metals might preclude hydrocarbon bioremediation. Soil particle size distribution might partly

dictate the potential rate and extent of hydrocarbon removal.

Biodegradability potential depends on function of hydrocarbon type, size, structure, and concentration. Polycyclic hydrocarbon concentrations must be within specific ranges. If concentrations are too low, indigenous microbes may not use PHCs as a primary source of organic carbon in preference to dissolved organic carbon; however, PHCs may be inhibitory if concentrations are too high. The availability of biodegradable PHCs, microbial viability is controlled by a variety of factors including oxygen, inorganic nutrients, osmotic/hydrostatic pressure, temperature, and pH.

Indigenous microbes use ambient inorganic nutrients and organic carbon to maintain cell tissue and increase biomass. Consequently, inorganic nutrient availability is reflected in microbial population densities within contaminant plumes in which intrinsic biodegradation is occurring. Although other factors that influence microbial viability are directly related to population density as inorganic nutrient and organic carbon availability. Population density is an indicator of ambient organic carbon and inorganic nutrient availability. According to USEPA (1987), groundwater samples collected from background locations hydraulically up-gradient/side-gradient of petroleum contaminant plumes typically contain total population densities of about 102–103 colony forming units per milliliter (cfu/ml). Microbial population densities within petroleum contaminant plumes typically increase in response to supplemental organic carbon supplied by dissolved/adsorbed-phase PHCs. Hence, there is a positive correlation between population densities and PHC concentrations within contaminant plumes under conditions in which intrinsic biodegradation is occurring. This correlation indicates that indigenous heterotrophs are stimulated to metabolize PHCs, and that ambient inorganic nutrient levels are not limiting biodegradation in situ. Other potential limiting factors include hydrostatic pressure, temperature, and pH, however, these factors are frequently within the range of microbial viability and typically do not limit intrinsic biodegradation, with the possible exception of pH.

Researchers determined the effects on biodegradation kinetics of a number of factors, including (i) intrinsic soil properties (particle size, carbon content, water holding capacity), (ii) soil contaminants (petroleum hydrocarbons, heavy metals), (iii) controllable conditions (temperature, nitrogen, and phosphorous content), and (iv) inoculation with hydrocarbon-degrading microorganisms. The hydrocarbon-degrading soil microfloras of polar regions are limited by N and P, as are such microflora in warmer regions. Addition of nitrogen and phosphorous stimulate hydrocarbon degradation.

1.8 Phytoremediation

Phytoremediation, the use of plants for environmental restoration is an emerging cleanup technology to exploit plant potential to remediate soil and water contaminated with a variety of compounds, several technological subsets have been proposed. Phytoextraction is the use of higher plants to remove inorganic contaminants, primarily metals, from polluted soil. In this approach, plants capable of accumulating high levels of metals are grown in contaminated soil. At maturity, metal-enriched above-ground biomass is harvested and a fraction of soil–metal contamination is removed. Plants have a natural propensity to take up metals. Some, such as Cu, Co, Fe, Mo, Mn, Ni, and Zn, are essential mineral nutrients. Others, however, such as Cd and Pb, have no known physiological activity. Perhaps, not surprisingly, phytoremediation as an environmental cleanup technology was initially proposed for the remediation of metal-contaminated soil. The general use of plants to remediate environmental media through in-situ processes which includes rhizofiltration (absorption, concentration, and precipitation of heavy metals by plant roots), phytoextraction (extraction and accumulation of contaminants in harvestable plant tissues such as roots and shoots), phytotransformation (degradation of complex organic molecules to simple molecules which are incorporated into plant

tissues), phytostimulation or plant-assisted bioremediation (stimulation of microbial and fungal degradation by release of exudates/enzymes into the root zone), and phytostabilization (absorption and precipitation of contaminants, principally metals, by plants). A wide range of organic and inorganic contaminants; most appropriate for sites where large volumes of groundwater with relatively low concentrations of contaminants must be remediate to strict standards. Most effective where ground-water is within 10 ft of the ground surface, and soil contamination is within 3 ft of the ground surface.

Use of native plants in phytoremediation provides advantages over other species and helps bring back the heritage of flora lost through human activity. In addition to restoring biodiversity in areas that have been disturbed, remediating superfund sites using native species provides for wildlife habitat enhancement and conservation and saves money over alternative cleanup methods. Unlike many introduced species, once established, native plants do not require fertilizers, pesticides, or watering. As encouraged by the Superfund Redevelopment Initiative, use of native plants in site restoration may serve to restore wetlands and other habitats and create nature parks, sanctuaries, and other green areas.

Phytoremediation is the use of specialized plants to clean up polluted soil. While most of the plants exposed to high levels of soil toxins will get injured or die, scientists have discovered that certain plants are resistant and even a smaller group actually thrive. Both groups of plants are of interest to researchers, but the thriving plants show a particular potential for remediation because it has been shown that some of them actually transport and accumulate extremely high levels of soil pollutants within their bodies. They are therefore aptly named hyperaccumulators.

Hyperaccumulators already are being used throughout the country to help clean up heavy metal-polluted soil. Heavy metals are some of the most stubborn soil pollutants. They can bond very tightly to soil particles, and they cannot be broken down by microbial processes. Most heavy metals are also essential plant nutrients, so plants have the ability to take up the metals and transport them throughout their bodies. However, on polluted soil, the levels of heavy metals are often hundreds of times greater than normal, and this overexposure is toxic to the vast majority of plants. Hyperaccumulators, on the other hand, actually prefer these high concentrations. Essentially, hyperaccumulators are acting as natural vacuum cleaners, sucking pollutants out of the soil and depositing them in their above-ground leaves and shoots. Removing the metals is as simple as pruning or cutting the hyperaccumulators' above-ground mass, not excavating tons of soil. Resistant, but not hyperaccumulating, plants also have a role in phytoremediation. Organic toxins, those that contain carbon such as the hydrocarbons found in gasoline and other fuels, can be broken down by microbial processes. Plants play a key role in determining the size and health of soil microbial populations. All plant roots secrete organic materials that can be used as food for microbes, and this creates a healthier, larger, more diverse, and active microbial population, which in turn causes a faster breakdown of pollutants. Resistant plants can thrive on sites that are often too toxic for other plants to grow. They in turn give the microbial processes the boost they need to remove organic pollution more quickly from the soil.

Both forms of phytoremediation have the added benefit of not disturbing the soil. While excavation is an effective way to get rid of pollution, it removes the organic matter rich topsoil and, because of the use of heavy machinery, compact the soil that is left behind. Phytoremediation does not degrade the physical or chemical health of the soil. Actually, it creates a more fertile soil. Soil organic matter is increased as a result of root secretions and falling stems and leaves, and the roots create pores through which water and oxygen can flow. Additionally, few would argue that a dusty excavation site is more aesthetically pleasing than a nicely planted field.

However, there are many limitations to phytoremediation. It is a slow process that may take many growing seasons before an adequate reduction of pollution is seen, whereas soil excavation and treatment clean up the site quickly. Also, hyperaccumulators can be a pollution

hazard themselves. For instance, animals can eat the metal rich hyperaccumulators and cause the toxins to enter the food chain. If the concentration of metals in the plants is thought to be high enough to cause toxicity, there must be a way to segregate the plants from humans and wildlife, which may not be an easy task. Additionally, phytoremediation is in its infancy, and its effectiveness in cleaning up various toxins compared to conventional means of treatment is not always known. However, with more research and practice, the practicality of using phytoremediation should increase.

Phytostabilization aims to retain contaminants in the soil and prevent further dispersal. Contaminants can be stabilized in the roots or within the rhizosphere. Revegetation of mine tailings is a common practice to prevent further dispersal of contaminants. Mine tailings have been stabilized using commercially available varieties of metal tolerant grasses such as *Agrostis tenuis* cv. *Goginan*

Phytodegradation involves the degradation of organic contaminants directly, through the release of enzymes from roots, or through metabolic activities within plant tissues (Fig. 1.5). In phytodegradation organic contaminants are taken up by roots and metabolized in plant tissues to less toxic substances. Phytodegradation of hydrophobic organic contaminants have been particularly successful. Poplar trees (*Populus* sp.) have been used successfully in phytodegradation of toxic and recalcitrant organic compounds.

Phytovolatilization involves the uptake of contaminants by plant roots and its conversion to a gaseous state, and release into the atmosphere. This process is driven by the evapotranspiration of plants. Plants that have high evapotranspiration rate are sought after in phytovolatilization (Fig. 1.5). Organic contaminants, especially volatile organic compounds (VOCs) are passively volatilized by plants. For example, hybrid poplar trees have been used to volatilize trichloroethylene (TCE) by converting it to chlorinated acetates and CO_2. Metals such as Se can be volatilized by plants through conversion into dimethylselenide [$Se(CH_3)_2$]. Genetic engineering has been used to allow plants to volatilize specific contaminants. For example, the ability of the tulip tree (*Liriodendron tulipifera*) to volatilize methyl-Hg from the soil into the atmosphere (as Hg_0) was improved by inserting genes of modified *Escherichia coli* that encode the enzyme mercuric ion reductase (merA).

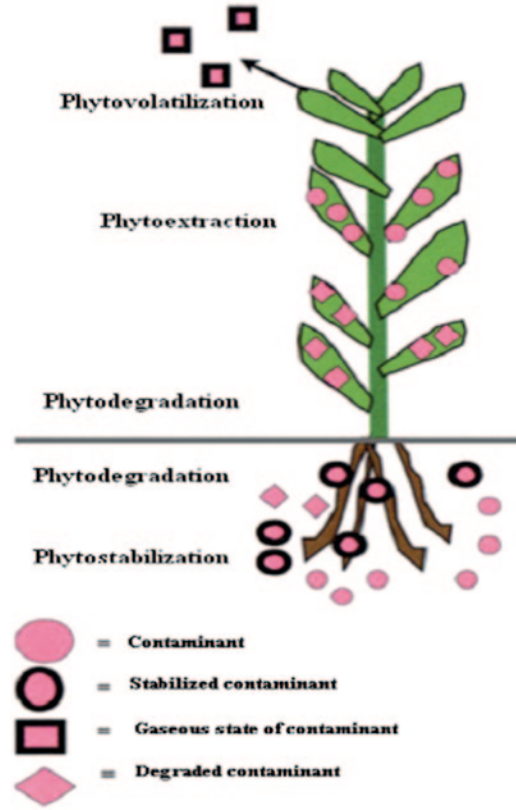

Fig. 1.5 Schematic model of different phytoremediation technologies involving removal and containment of contaminants. (Source: Greipsson 2011)

Phytoextraction uses the ability of plants to accumulate contaminants in the above-ground, harvestable biomass. This process involves repeated harvesting of the biomass in order to lower the concentration of contaminants in the soil. Phytoextraction is either a continuous process (using metal-hyperaccumulating plants, or fast growing plants), or an induced process (using chemicals to increase the bioavailability of metals in the soil). Continuous phytoextraction is based on the ability of certain plants to gradually accumulate contaminants (mainly metals) into their biomass.

Certain plants can hyperaccumulate metals without any toxic effects. These plants are adapted to naturally occurring, metalliferous soils. More than 400 plant species can hyperaccumulate various metals. However, most plants can only hyperaccumulate one specific metal.

Hyperaccumulating plants can contain more than 1% of a metal in their dry biomass. For example, the hyperaccumulating plant *Berkheya coddii* was found to contain as much as 3.8% of Ni in the dry, above-ground biomass, when grown in contaminated soil. It is possible to extract metals from the harvested biomass in a process termed phytomining. The underlying mechanism of hyper-accumulation of metals in plants is the overexpression of genes that regulate cell membrane transporters. These include the Cu-transporter (COPT1) and Zn-transporter (ZNT1). The main limitations on the use of hyperaccumulating plants in phytoextraction are slow growth and low biomass production. The effectiveness of phytoextraction is a function of a plant's biomass production and the content of contaminants in the harvested biomass.

Therefore, fast-growing crops that accumulate metals have a great potential in phytoextraction. The use of crops in phytoextraction can be improved by manipulation of their associated soil microbes. Inoculation of plant growth-promoting bacteria (PGPR) and arbuscular mycorrhizal fungi (AMF) can increase plant biomass. The AMF–plant symbiosis usually results in reduced accumulation of metals in the above-ground biomass of plants. Therefore, suppressing AMF activity, by using specific soil fungicides, has resulted in increased metal accumulation in plants. The role of AMF in regulating metal uptake by plants appears to vary depending on numerous factors, such as AMF populations, plant species, nutrient availability, and metal content in the soil. Also, this regulation of AMF is usually metal-specific; where the uptake of essential metals is generally increased, but the uptake of nonessential metals is inhibited. However, exceptions have been found where AMF increases uptake of Ni, Pb, and As in plants. Induced phytoextraction involves the use of fast-growing crops and chemical manipulation of the soil. Low bioavailability of metals in the soil is a limiting factor in phytoextraction. The bioavailability of metals can be increased by the use of synthetic chelates such as ethylene diamine tetracetic acid (EDTA) or acidifying chemicals (e.g., NH_4SO_4). The use of synthetic chelates increases the absorption of metals to the root and the translocation of metals from the roots to the foliage. The timing of chelate application is critical, and should ideally take place at the peak of biomass production. The effectiveness of using EDTA was demonstrated by growing corn (*Zea mays*) in Pb-contaminated soil treated with 10 mmol kg^{-1} EDTA. This resulted in a high accumulation of Pb (1.6% of shoot dry weight), and facilitated the translocation of Pb from the roots to the foliage. Some drawbacks of using synthetic chelates in phytoremediation are the result of increased solubility of the metals within the soil. In turn, this increases the risk of metal migration through the soil profile and into the groundwater. However, a possible solution is to treat contaminated soil ex-situ in a confined site with an impervious surface. Also, periodic application of low doses of synthetic chelates reduces the risk of metal migration.

1.9 Molecular Approach of Bioremediation

Microbial removal of contaminants from the environment often takes place without human intervention. This has been termed intrinsic bioremediation. Relying on intrinsic bioremediation is increasingly the bioremediation option of choice if it can be shown that the contamination does not pose an immediate health threat and it remains localized. If the rate of intrinsic bioremediation is too slow, then environmental conditions can be manipulated to stimulate the activity of microorganisms that can degrade or immobilize the contaminants of concern. Engineered bioremediation strategies include: the addition of electron donors or acceptors that will stimulate the growth or metabolism of microorganisms that are involved in the bioremediation processes; the addition of nutrients that limit the growth or activity of the

microorganisms; and amendments to microorganisms with desired bioremediation capabilities.

The 16S rRNA Approach A significant advance in the field of microbial ecology was the finding that the sequences of highly conserved genes that are found in all microorganisms, most notably the 16S rRNA genes could provide a phylogenetic characterization of the microorganisms that comprise microbial communities. This was a boon to the field of bioremediation because it meant that by analyzing 16S rRNA sequences in contaminated environments, it was possible to determine definitively the phylogenetic placement of the microorganisms that are associated with bioremediation processes.

Analysis of Genes Involved in Bioremediation Examining the presence and expression of the key genes involved in bioremediation can yield more information on microbial processes than analysis of 16S rRNA sequences. In general, there is a positive correlation between the relative abundance of the genes involved in bioremediation and the potential for contaminant degradation. However, the genes for bioremediation can be present but not expressed. Therefore, there has been an increased, emphasis on quantifying the levels of mRNA for key bioremediation genes. Often, increased mRNA concentrations can be, at least qualitatively, associated with higher rates of contaminant degradation. For example, the concentrations of mRNA for *nahA*, a gene involved in aerobic degradation of naphthalene were positively correlated with rates of naphthalene degradation in hydrocarbon-contaminated soil. The reduction of soluble ionic mercury, Hg(II), to volatile Hg(0), is one mechanism for removing mercury from water; the concentration of mRNA for *merA*, a gene involved in Hg(II) reduction was highest in mercury contaminated waters with the highest rates of Hg(II) reduction. However, the concentration of *merA* was not always proportional to the rate of Hg(II) reduction illustrating that factors other than gene transcription can control the rates of bioremediation processes. Highly sensitive methods that can detect mRNA for key bioremediation genes in single cells are now available. This technique, coupled with 16S rRNA probing of the same environmental samples, could provide data on which phylogenetic groups of organisms are expressing the genes of interest.

Application of Genomics Although the molecular techniques have outlined to improve our understanding of bioremediation, investigations in this field are on the cusp of a new era which promises for the first time to provide a global insight into the metabolic potential and activity of microorganisms living in contaminated environments. This is the "genomics era" of bioremediation. With the application of genome-enabled techniques to the study of not only pure cultures, but also environmental samples, it will be possible to develop the models that are needed to model microbial activity predicatively under various bioremediation strategies (Fig. 1.6).

The application of genomics to bioremediation initially revolutionized the study of pure cultures, which serve as models for important bioremediation processes (Nierman and Nelson 2002). Complete, or nearly complete, genome sequences are now available for several organisms that are important in bioremediation (Table 1.1). Whole genome sequencing is especially helpful in promoting the understanding of bioremediation-relevant microorganisms, whose physiology has not previously been studied in detail. For example, as noted earlier, molecular analyses have indicated that *Geobacter* species are important in the bioremediation of organic and metal contaminants in subsurface environments. The sequencing of several genomes of microorganisms of the genus *Geobacter*, as well as closely related organisms, has significantly altered the concept of how *Geobacter* species function in contaminated subsurface environments. For instance, before the sequencing of the *Geobacter* genomes, *Geobacter* species were thought to be nonmotile, but genes encoding flagella were subsequently discovered in the *Geobacter* genomes. Further investigations revealed

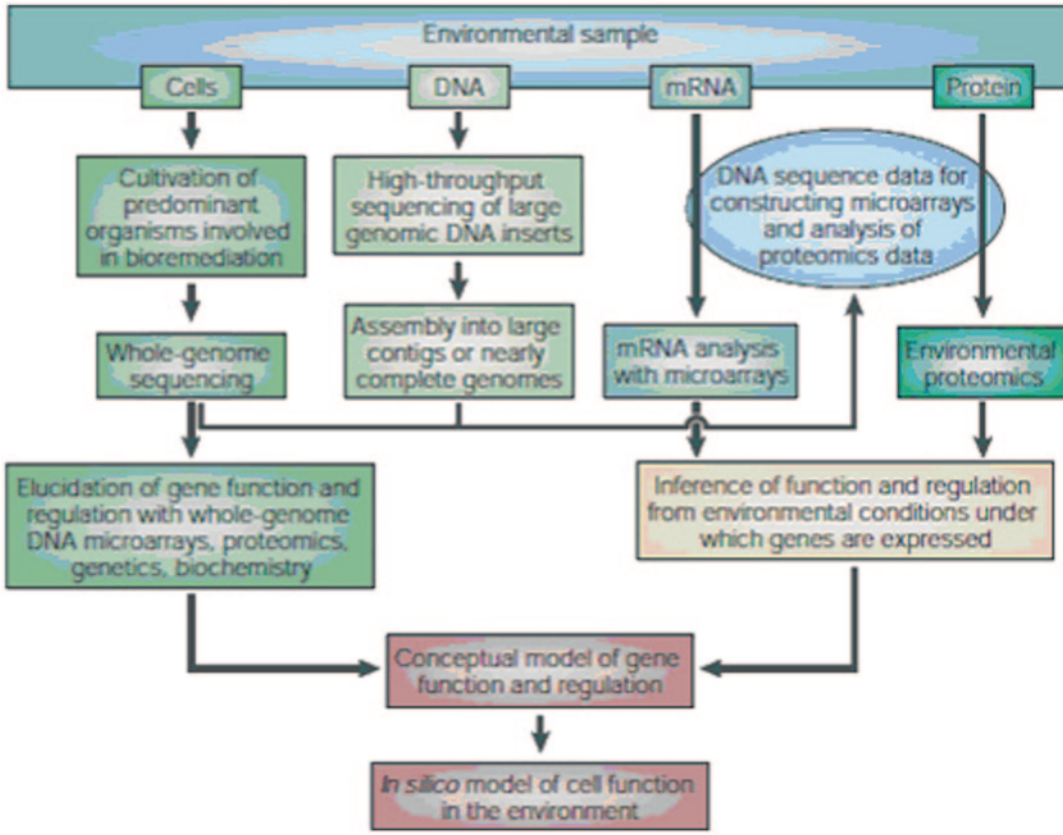

Fig. 1.6 Genome-enabled techniques contribute to the development of models of how microorganisms function in contaminated environments. (Source: Derek R. Lovley 2003 Nature Reviews)

that *Geobacter metallireducens* specifically produces flagella only when the organism is growing on insoluble Fe(III) or Mn(IV) oxides. Genes for chemotaxis were also evident in the *Geobacter* genomes, and experimental investigations have revealed that *G. metallireducens* has a novel chemotaxis to Fe(II),which could help guide it to Fe(III) oxides under anaerobic conditions (Nevin and Lovley 2002). Pili genes are present and are also specifically expressed during growth on insoluble oxides. Genetic studies have indicated that the role of the pili is to aid in attachment to Fe(III) oxides, as well as facilitating movement along sediment particles in search of Fe(III) (Fig. 1.7).

This energy-efficient mechanism for locating and reducing Fe(III) oxides in *Geobacter* species contrasts with the strategies for Fe(III) reduction in other well-studied organisms, such as *Shewanella* and *Geothrix* species. These other organisms release Fe(III) chelators, which solubilize Fe(III) from Fe(III) oxides, and electron shuttling compounds, which accept electrons from the cell surface and then reduce Fe(III) oxides. These strategies make it possible for *Shewanella* and *Geothrix* species to reduce Fe(III) without directly contacting the Fe(III) oxide.

Table 1.1 Examples of genomes available for microorganisms relevant to bioremediation

Microorganism	Relevance to bioremediation
Dehalococcoides ethanogenes	Reductive dechlorination of chlorinated solvents to ethylene. The 16S rRNA gene *ethanogenes s*equence of *D. ethanogenes* is closely related to sequences that are enriched in subsurface environments in which chlorinated solvents are being degraded
Geobacter sulfurreducens, Geobacter metallireducens	Anaerobic oxidation of aromatic hydrocarbons and reductive precipitation of uranium. *Sulfurreducens,* 16S rRNA gene sequences closely related to known *Geobacter* species predominate during anaerobic in situ bioremediation of aromatic hydrocarbons and uranium
Rhodopseudomonas	Main organism for elucidating pathways of anaerobic metabolism of aromatic *palustris* compounds, and regulation of this metabolism.
Pseudomonas putida	Metabolically versatile microorganism capable of aerobically degrading a wide variety of organic contaminants. Excellent organism for genetic engineering of bioremediation capabilities
Dechloromonas aromatic	Representative of ubiquitous genus of perchlorate-reducing microorganisms and capable of the anaerobic oxidation of benzene coupled to nitrate reduction
Desulfitobacterium hafniense	Reductive dechlorination of chlorinated solvents and phenols. *Desulfitobacterium* species are widespread in a variety of environments
Desulfovibrio vulgaris	Shown to reductively precipitate uranium and chromium. An actual role in contaminated environments is yet to be demonstrated
Shewanella oneidensis	A closely related *Shewanella* species was found to reduce U(VI) to U(IV) in culture, but *Shewanella* species have not been shown to be important in metal reduction in any sedimentary environments
Deinococcus radiodurans	Highly resistant to radiation and so might be genetically engineered for bioremediation of highly radioactive environments

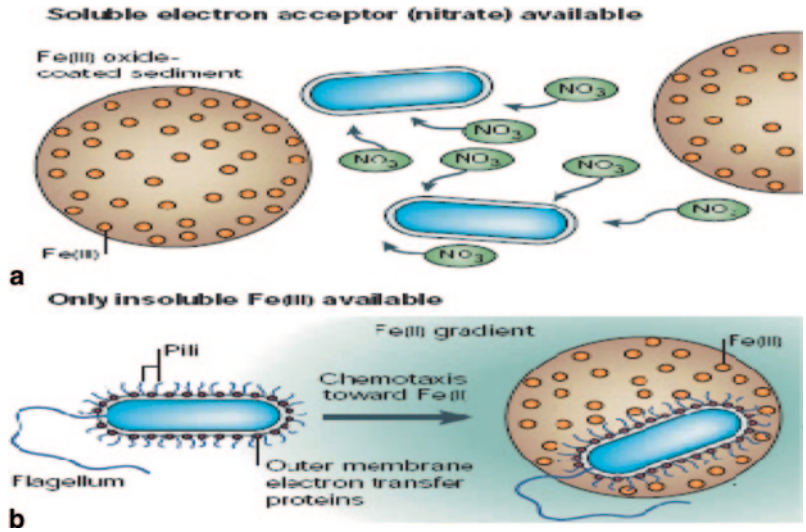

Fig. 1.7 Genome-derived model for physiological differences in *Geobacter* during growth on soluble electron acceptors or insoluble Fe(III) oxide. (Source: Derek R. Lovley 2003, Nature Reviews)

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Bioremediation of Industrial Effluents: Distillery Effluent

2

Garima Kaushik

Abstract

Distilleries are one of the most polluting industries generating enormous amount of wastewater from which an average of 10–15 L of effluent is released with the production of 1 L of alcohol. The distillery wastewater known as spent wash is characterized by its dark brown color, high temperature, low pH, and high percentage of dissolved organic and inorganic matter. It also contains nearly 2 % of the dark brown recalcitrant pigment called melanoidin which imparts dark brown color to the effluent. Various physical, chemical, and alternate treatment methods have been adopted for the removal of color from this wastewater. But these methods only change the form of contaminants rather than degrading them completely.

Biological methods produce relatively little amount of product after treatment by resolving a large amount of organism elements into carbon dioxide to be stabilized, or by removing organic matters contained in wastewater with the generation of methane gas. In the biological treatment methods, pollutants in wastewater can be resolved, detoxified, and separated by using mainly microorganisms. Due to the relatively low cost and the variations of work progress, the biological methods have been most widely used all over the world. A number of fungi, bacteria, yeast, and algae have been reported to have effluent treatment capabilities by the process of absorption, adsorption, and enzymatic degradation techniques. Toxicity studies of the biologically treated wastewaters also suggested that the process is efficient enough to reduce the toxicity of the spent wash by around 80 %. Hence, compared to the common and expensive physical or chemical ways for decolorization, an efficient bioremediation system has been found successful through biosorption and enzymatic ways of decolorization.

Keywords

Biodegradation · Distillery wastewater · Melanoidin

2.1 Introduction

Alcohol distilleries in India are one of the most polluting industries; in addition, they are high consumers of raw water. In India, major distill-

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Fig. 2.1 Detailed process of alcohol production

eries are an agro-based industry with around 300 units located mainly in rural, sugarcane-growing regions. The total installed capacity is 3250 million L alcohol per annum with an estimated production of 2300.4 million L in 2006–2007 (Ethanol India 2007). Bioethanol is produced worldwide for beverage, industrial, chemical, and some fuel use, by fermenting agricultural products such as molasses, sucrose-containing juices from sugarcane or sugarbeets, potatoes, fruits, and grains (notably maize, wheat, grain sorghum, barley, and rye). With growing population, industrialization, and energy consumption, coupled with an increasing reliance on fossil fuels, the energy security needs of the world continue to escalate.

2.2 Critical Review

2.2.1 Process of Ethanol Production

Alcohol manufacture in distilleries consists of four main steps, viz., feed preparation, fermentation, distillation, and packaging (Fig. 2.1).

a. Feed Preparation

Ethanol can be produced from a wide range of feedstock. These include sugar-based (cane and beet molasses, cane juice), starch-based (corn, wheat, cassava, rice, barley), and cellulosic (crop residues, sugarcane bagasse, wood, municipal solid wastes) materials. In general, sugar-based feedstock containing readily available fermentable sugars are preferred while Indian distilleries almost exclusively use sugarcane molasses The composition of molasses varies with the variety of cane, the agroclimatic conditions of the region, sugar manufacturing process, and handling and storage (Godbole 2002).

b. Fermentation

Yeast culture is prepared in the laboratory and propagated in a series of fermenters. The feed is inoculated with about 10% by volume of yeast (*Saccharomyces cerevisiae*) inoculum. This is an anaerobic process carried out under controlled conditions of temperature and pH wherein reducing sugars are broken down to ethyl alcohol and carbon dioxide. The reaction is exothermic. To maintain the temperature be-

tween 25 and 32 °C, plate heat exchangers are used; alternatively some units spray cooling water on the fermenter walls. Fermentation can be carried out in either batch or continuous mode. Fermentation time for batch operation is typically 24–36 h with an efficiency of about 95%. The resulting broth contains 6–8% alcohol. The sludge (mainly yeast cells) is separated by settling and discharged from the bottom, while the cell free fermentation broth is sent for distillation.

c. Distillation

Distillation is a two-stage process and is typically carried out in a series of bubble cap fractionating columns. The first stage consists of the analyzer column and is followed by rectification columns. The cell free fermentation broth (wash) is preheated to about 90 °C by heat exchange with the effluent (spent wash) and then sent to the degasifying section of the analyzer column. Here, the liquor is heated by live steam and fractionated to give about 40–45% alcohol. The bottom discharge from the analyzer column is the spent wash. The alcohol vapors are led to the rectification column where by reflux action, 96% alcohol is tapped, cooled, and collected. The condensed water from this stage, known as spent lees is usually pumped back to the analyzer column.

d. Packaging

Rectified spirit (~96% ethanol by volume) is marketed directly for the manufacture of chemicals such as acetic acid, acetone, oxalic acid, and absolute alcohol. Denatured ethanol for industrial and laboratory use typically contains 60–95% ethanol as well as between 1–5% each of methanol, isopropanol, methyl isobutyl ketone (MIBK), ethyl acetate, etc. (Skerratt 2004). For beverages, the alcohol is matured and blended with malt alcohol (for manufacture of whisky) and diluted to requisite strength to obtain the desired type of liquor. This is bottled appropriately in a bottling plant. Anhydrous ethanol for fuel-blending applications (power alcohol) requires concentration of the ethanol to >99.5 wt% purity.

Table 2.1 Wastewater generation in various operations in distillery unit. (Tewari et al. 2007)

Distillery operations	Average wastewater generation[a] (kLD/distillery)	Specific wastewater generation (kL wastewater/kL alcohol)
Spent wash (distillation)	491.9	11.9
Fermenter cleaning	98.2	1.6
Fermenter cooling	355.1	2.0
Condenser cooling	864.4	7.9
Floor wash	30.8	0.5
Bottling plant	113.8	1.3
Others[b]	141.6	1.2

[a] Data based on 36 distilleries, with average installed capacity of 53.5 kLD

[b] Domestic wastewater in sugar-distillery complex, boiler-blow down, leakages, and laboratory

The quantum and characteristics of wastewater generated at various stages in the manufacturing process are provided in Tables 2.1 and 2.2, respectively. The main source of wastewater generation is the distillation step wherein large volumes of dark brown effluent (termed as spent wash, stillage, slop, or vinasse) is generated in the temperature range of 71–81 °C (Yeoh 1997; Nandy et al. 2002; Patil et al. 2003). The characteristics of the spent wash depend on the raw material used (Mall and Kumar 1997), and also it is

Table 2.2 Typical characteristics of distillery wastewater streams. (Tewari et al. 2007)

Parameter	Spent wash	Fermenter cooling	Fermenter cleaning	Condenser cooling	Fermenter wash	Bottling plant
Color	Dark brown	Colorless	Colorless	Colorless	Faint	Colorless
pH	4–4.5	6.26	5.0–5.5	6.8–7.8	6	7.45
Total solids (mg/L)	100,000	1000–1300	1000–1500	700–900	550	400
Suspended solids (mg/L)	10,000	220	400–600	180–200	300	100
BOD (mg/L)	45,000–60,000	100–110	500–600	70–80	15	5
COD (mg/L)	80,000–120,000	500–1000	1200–1600	200–300	25	15

BOD biochemical oxygen demand, *COD* chemical oxygen demand

estimated that 88 % of the molasses constituents end up as waste (Jain et al. 2002).

The spent wash is the most polluting stream and contains practically all unfermentable soluble matter present in the molasses. Apart from the extremely high chemical oxygen demand (COD) and biochemical oxygen demand (BOD) load, the dark color is also a key concern. This dark color is mainly imparted by melanoidins that are low and high molecular weight polymers formed as one of the final products of Maillard reaction, which is a nonenzymatic browning reaction resulting from the reaction of reducing sugars and amino compounds (Martins and van Boekel 2004). This reaction proceeds effectively at temperatures above 50 °C and pH 4–7. These are complex organic compounds, when released in environment without treatment, react with a wide variety of other chemicals in presence of light and heat to form highly toxic and recalcitrant compounds (Kinae et al. 1981; Zacharewski et al. 1995). Thus, it is obligatory to treat the effluent before disposal into the environment.

2.3 Bioremediation

Generally, methods of treating wastewater include physical–chemical methods and biological methods. Methods such as sedimentation, flotation, screening, adsorption, coagulation, oxidation, ozonation, electrolysis, reverse osmosis, ultrafiltration, and nanofiltration technologies have been used for treatment of suspended solids, colloidal particles, floating matters, colors, and toxic compounds (Pokhrel and Viraraghavan 2004). The drawbacks of the physical–chemical methods include high costs and the need to re-treat the products, which further increases the cost of treatment. Biological method produces relatively little amount of product after treatment by resolving a large amount of organism elements into carbon dioxide to be stabilized, or by removing organic matters contained in wastewater with the generation of methane gas. In the biological treatment method, pollutants in wastewater can be resolved, detoxified, and separated by using mainly microorganisms. Due to the relatively low cost and the variations of work progress, the biological methods have been most widely used all over the world.

2.4 Treatment of Distillery Spent Wash

Biological treatment can be divided into aerobic and anaerobic depending on the availability of oxygen. Aerobic treatment involves activated sludge treatment, aerated lagoons, and aerobic biological reactors. Anaerobic filter, upflow sludge blanket (UASB), fluidized bed, anaerobic lagoon, and anaerobic contact reactors are anaerobic processes, that are commonly used to treat distillery mill effluents. Among these treatments one thing is common, use of microbes (Pokhrel and Viraraghavan 2004). A number of fungi, bacteria, yeast, and algae have been reported to have effluent-treatment capabilities.

2.4.1 Decolorization of Effluent by Fungi

In recent years, several basidiomycetes and ascomycetes type fungi have been used in the decolorization of wastewaters from distilleries. Filamentous fungi have lower sensitivity to variations in temperature, pH, nutrients, and aeration, and have lower nucleic acid content in the biomass (Knapp et al. 2001). *Coriolus* sp. no. 20, in class basidiomycetes, was the first strain for the application of its ability to remove melanoidins from molasses wastewater (Watanabe et al. 1982). Published papers report the use of wide variety of fungi like *Aspergillus fumigatus* G-2-6 (Ohmomo et al. 1987), *Emericella nidulans var. lata* (Kaushik and Thakur 2009a), *Geotrichum candidum* (Kim and Shoda 1999), *Trametes* sp. (González et al. 2000), *Aspergillus niger* (Patil et al. 2003), *Citeromyces* sp. (Sirianuntapiboon et al. 2003), *Flavodon flavus* (Raghukumar et al. 2004), and *Phanerochaete chrysosporium* (Thakkar et al. 2006) for decolorization of distillery mill effluent.

White rot fungi is another group of widely exploited microorganism in distillery effluent bioremediation. White rot fungi produce various isoforms of extracellular oxidases including laccases, manganese peroxidases and lignin peroxidase, which are involved in the degradation of various xenobiotic compounds and dyes. Another important mechanism involved in decolorization of the distillery mill effluent by fungi is adsorption.

2.4.2 Decolorization of Effluent by Bacteria

Different bacterial cultures capable of both bioremediation and decolorization of distillery spent wash have been isolated. Different researchers have reported isolation of various bacterial strains acclimatized on higher concentrations of distillery mill effluent. These are *Lactobacillus hilgardii* (Ohmomo et al. 1988), *Bacillus* sp. (Kambe et al. 1999; Kaushik and Thakur 2009b), *Pseudomonas putida* (Ghosh et al. 2002), *Bacillus thuringiensis* (Kumar and Chandra 2006), and *Pseudomonas aeruginosa* (Mohana et al. 2007). Some researchers carried out melanoidin decolorization by using immobilized whole cells. These strains were able to reduce significant levels of BOD and COD. The major products left after treatment were biomass, carbon dioxide, and volatile acids.

Besides fungi and bacteria, yeast (Moriya et al. 1990; Sirianuntapiboon et al. 2003) and algae (Valderrama et al. 2002; Kumar and Chandra 2004) have also been utilized widely since long back for biodegradation of complex, toxic, and recalcitrant compounds present in distillery spent wash.

2.4.3 Decolorization of Effluent by Algae

Cyanobacteria are considered ideal for treatment of distillery effluent as they apart from degrading the polymers also oxygenate water bodies, thus reduce the BOD and COD levels. Kalavathi et al. (2001) explored the possibility of using a marine cyanobacterium for decolorization of distillery spent wash and its ability to use melanoidins as carbon and nitrogen source. A marine filamentous, nonheterocystous form *Oscillatoria boryana* BDU 92181 used the recalcitrant biopolymer melanoidin as nitrogen and carbon source leading to decolorization. The mechanism of color removal is postulated to be due to the production of hydrogen peroxide, hydroxyl anions, and molecular oxygen, released by the cyanobacterium during photosynthesis.

2.5 Role of Bioreactors in Effluent Treatment

a. Anaerobic Reactors

Wastewater treatment using anaerobic process is a very promising reemerging technology, produces very little sludge, requires less energy, and can become profitable by cogeneration of useful biogas (Mailleret et al. 2003). However, these processes have been sensitive to organic shock loadings, low pH, and show slow growth rate of anaerobic microbes resulting in longer hydraulic retention times (HRT). This often results in poor performance of conventional mixed reactors. Biomethanation using biphasic system is most appropriate treatment method for high strength wastewater because of its multiple advantages viz., possibility of maintaining optimal conditions for buffering of imbalances between organic acid production and consumption, stable performance, and higher methane concentration in the biogas produced (Seth et al. 1995). In recent years, the UASB process has been successfully used for the treatment of various types of wastewaters (Lettinga and Hulshoff Pol 1991). Jhung and Choi (1995) performed a comparative study of UASB and anaerobic fixed film reactors for treatment of molasses wastewater. The UASB technology is well suited for high strength distillery wastewaters only when the process has been successfully started up and is in stable operation. However, the conventional UASB reactors showed

severe limitations mainly related to mass transfer resistance or the appearance of concentration gradients inside the systems, slow primary startup requiring several weeks, and difficulty in controlling granulation process which depends upon a large number of parameters.

b. Aerobic reactors

Anaerobically treated distillery spent wash still contains high concentrations of organic pollutants and as such cannot be discharged directly. Aerobic treatment of anaerobically treated distillery spent wash has been attempted for the decolorization of the major colorant, melanoidin and for further reduction of the COD and BOD. A large number of microorganisms such as bacteria (pure and mixed culture), cyanobacteria, yeast, fungi, etc. have been isolated in recent years that are capable of degrading melanoidin and ultimately decolorizing the wastewater.

2.6 Enzymatic Processes for Decolorization

A large number of enzymes (e.g., peroxidases, oxidoreductases, cellulolytic enzymes, proteases amylases, etc.) from a variety of different sources have been reported to play an important role in an array of waste treatment applications (Ferrer et al. 1991; Dec and Bollag 1994). Paper and pulp mills, textiles and dye-making industries, alcohol distilleries, and leather industries are some of the industries that discharge highly colored effluents. The ligninolytic system consists of two main groups of enzymes: peroxidases (lignin peroxidases and manganese peroxidases) and laccases (Leonowicz et al. 2001; Arana et al. 2004; Baldrian 2006). Although the enzymatic system associated with decolorization of melanoidin containing wastewater appears to be related to the presence and activity of fungal ligninolytic mechanisms, this relation is as yet not completely understood. Laccase is a multicopper blue oxidase capable of oxidizing *ortho*- and *para* diphenols and aromatic amines by removing an electron and proton from a hydroxyl group to form a free radical. These enzymes lack substrate specificity and are thus capable of degrading a wide range of xenobiotics including industrial colored wastewaters. The mechanism of action of these enzymes is as follows:

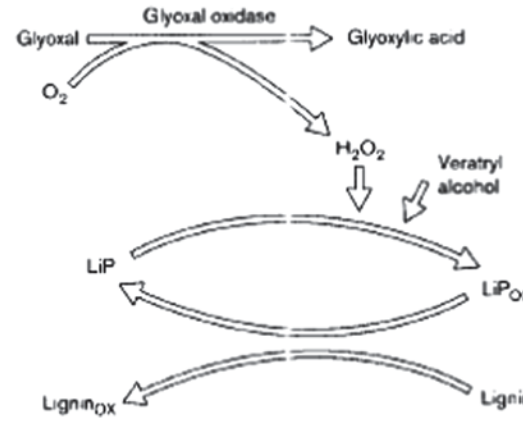

Fig. 2.2 Mechanism of action for lignin peroxidase (LiP). *ox* oxidized state of enzyme. (Breen and Singleton 1999)

a. Lignin Peroxidase (LiP)

LiP is a heme-containing glycoprotein, which requires hydrogen peroxide as an oxidant. LiP from different sources was shown to mineralize a variety of recalcitrant aromatic compounds and to oxidize a number of polycyclic aromatic and phenolic compounds (Karam and Nicell 1997).

Fungi secrete several isoenzymes into their cultivation medium, although the enzymes may also be cell wall-bound (Lackner et al. 1991). LiP oxidizes nonphenolic lignin substructures by abstracting one electron and generating cation radicals, which are then decomposed chemically (Fig. 2.2). LiP is secreted during secondary metabolism as a response to nitrogen limitation. They are strong oxidizers capable of catalyzing the oxidation of phenols, aromatic amines, aromatic ethers, and polycyclic aromatic hydrocarbons (Breen and Singleton 1999).

b. Manganese Peroxidase (MnP)

MnP is also a heme-containing glycoprotein which requires hydrogen peroxide as an oxidant. MnP oxidizes Mn(II) to Mn(III) which then oxidizes phenol rings to phenoxy radi-

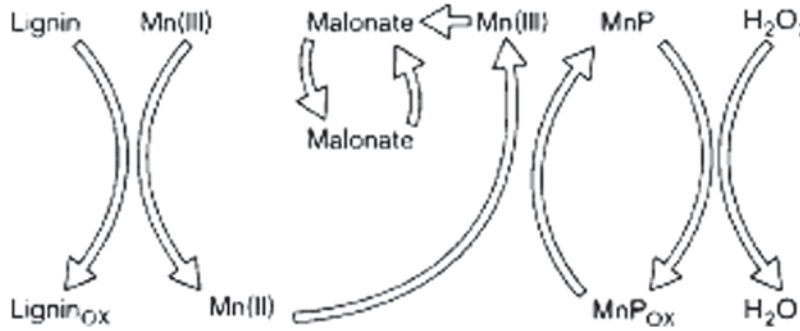

Fig. 2.3 Mechanism of action for manganese peroxidase (MnP). *ox* oxidized state of enzyme. (Breen and Singleton 1999)

cals, which lead to decomposition of compounds (Fig. 2.3). MnP catalyzes the oxidation of several monoaromatic phenols and aromatic dyes, but depends on both divalent manganese and certain types of buffers. The enzyme requirement for high concentrations of Mn(III) makes its feasibility for wastewater treatment application doubtful (Karam and Nicell 1997). Evidence for the crucial role of MnP in lignin biodegradation are accumulating, e.g., in depolymerization of lignin (Wariishi et al. 1991) and chlorolignin (Lackner et al. 1991), in demethylation of lignin and delignification and bleaching of pulp (Paice et al. 1993), and in mediating initial steps in the degradation of high-molecular mass lignin (Perez and Jeffries 1992).

c. Laccase

Laccase (EC 1.10.3.2, benzenediol:oxygen oxidoreductase) is a multicopper blue oxidase capable of oxidizing *ortho*- and *para*-diphenols and aromatic amines by removing an electron and proton from a hydroxyl group to form a free radical. Laccase in nature can be found in eukaryotes as fungi (principally by basidiomycetes), plants, and insects. However, in recent years, there is an increasing evidence for the existence in prokaryotes (Claus 2003). Corresponding genes have been found in gram-negative and gram-positive bacteria *Azospirillum lipoferum* (Bally et al. 1983), *Marinomonas mediterranea* (Sánchez-Amat and Solano 1997), and *Bacillus subtilis* (Martins et al. 2002).

Laccases not only catalyze the removal of a hydrogen atom from the hydroxyl group of methoxy-substituted monophenols, *ortho*- and *para*-diphenols, but can also oxidize other substrates such as aromatic amines, syringaldazine, and nonphenolic compounds to form free radicals (Bourbonnais et al. 1997; Li et al. 1999). After long reaction times there can be coupling reactions between the reaction products and even polymerization. It is known that laccases can catalyze the polymerization of various phenols and halogen, alkyl- and alkoxy-substituted anilines (Hoff et al. 1985). The laccase molecule, as an active holoenzyme form, is a dimeric or tetradimeric glycoprotein, usually containing four copper atoms per monomer, bound to three redox sites (Fig. 2.4). The molecular mass of the monomer ranges from about 50–100 kDa. Typical fungal laccase is a protein of approximately 60–70 kDa with acidic isoelectric point around pH 4.0. Several laccase isoenzymes have been detected in many fungal species. Several laccases, however, exhibit a homodimeric structure, the enzyme being composed of two identical subunits with a molecular weight typical for monomeric laccase.

Application of Laccases The interest in laccases as potential industrial biocatalysts has particularly increased after the discovery of their ability to oxidize recalcitrant nonphenolic lignin compounds (Li et al. 1999). This capability has later been shown to be generally applicable to a number of biotechnological problems; all of them are related to the degradation or chemical modification of structurally diverse compounds, being either xenobiotic or naturally occurring aromatic compounds. Laccase is currently being investigated by a number of research

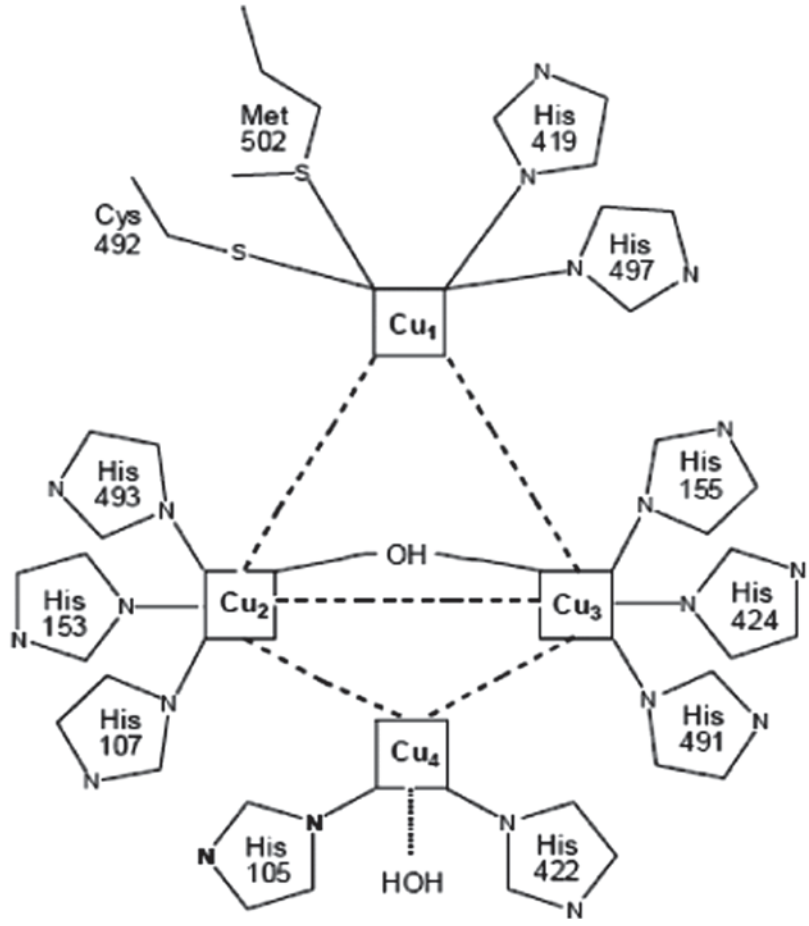

Fig. 2.4 Copper centers of the laccase. (Adapted from Claus 2004)

groups, e.g., with respect to litter mineralization (Dedeyan et al. 2000), dye detoxification, and decolorization (Abadulla et al. 2000; Kaushik and Thakur 2013). Laccases in both free and immobilized form as well as in organic solvents have found various biotechnological applications such as analytical tools—biosensors for phenols, development of oxygen cathodes in biofuel cells, organic synthesis, immunoassays labeling, delignification, demethylation, and thereby bleaching of craft pulp (Bourbonnais and Paice 1992; Bourbonnais et al. 1995) In addition, laccases have also shown to be useful for the removal of toxic compounds through oxidative enzymatic coupling of the contaminants, leading to insoluble complex structures (Wang et al. 2002). Laccase was found to be responsible for the transformation of 2,4,6-trichlorophenol to 2,6-dichloro-1,4-hydroquinol and 2,6-dichloro-1,4-benzoquinone (Leontievsky et al. 2000). Laccases from white rot fungi have been also used to oxidize alkenes, carbazole, N-ethylcarbazole, fluorene, and dibenzothiophene in the presence of hydroxybenzotriole (HBT) and 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as mediators (Niku-Paavola and Viikari 2000; Bressler et al. 2000). An isolate of the fungus *Flavodon flavus* was shown to be able to decolorize the effluent from a Kraft paper mill bleach plant. *F. flavus* decolorized several synthetic dyes like azure B, brilliant green, congo red, crystal violet, and Remazol brilliant blue R in low nitrogen medium (Raghukumar 2000). Partial decolorization of two azo dyes (orange G and amaranth) and complete decolorization of two triphenylmethane dyes (bromophenol blue and malachite green) was achieved by cultures of *Pycnoporus sanguineus* producing laccase as the

sole phenoloxidase (Pointing et al. 2000). Laccase purified from *Trametes hirsuta*, was able to degrade triarylmethane, indigoid, azo, and athraquinonic dyes used in dyeing textiles (Abadulla et al. 2000) as well as 23 industrial dyes (Rodriguez et al. 1999).

2.7 Adsorption-Assisted Decolorization

Several methods for the treatment of colored wastewaters have been proposed in the literature. These include physicochemical treatment processes, chemical oxidation, and biological degradation. Among various physicochemical treatment processes, adsorption has been found to be an attractive technique for the removal of most organic compounds in wastewaters, especially at lower concentrations. Activated carbon has been the most commonly used adsorbent. However, high cost of activation, regeneration, and the disposal of the concentrate from the cleaning cycles pose problems in the use of activated carbon. Hence, the search of new low cost adsorbents has attracted a number of investigators. Several low cost adsorbents like wood, coir pith, coal fly ash, bagasse fly ash (BFA), and coal-fired boiler bottom ash have been used for the treatment of a wide variety of wastewaters.

An efficient, cost-effective, and environmentally friendly technique; biosorption is mainly a physicochemical process involving the use of biological material-live or nonviable, can be used to concentrate and recover or eliminate the pollutants from aqueous solutions. Various workers have investigated the biosorption of various organic pollutants and color from wastewaters (Tsezos and Bell 1989; Fu and Viraraghavan 2001). Biomass of some natural microbial species, including bacteria, fungi, and algae, is capable of removing the different textile dyes by biosorption, biodegradation, or mineralization (Carliell et al. 1995). Some low-cost fungal biomass has been used as biosorbent for the removal of dye and metal ions from or wastewater, which included *Trametes versicolor* (Bayramoglu et al. 2003), and *Corynebacterium glutamicum* (Won et al. 2004).

a. **Mechanism of Biosorption**

According to the dependence on the cell's metabolism, biosorption mechanisms can be divided into:

1. Metabolism-dependent
2. Nonmetabolism-dependent

According to the location where the sorbate removed from solution is found, biosorption can be classified as

1. Extracellular accumulation/precipitation
2. Cell surface sorption/precipitation and
3. Intracellular accumulation

Microbial biomass consists of small particles with low density, poor mechanical strength, and little rigidity. This phenomenon is generally based on a set of chemical and physical mechanisms (involving physicochemical interactions such as electrostatic interactions, ion exchange, complexation, chelation, and precipitation) leading to the immobilization of a solute component on the microbial cell wall components. The complexity of the microbial structure implies that there are many ways for the pollutant to be captured by the cells. Biosorption mechanisms are therefore various (physical adsorption, chemical binding of ionic groups, ion exchange, etc.) and in some cases they are still not very well understood (Veglio and Beolchini 1997). Cell surface sorption is a physicochemical interaction, which is not dependent on metabolism. Cell walls of microbial biomass mainly composed of polysaccharides, proteins, and lipids, offer abundant functional groups such as carboxyl, hydroxyl, phosphate, and amino groups, as well as hydrophobic adsorption sites such as aliphatic carbon chains and aromatic rings (Ringot et al. 2005). This physicochemical phenomenon is quick and can be reversible.

Physical Adsorption If the attraction between the solid surface and the adsorbed molecules is physical in nature, the adsorption is referred to as physical adsorption (physiosorption). Generally, in physical adsorption the attractive forces between adsorbed molecules and the solid surface are van der Waals forces and they being weak in

nature result in reversible adsorption. Electrostatic interactions have been demonstrated to be responsible for copper biosorption by bacterium *Zoogloea ramigera* and alga *Chlorella vulgaris* (Aksu et al. 1992), and for chromium biosorption by fungi *Ganoderma lucidum* and *Aspergillus niger* (Srivastava and Thakur 2006).

Chemical Adsorption If the attraction forces are due to chemical bonding, the adsorption process is called chemisorption. In view of the higher strength of the bonding in chemisorption, it is difficult to remove chemisorbed species from the solid surface. Aksu et al. (1992) hypothesized that biosorption of copper by *C. vulgaris* and *Z. ramigera* takes place through both adsorption and formation of coordination bonds between metals and amino and carboxyl groups of cell wall polysaccharides. Microorganisms may also produce organic acids (e.g., citric, oxalic, gluonic, fumaric, lactic, and malic acids), which may chelate toxic metals resulting in the formation of metalloorganic molecules. These organic acids help in the solubilization of metal compounds and their leaching from the surfaces.

Ion Exchange Ion exchange is basically a reversible chemical process wherein an ion from solution is exchanged for a similarly charged ion attached to an immobile solid particle. Ion exchange shares various common features along with adsorption, in regard to application in batch and fixed-bed processes and they can be grouped together as ''sorption processes'' for a unified treatment to have high water quality. Ion exchange has been fruitfully used too for the removal of colors. By far the largest application of ion exchange (Clifford 1999) to drinking water treatment is in the area of softening that is the removal of calcium, magnesium, and other polyvalent cations in exchange for sodium. Various studies have been carried out using ion exchange for the removal of dyes (Liu et al. 2007; Wu et al. 2008). Delval et al. (2005) prepared starch-based polymers by a crosslinking reaction of starch-enriched flour using epichlorohydrin as a crosslinking agent in the presence of NH_4OH.

b. **Factors Affecting Biosorption**
   The following factors affect the biosorption process:
   1. Temperature seems not to influence the biosorption performances in the range of 20–35 °C (Aksu et al. 1992).
   2. pH seems to be the most important parameter in the biosorptive process: it affects the solution chemistry of the metals, the activity of the functional groups in the biomass (Galun et al. 1987).
   3. Biomass concentration in solution seems to influence the specific uptake: for lower values of biomass concentrations there is an increase in the specific uptake. Interference in between the binding sites due to increased biomass was suggested as a possible reason (Gadd et al. 1988).
c. **Biosorption Equilibrium Models**
   One of the most important characteristics of an adsorbent is the quantity of adsorbate it can accumulate which is usually calculated from the adsorption isotherms. The adsorption isotherms are constant-temperature equilibrium relationship between the quantity of adsorbate per unit of adsorbent (q_e) and its equilibrium solution concentration (C_e). Several equations or models are available that describe this function like the Freundlich and the Langmuir equations.

2.8 Future Prospects

The present status described in the chapter has allowed important information on the types of species involved in decolorization and degradation of distillery spent wash in the various lab scale to pilot scale studies but their interaction with the native microbial communities is still being questioned. Future studies should, therefore, focus not only on identification of other communities as observed in denaturing gradient gel electrophoresis (DGGE) band pattern but also their quantification using reliable quantitative methods. Assessment of activity and the interactions between the introduced organisms will also be

important for the design and control of biological reactors. Another area of study scope lies with isolated and purified microbial enzymes and the focus lie on investigation of production strategies such as recombinant expression in another organism.

2.9 Conclusion

Ethanol manufacture from molasses based industries generates large volumes of high strength wastewater, which is of serious environmental concern. It is estimated that in a large scale unit approximately 0.2 million L of molasses spent wash (MSW) is generated each day. The main source of wastewater generation is the distillation step wherein large volumes of dark brown effluent (termed as spent wash) is generated in the temperature range of 71–81 °C. This spent wash is dark brown colored polluting stream and contains practically all unfermentable soluble matter apart from the extremely high COD and BOD load. This dark color is mainly imparted by melanoidin, that are low and high molecular weight polymers formed as one of the final products of Maillard reaction. This colored waste stream contains highly toxic and recalcitrant compounds and when released untreated in any nearby water stream causes eutrophication and blocks the sunlight (due to color), ultimately creating a toxic environment to the aquatic biota. Therefore, a comprehensive treatment strategy is required for decolorization and detoxification of distillery spent wash before its disposal into the environment. Compared to the common and expensive physical or chemical ways for decolorization, an efficient bioremediation system has been found successful through biosorption and enzymatic ways of decolorization. However, pollution from distillery effluents is a complex environmental problem; its permanent solution will require comprehensive system considerations as well as multidisciplinary and holistic approaches.

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In Silico Approach to Study the Regulatory Mechanisms and Pathways of Microorganisms

3

Arun Vairagi

Abstract

Metabolic pathways and extreme pathways are the central paradigm of any life form. The detailed study and analysis of these pathways can yield better and engineered biological systems. This is the time when conventional methods of studying microorganisms are no longer practiced because of their limited productivity and higher time consumption. Bioinformatics has fulfilled the need for high-throughput experimental technologies, which are reliable and less time consuming too. With the help of computational biology, it is easy to study the whole microorganism's metabolic and extreme pathways network and to obtain authentic results. Some in silico tools are designed to fulfill the need for high-throughput analysis of different pathways in microorganisms, like metagenome analyzer (MEGAN) which works on short-read data, the Pathways Tool which helps in constructing the pathway database and the Model SEED, a resource for the generation, optimization, duration and analysis of genome-scale metabolic models. Thus, network-based pathways are emerging as an important paradigm for analysis of biological systems.

Keywords

Bioinformatics · Microbial metabolism · In silico tools · Biological systems

3.1 Introduction

3.1.1 Pathway Analysis

A pathway is a sequence of activities among molecules in a cell that leads to a certain product or change in the cell. Such a pathway can activate the assemblage of new molecules, such as a fat or protein. Pathways can also turn genes on and off. For a life form to develop correctly and stay well, many

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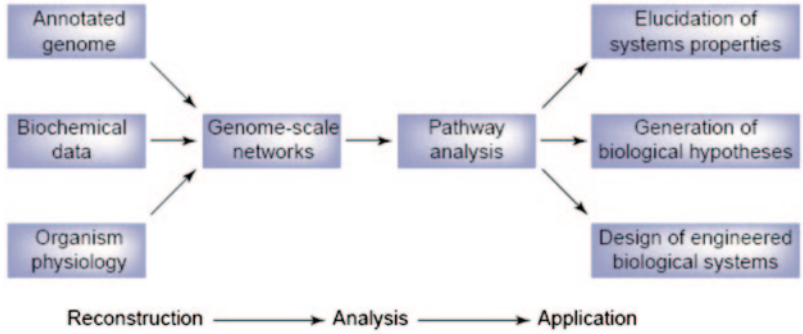

Fig. 3.1 An overview of pathway analysis. (Reproduced from http://www2.bio.ifi.lmu.de/lehre/SS2007/SEM_Advanced/Folien/Extreme_pathways.pdf)

things must work together at many different levels—from organs to cells to genes. Cells are constantly receiving cues from both inside and outside the body, which are prompted by such things as injury, infection, stress or even food. To react and adjust to these cues, cells send and receive signals through biological pathways. The molecules that make up biological pathways interact with signals, as well as with each other, to carry out their chosen tasks. Biological pathways can also produce small or large outcomes. Scientists are researching that biological pathways are far more complex than once believed. Most pathways do not start at point X and end at point Y. In fact, many pathways have no real restrictions, and they often work together to complete tasks. When multiple biological pathways interact with each other, it is known as a biological network (Fig. 3.1). These pathways have then been grouped conceptually as functional units such as glycolysis or the tricarboxylic acid cycle (TCA) cycle. This type of pathway definition is useful for identifying portions of the metabolic network, but the divisions are somewhat vague between the point where one pathway ends and another begins. In addition, this type of pathway definition does not relate to the overall functions of the network as a whole.

3.1.2 A Brief History of the Field of Pathway Analysis

The first work on pathways can be traced back to 1980 with the development of SNA by Bruce Clarke (Fig. 3.2). The theory was developed to study instability in inorganic chemical networks. This was the first attempt to apply convex analysis to reaction networks but it was never extended to living systems. This was followed by some work using AI to search through reaction networks following along the lines of graph theory and was taken another step further by Mavro with the introduction of stoichiometric constraints. Both of these approaches lacked a sound theoretical basis. In 1994, Schuster became the first to apply convex analysis to metabolic networks with the introduction of a non-unique set of elementary modes. This theory was applied a few years later by Liao to optimize bacterial strain design for the high-efficient production of aromatic amino acids. So at this point in time, pathway analysis was just beginning to be applied but still lacked a unified theoretical foundation, which is where the present work comes in.

3.2 Extreme Pathways

3.2.1 Definition

Extreme pathways are defined as vectors derived mathematically and can be used to characterize the phenotypic potential of a defined metabolic network (Schilling et al. 1999, 2000). Extreme pathway analysis has the following characteristics:

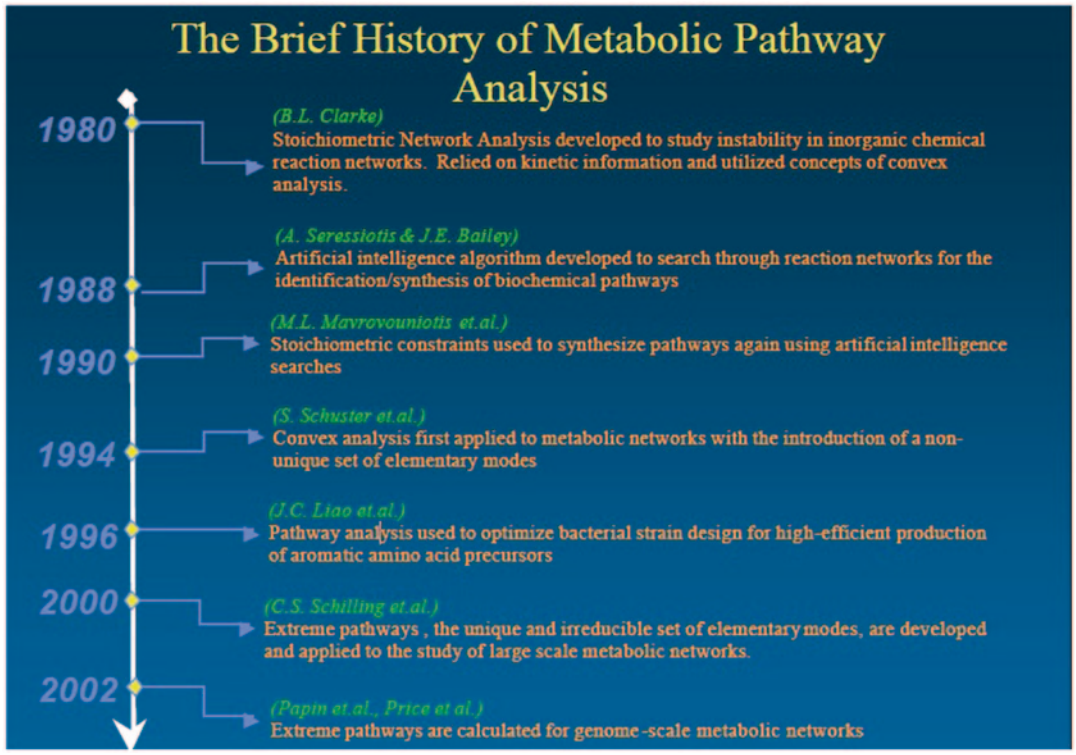

Fig. 3.2 Schematic representation of historical footsteps in metabolic pathways analysis. (Reproduced from http://gcrg.ucsd.edu/sites/default/files/Attachments/Images/classes/taiwan_notes/5_slides_expa. pdf)

(1) It generates a unique and minimal set of systemic pathways
(2) It describes all possible steady-state flux distributions that the network can achieve by non-negative linear combinations of the extreme pathways
(3) It enables the determination of time-invariant, topological properties of the network

The calculation of extreme pathways is computationally challenging and for large networks, generates a tremendous amount of numerical data (Schilling et al. 2000; Samatova et al. 2002).

The phenotypic capabilities of a genome-scale metabolic network can be characterized by a set of systemically independent and unique extreme pathways (Schilling et al. 2000). Extreme pathways correspond to steady-state flux distributions through a metabolic network (Fig. 3.3). Thus, extreme pathways do not simply describe a linear set of reactions linking substrate to product, but instead, characterize the relative flux

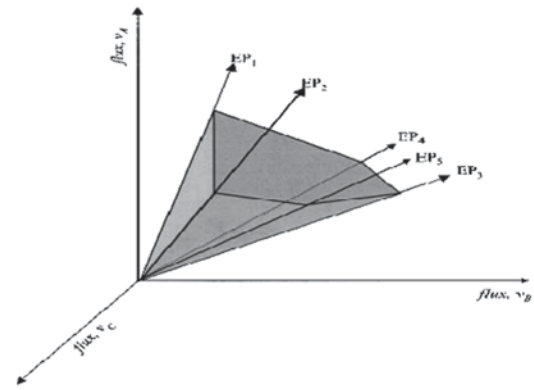

Fig. 3.3 Schematic representation of a *convex cone* characterized by five extreme pathways. Extreme Pathways 1–5 (EP_1, EP_2, EP_3, EP_4, and EP_5) circumscribe the solution space for the three fluxes indicated (v_A, v_B, and v_C). EP4 lies in the plane formed by fluxes v_A and v_B. Consequently, flux v_C does not participate in that extreme pathway. EP_3, EP_4, and EP_5 are all close and represent different uses of a network to achieve a similar overall result. All points within the *convex cone* can be described as a nonnegative linear combination of the extreme pathways. (Reproduced from Papin et al. 2002)

levels through all the reactions necessary to convert substrates to products, to balance all cofactor pools, and to secrete any byproducts needed to maintain the network in a homeostatic state. The sets of extreme pathways studied here lead to the synthesis of a target product, such as an individual amino acid or all the protein in a cell. Therefore, each extreme pathway in the set corresponds to a complete flux map that synthesizes the target product within the metabolic network. Extreme pathways are so named because they are the edges of a solution space and thus characterize the extreme functions of the network. The extreme pathways can be thought of as generating a convex cone in high-dimensional space, circumscribing all possible steady-state metabolic phenotypes. (Papin et al. 2002)

It should be noted that the extreme pathways are an irreducible, non-redundant subset of elementary modes (Pfeiffer et al. 1999; Schuster et al. 1999, 2000). Elementary modes for a given network are more numerous than the extreme pathways, but can all be represented by non-negative, linear combinations of the extreme pathways.

3.2.2 Types of Extreme Pathways

Extreme pathways can be classified into three types, based upon the metabolites that enter and leave the particular network, known as exchange fluxes (Fig. 3.4).

Type I extreme pathways have exchange fluxes that cross system boundaries, and represent primary metabolic pathways. These extreme pathways detail the conversion of substrates into products and byproducts.

Type II extreme pathways also have exchange fluxes that cross system boundaries, but these exchange fluxes only correspond to "currency" metabolites, such as ATP, NADH and so forth. *Type II* pathways can be through of as "futile" cycles, and must proceed "downhill" in terms of free energy.

Type III extreme pathways have no active exchange fluxes, and therefore represent internal cycles. Based upon thermodynamics, these cycles cannot be active because there is no energy source to drive them. Thus, these type III extreme pathways can be eliminated from the convex basis without loss of phenotypic potential. (http://gcrg.ucsd.edu/sites/default/files/Attachments/Images/classes/taiwan_notes/5_slides_expa.pdf).

3.2.3 Pathway Tool

The Pathway Tools are the software environment for the quality production of model-organism databases (MODs), and are also reusable. The Model-Organism Databases formed are called pathways/genome databases (PGDB). Information related to genes, proteins and the genetic and metabolic networks of an organism is stored in the PGDB. The Pathway Tool gives two different modalities for interacting with a PGDB:

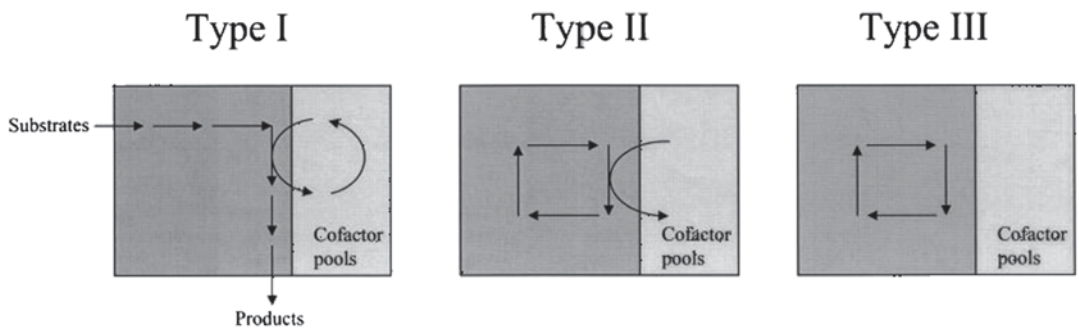

Fig. 3.4 Showing the types of extreme pathways based on their exchange fluxes. (Reproduced from http://www2.bio.ifi.lmu.de/lehre/SS2007/SEM_Advanced/Folien/Extreme_pathways.pdf)

- A graphical environment is provided so that the user can easily interact with the PGDB
- It provides a sophisticated ontology and database API that allows a program to perform complex queries, symbolic computations and data mining on the content of the PGDB (Karp et al. 2002)

Pathway Tools are the combination of four tools working on different aspects of the same query. They are known as the four components of the complete Pathway Tools software.

The first component is the Pathway/Genome Navigator which provides query, visualization and analysis services to PGDBs. It can serve both as a local application as well as a Web server. It helps in facilitating information fast, and allows the scientific community to exhibit information in various forms such as graphical and to distribute a PGDB to others via the web. The second component, known as PathoLogic, has a function for users to create a new PGDB, which contains information about genes, proteins and other predicted metabolic networks of the organism. The third component is known as the Pathway/Genome Editor, which provides the facility of editing the PGDB and its content in an interactive mode. It has a function for creating new pathways and establishing relationships amongst the newly discovered components. The fourth component is Pathway Tool Ontology, which defines the rich set of classes, attributes and relationships for high-fidelity modelling of biological data. (Karp et al. 2002)

3.3 Metagenomics

Metagenomes are studied under this heading, as well as the genetic material obtained directly from the environmental samples. It is the analysis of genomes of microorganisms by direct extraction and DNA cloning from an assemblage of microorganisms. The development of metagenomics stemmed from the ineluctable evidence that as-yet-uncultured microorganisms represent the vast majority of organisms in most environments on earth. The approach of microbiologists towards many problems has been changed by the subject of metagenomics, which redefined the concept of a genome, and accelerated the rate of gene discovery. (Handlesman 2004). In addition, the metagenomic libraries derived from environmental DNA are useful for characterizing uncultured microorganisms. However, conventional library-screening techniques permit characterization of relatively few environmental clones. (Sebat et al. 2003). Jo Handelsman coined the term in 1998. There are a vast number of metagenome projects currently active, producing a huge amount of data related to DNA. Advances in the throughput and cost-efficiency of sequencing technology are fueling a rapid increase in the number and size of metagenomic datasets being generated. Researchers are now able to study the DNA of a wider range of microorganisms and genes on a more complete and detailed scale. The basic questions of interest are: which species are present in a given environment, and what types of genes, functions or pathways are present in the DNA or actually active in the sample? As research begins to answer these basic questions, the focus will shift to the comparison of different datasets, because researchers will want to determine and understand the similarities and differences between the metagenomes of different environments (Fig. 3.5).

Metagenomics has been defined as “the genomic analysis of microorganisms by direct extraction and cloning of DNA from an assemblage of microorganisms.” (Handelsman 2004) The fact which made metagenomics more important is that 99% of microorganisms are not culturable. The goal of this new area is to achieve a better insight into the existence of different varieties of microorganisms. The identification of these unknown microorganisms obtained from the environment is done by comparing them with a known sequence database. Developing sequencing-by-synthesis technologies with very high throughput are flagging the way to low-cost random “shotgun” approaches like MEGAN, a computer program that allows in silico analysis of large metagenomic datasets.

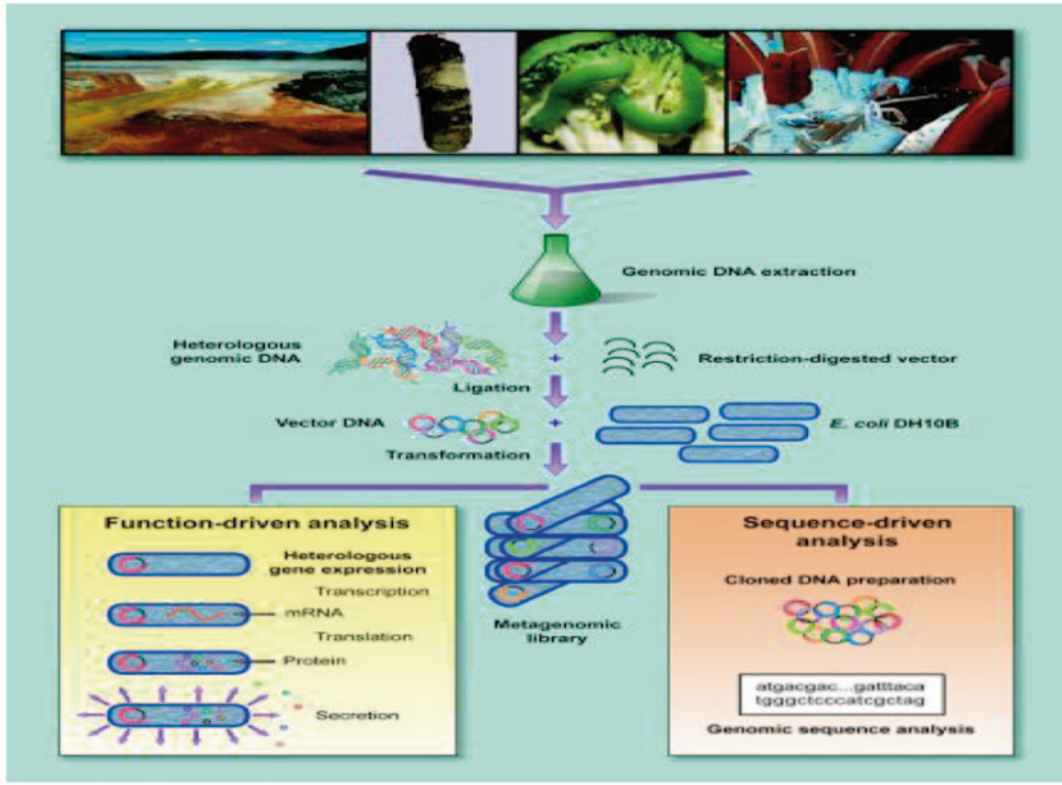

Fig. 3.5 Construction and screening of metagenomic libraries. (Reproduced from Handlesman 2004)

3.4 MEGAN (MetaGenome Analyzer)

MEGAN is the first stand-alone tool for metagenome analysis. It allows the investigation of very large datasets from environmental samples using shotgun sequencing techniques in particular, and is designed to sample and investigate the unknown biodiversity of environmental samples where more precise techniques with smaller, better known samples cannot be used. In MEGAN's processing pipeline, it initially performs the analysis of the metagenomic sample. First, reads are collected from the sample using any random shotgun protocol. Second, a sequence comparison of all reads against one or more databases of known reads is performed, using **basic local alignment search tool** (BLAST) or a similar comparison tool. Third, MEGAN processes the results of the comparison to collect all hits of reads against known sequences, and assigns a taxon ID to each sequence based on the National Center for Biotechnology Information (NCBI) taxonomy. This produces a MEGAN file that contains all information needed for analyzing and generating graphical and statistical output. Fourth, the user interacts with the program to run the lowest common ancestor (LCA) algorithm to analyze the data, inspect the assignment of individual reads to taxa based on their hits and to produce summaries of the results at different levels of the NCBI taxonomy (Fig. 3.6) (Huson et al. 2007)

MEGAN can be used to interactively explore the dataset in the following manner:

a. Comparative visualization

To compare a collection of different datasets visually, MEGAN provides a comparison view that is based on a tree in which each node shows the number of reads assigned to it for each of the

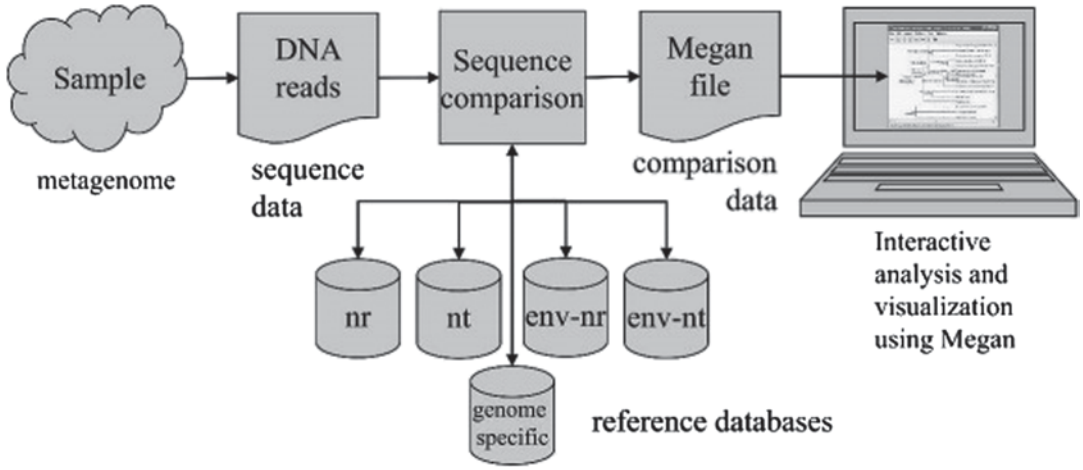

Fig. 3.6 For a given sample of organisms, a randomly selected collection of DNA fragments is sequenced. The resulting reads are then compared with one or more reference databases using an appropriate sequence comparison program such as BLAST. The resulting data are processed by MEGAN to produce an interactive analysis of the taxonomical content of the sample. (Reproduced from http://ab.inf.uni-tuebingen.de/software/megan5/)

datasets. This can be done either as a pie chart, a bar chart or as a heat map. To construct such a view using MEGAN, first, all the datasets must be individually opened in the program. Using a provided "compare" dialog, one can then set up a new comparison document containing the datasets of interest. The following figure shows the taxonomic comparison of all eight marine datasets. Here, each node in the NCBI taxonomy is shown as a bar chart indicating the number of reads (normalized, if desired) from each dataset that have been assigned to the node (Fig. 3.7).

b. Taxonomic analysis

MEGAN can be used to interactively explore the dataset. The following figure shows the assignment of reads to the NCBI taxonomy (Fig. 3.8). Each node is labeled by a taxon and the number of reads assigned to the taxon. The size of a node is scaled logarithmically to represent the number of assigned reads. Optionally, the program can also display the number of reads summarized by a node, that is, the number of reads that are assigned to the node or to any of its descendants in the taxonomy. The program allows one to interactively inspect the assignment of reads to a specific node, to drill down to the individual BLAST hits that support the assignment of a read to a node, and to export all reads (and their matches, if desired) that were assigned to a specific part of the NCBI taxonomy. Additionally, one can select a set of taxa and then use MEGAN to generate different types of charts for them. (http://ab.inf.uni-tuebingen.de/software/megan5/ Dated 5/16/2014)

MEGAN5 also provides a number of new plots and charts including a co-occurrence plot, space-filling radial trees, etc.

3.5 Conclusion

Pathway Analysis and Comparative Metagenomics is a rapidly emerging field. Therefore, time saving and comprehensible tools are needed to study various sequences and datasets related to microorganisms. In this chapter, we have discussed some really good, user-friendly tools which provide a better understanding of relationships among individual microorganisms' pathways as well as in the community of microorganisms. Pathway Tool, the combination of four different softwares, provides the user with the facility for making a whole new pathways-related database

Fig. 3.7 Phylogenetic diversity of the *Sargasso* Sea sequences computed by MEGAN. The microheterogeneity of sample 1 was investigated by comparing it to pooled samples 2, 3, and 4 (Venter et al. 2004). **a** Analysis of 10,000 reads randomly chosen from Sample 1. **b** Analysis of 10,000 reads randomly chosen from Sample 2. **c, d** A more detailed view of sample 1 and samples 2–4, respectively, illustrating a significant difference of relative frequencies of *Shewanella* and *Burkholderia* species in the two data sets. In all such Figs. (Fig. 3.7), each *circle* represents a taxon in the NCBI taxonomy and is labeled by its name and the number of reads that are assigned either directly to the taxon, or indirectly via one of its subtaxa. The size of the circle is scaled logarithmically to represent the number of reads assigned directly to the taxon. (Huson et al. 2007)

Fig. 3.8 Assignment of reads to the NCBI taxonomy. (Reproduced from http://ab.inf.uni-tuebingen.de/software/megan5/)

of microorganisms. It provides pathway-related information in various simpler forms; it also provides a graphical view of the output, which can be grasped easily. For studies related to metagenomics, we discussed the MEGAN tool which simplifies huge datasets into simple short ones by using shotgun techniques. MEGAN provides a simple solution to the complex problem of metagenomic study. Due to these fast and reliable computational tools, various advancements are taking place in the field of Pathways Analysis and Metagenomics, making them the central paradigm of biological research.

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Microbial Diversity: Its Exploration and Need of Conservation

4

Monika Mishra

Abstract

Microbial diversity is fundamental to maintenance and conservation of global genetic resources. Actions must be taken to estimate, record, and conserve microbial diversity, not only to sustain human health, but also to enhance the human condition globally through sensible use and conservation of genetic resources of the microbial world. The microbial world is the largest unexplored reservoir of biodiversity on the earth. The exploration of microbial diversity has been prompted by the fact that microbes are essential for life, since they perform numerous functions essential for the environment that include nutrient recycling and environmental detoxification. Priceless contribution of microbial diversity in commercial and industrial applications promoted the management of the same for sustainable use. Natural environment is diverse and the enormous potential of microorganisms to provide novel pharmaceuticals, fine chemicals, and new technologies, is used by the biotechnology industry. Unfortunately, despite the evident economic value of microbial diversity, microorganisms have been mostly ignored in debates on the conservation and management of global diversity. There is, therefore, an urgent need to motivate researchers to be more apprehensive about the conservation, management, and exploitation of microbial diversity.

Keywords

Genetic · Resources · Industry · Microbial diversity · Research

4.1 Introduction

The microorganisms play a vital and often distinctive role in the functioning of the ecosystems in maintaining a sustainable environment and its productivity. The loss of biodiversity and their ability to provide ecological services to humans has now become a central thought to be

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considered in ecology. A number of major experiments have recently shown that declining plant diversity may impair plant biomass, primary production and nutrient retention, and so many ecosystem properties. Presently, the relationship between biodiversity and ecosystem functioning in ecological and environmental sciences has emerged as a central issue. Microorganisms are invisible, less familiar and apparently considered primarily as agents of disease and these may be the few reasons for ignoring their management.

However, few experiments have directly tested the consequences of changing the diversity of ecosystem components other than plants, and simultaneously manipulated the diversity of primary producers (algae) and decomposers (bacteria) in aquatic microorganisms and found complex interactive effects of algal and bacterial diversity on algal and bacterial biomass production. Both algal and bacterial diversity had significant effects on the number of the carbon source used by bacteria, suggesting nutrient cycling associated with microbial exploitation of organic carbon source as the link between bacterial diversity and algal production. There are several explanations but the exact theory is greatly missing.

Producers and decomposers are the two key functional groups that form the basis of all ecosystems interactions. Obviously, their diversity might have major consequences on the functioning of ecosystems. Thus, it is now generally accepted that the extent of microbial diversity has not been adequately characterized and there is a huge mismatch between the knowledge of that diversity and its importance in both ecosystem process and economic development. Soil quality has been defined as the capacity of the soil to function within ecosystem limitations to sustain biological productivity, maintain environmental quality, and promote plant and animal health. Nutrient immobilization by decomposers and competition for inorganic nutrients between plants and decomposers are known to occur, but at equilibrium, the two functional groups must be limited by different factors in order to allow their consistence and ecosystem persistence. Microbial diversity is an unseen national resource that deserves greater attention. It is too small to be seen and studied or valued. Microbial diversity includes the spectrum of variability among all types of microorganisms (bacteria, fungi, viruses, and many more) in the world and is greatly changed by human intervention. Microorganisms are the ubiquitous custodians of the Earth occurring in all climate areas including Arctic and Antarctic, the heat of geysers etc. They are decomposers, converting nutrients in the organic wastes from dead organisms into molecules that are reused within ecosystems.

4.2 Microbes: Necessity of Life

Conserving microbial diversity will often, in a practical sense, equate to the conservation of the ecosystem microbial gene pool. From a rational point of view, the conservation of the gene pool and microbial diversity itself equates to the conservation of the physical and chemical conditions within an environment that best support the indigenous microbiota. Figure 4.1 clearly depicts the relationship between various microbial species.

Extremophile Life Forms Majority of life forms found in extreme environmental conditions are microbes. These extreme physico-chemical conditions may be pH, heat, salinity, pressure, radiation, etc. These microbes can be characterized by using r-RNA comparative sequencing technology. These microbes may be capable of producing a wide array of enzymes in extreme conditions which may be used in various industrial applications such as lipase, protease, DNA polymerase, etc (Tripathi et al. 2007). These microorganisms hold many secrets such as genetic instructions which make them able to produce these enzymes in extreme conditions.

4.3 What Are the Drivers Causing Decrease of Microbial Biodiversity

The diversity of microscopic life forms (including viruses, archaea, bacteria, and small eukaryotic microorganism) are recently coming to light,

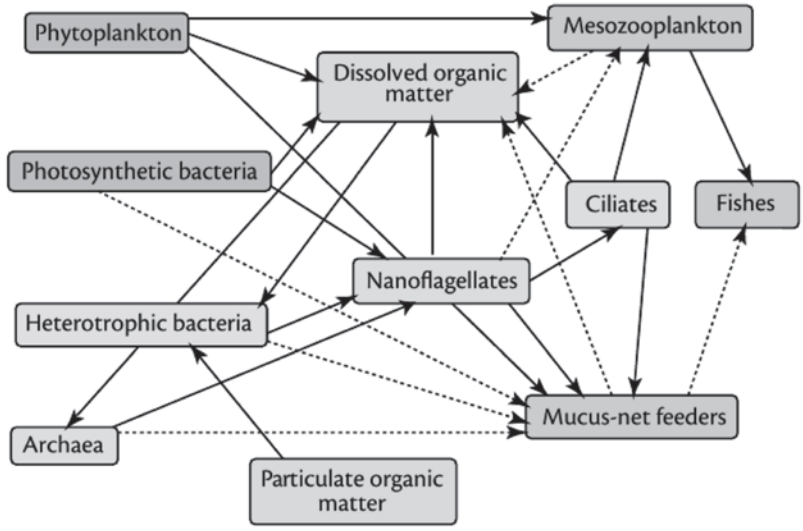

Fig. 4.1 Microbial loop and aquatic food web. (Nardini et al. 2010)

and their varieties, abilities, distributions, ecosystem functions, and conservation status need to be further investigated. The primary cause is habitat fragmentation, degradation, and destruction due to land use, change arising from conversion, strengthening of production systems, abandonment of traditional (which were often biodiversity friendly) practices, construction, and catastrophic events including fires. There are some other key causes which include excessive exploitation of the environment, pollution, and the spread of invasive alien species (Nardini et al. 2010).

Commonly used measures of biodiversity, such as the number of species present, are strongly scale dependent and only report a change after a species is lost. There is no worldwide accepted set of methods to assess biodiversity. The main problem is that the data is much diverse and it is physically discrete and disorganized. The solution is to organize the information, and create systems whereby data of different kinds, from many sources, can be pooled. This will improve our understanding of biodiversity and will allow the development of measures of its condition over time.

4.3.1 Impact of Anthropogenic Activities

There are many reports depicting effect of chemical pollutants such as polycyclic aromatic hydrocarbons (PAHs) on microbial community structure. PAHs are present in oil and coal and produced by incomplete combustion of wood and coal. They are widespread over the world and are considered as heavy pollutants due to their toxic, carcinogenic, and mutagenic effects on organisms. The study of bacterial communities in PAH contaminated soils at an electronic waste processing center in China shows that different levels of PAHs might affect the bacterial community by suppressing or favoring certain groups of bacteria, for instance, uncultured *Clostridium sp.* and *Massilia sp.*, respectively (Zhang et al. 2010).

4.3.2 Reef Ecosystem

Most coral reefs are moderately to severely degraded by local human activities such as fishing and pollution as well as global change; hence, it is difficult to separate local from global effects.

Sandin et al. (2008) surveyed coral reefs on deserted islands in the northern Line Islands to provide a baseline of reef community structure, and on increasingly populated islands to document changes associated with human activities.

4.3.3 Climate Change

Effects of climate change on biodiversity (such as changing distribution, migration, and reproductive patterns) are already observable. Average temperature is expected to rise between 2 and 6.3 °C by the year 2100. Predicted impacts associated with such temperature increase include a further rise in global mean sea level of 9–88 cm, more precipitation in temperate regions and Southeast Asia, in turn a higher probability of floods (Nardini et al. 2010). On the contrary Central Asia, the Mediterranean region, Africa, parts of Australia and New Zealand will get less precipitation which can result in greater probability of droughts, more frequent and powerful extreme climatic events, such as heat waves, storms, and hurricanes, an prolonged range of some dangerous "vector-borne diseases", such as malaria, and further warming of the Arctic region (Nardini et al. 2010). Pollution from nutrients such as nitrogen, introduction of invasive species, over harvesting of wild animals can all reduce resilience of ecosystems. In the atmosphere, greenhouse gases such as water vapor, carbon dioxide, ozone, and methane act like the glass roof of a greenhouse by trapping heat and warming the planet. The natural levels of greenhouse gases are being supplemented by emissions resulting from human activities, such as the burning of fossil fuels, farming activities, and land-use changes. As a result, the Earth's surface and lower atmosphere are warming. This will have profound effects on the biodiversity.

4.3.4 Effect of Temperature on Microbial Communities

Pearce (2008) and Rodriguez-Blanco et al. 2009 has demonstrated the effects of factors such as temperature, nutrient availability, grazing, salinity, seasonal cycle, and carbon dioxide concentration on bacterial community structure in the polar and alpine ecosystems. The results suggest that the spatial distribution of genetic variation and, hence, comparative rates of evolution, colonization, and extinction are particularly important when considering the response of microbial communities to climate change. Although the direct effect of a change in, e.g., temperature is known for very few Antarctic microorganisms, molecular and genomic techniques are starting to give us an insight into what the potential effects of climate change might be at the molecular/cellular level (Friedmann 1993).

4.4 Utility of Microbial Diversity

Microbial diversity existing in natural ecosystems has the following major applications:

4.4.1 Biogeochemical Cycling of Matter

Soil acts as the source of nutrition for the growth of a spectrum of microorganisms which have remarkable ability to degrade a vast variety of complex organic compounds due to their metabolic bioremediation agents. They also play a vital role in providing conditions for functions of humans and animals and for the continuation of all lifeforms on Earth. Many microorganisms carry out unique geochemical processes critical to the operation of the biosphere (Gruber and Galloway 2008) and no geochemical cycle is carrying out without their involvement. Metabolic variety of microbes is enormous, ranging from being photo- and chemosynthetic and to degrade various anthropogenic xenobiotic compounds. For example, the global nitrogen cycle in nature is dependent on microorganisms. Unique processes carried out by microorganisms include nitrogen fixation, oxidation of ammonia and nitrite to nitrate, and nitrate reduction with formation of dinitrogen and nitrous oxide gases (Gruber and Galloway 2008). Similar important and unique roles are played in other cycles, such as the sulfur and carbon cycles. In addition, microbes run

less visible elemental cycles of metals, carrying out oxidation/reduction of metals (e.g., manganese and iron). Microorganisms are the primary organisms responsible for degradation of a great variety of natural organic compounds, including cellulose, hemicellulose, lignin, and chitin, which are the most abundant organic matter on Earth (Mishra and Thakur 2012). Due to their versatility, microbes not only provide ecological services but also play a major role in semiartificial systems such as sewage treatment plants, landfills, and in toxic waste bioremediation. To mention few examples in which microbes are responsible for degradation of toxic chemicals derived from anthropogenic sources such as PAH, PCBs (polychlorinated biphenyls), dioxins, pesticides, etc (Jaiswal et al. 2011). In most cases these microbes are genuine members of natural communities. Some organisms are obligatory degraders, frequently switching their metabolism on degradation and consumption to acquire carbon and/or energy.

4.4.2 Microbes in Industrial Products

Many products useful to mankind are synthesized at commercial level using microbes. Beverages, antibiotics, alcohol, enzymes (glucose oxidase, amylase, protease, lipase, cellulose, xylanase, etc.), proteins, vaccines, steroids, amino acids are the few important examples. Microbial biochemicals are also used as biocontrol agents as an alternative to insecticides, pesticides etc.

4.4.3 Biodegradation of Xenobiotics

Human kind is increasingly using pesticides such as BHC, DDT, 2,4-D, 2,4,5-T for getting rid of unwanted weeds, insect pests, or pathogenic microorganisms. Removing chemicals from the environment can be achieved easily and in an environment-friendly manner by biological methods that involve use of microbes and plants to degrade xenobiotic compounds and thus decontamination of the polluted site.

It also participates in bioremediation and purification of hazardous wastes in water. Biological treatments are more effective as these methods convert toxic chemicals to less toxic ones and possess a significant degree of self-regulation (Mishra et al. 2013). Microorganisms have diverse capacities to biotransform and, in some cases, completely destroy toxic chemicals from our environment. Since these transformations alter the chemistry of the hazardous chemicals, they may also alter toxicity, environmental fate, and bioaccumulation potential (Das et al. 2012). Several halogenated chemicals such as the chlorinated aromatic compounds, which are major contaminants, nitro aromatics and other conjugated hydrocarbons-polluted contaminated sites could be reclaimed by use of the vanguard organisms isolated from contaminated sites by enrichment cultures. *Spingomonas paucimobilis* BPSI-3 that was isolated from PCB contaminated soil was observed to degrade halogenated PAHs and biphenyls (Davison et al. 1999). Head and Swannell (1994) reported bioremediation of petroleum hydrocarbon contaminates in marine habitats by anaerobic hydrocarbon metabolism via bioaugmentation and stressed to reject the approach of nutrient amendment as it can potentially exert an oxygen demand due to biological ammonia oxidation. Samanta et al. isolated *Ralstonia sp*. SJ98 from pesticide-contaminated agricultural soil using a chemotactic enrichment technique (Samanta et al. 2000).

In nature majority reactions result in mineralization of the contaminant but sometime recalcitrant formed during the process act as potent toxic compound than the original xenobiotic chemical. *Pseudomonas putida* and *Burkholderia cepacia* have even been genetically engineered to cover a wider range of contaminants though *Pseudomonas sp.* possesses metabolic plasmids too. Lajoie et al. (1994) studied the use of surfactant based field application vectors for PCB degradation, as single microbe barely possesses all the enzymes for mineralization of a xenobiotic chemical. The specificity of the pollutant and the microbe degrading it depends upon the enzymes involved in the selective chemotaxis of the microbe toward

the contaminant. The second phenomenon is of great interest as it increases the bioavailability of a pollutant to bacteria. As heavy metals are common contaminants worldwide and are a threat to the quality and sustainability of natural soil resource, rescuing of the heavy metal contaminated soils by microbes (in situ bioremediation) is a low cost and effective tool to minimize environment pollution and is in use today. Evdokimova (2000) have shown that in copper, nickel, cobalt, and sulphur compound contaminated sites in Kola Peninsula, the microbial diversity decreased a lot. But the fungi, bacteria, and actinomycetes were found to bioconcentrate these heavy metals by volatilizing or accumulating in cell capsules etc. *P. flourescens* AF39 accumulated heavy metals such as nickel and others and the whole process was observed to be rapid and pH dependent. Several biomarkers or technically biosensors are available now to obtain the presence of specific contaminant at a particular site.

4.4.4 Microbial Products Used in Novel Chemical Synthesis

Bioprocesses, which involve biocatalysts for the production of useful compounds, are expected to play a key role in green chemistry. Microbial diversity constitutes an infinite pool of novel chemistry, making up a valuable source for innovative biotechnology. So far we have only scratched the surface of it. The most recent estimates suggest that by now we only know approximately 5 % of the total species of fungi and may be as little as 0.1 % of the bacteria and among the ones already described, only a small fraction has been examined for metabolite profile.

The microbial secondary metabolites can be brought in use in three different ways: the bioactive molecule can be produced directly by fermentation; or the fermentation product can be used as starting material for subsequent chemical modification (derivatization); or thirdly the molecules can be used as lead compounds for a chemical synthesis. Remarkable milestone in the medicinal use of microbial metabolites and their derivatives was the introduction of the immune suppressants cyclosporin A, and rapamycin (Chen et al. 1995, Van Middlesworth and Cannell 1998). Other examples are the commercialization of the antihyperlipidemic lovastatin and guggulsterone (Urizar et al. 2002). Microbial natural products have also been developed as antidiabetic drugs, hormone (ion-channel or receptor) antagonists, anticancer drugs, and agricultural and pharmaceutical agents (Zhang 2005).

4.5 Genetic Diversity and Metagenomics

Genetic diversity is manifested as biological diversity through the structure, organization, regulation, and expression of DNA. Presence and expression of DNA in the biological systems of a given environment determine the physiological functions of the biotic and abiotic components of the environment. Metagenomics (also referred to as environmental and community genomics) is the genomic analysis of microorganisms by direct extraction and cloning of DNA from an assemblage of microorganisms. It is a new field combining molecular biology and genetics to isolate, identify, and characterize the genetic material from environmental samples and express it in suitable host. The metagenomic DNA is inserted into a model organism that lacks a specific gene function. Restoration of a physical or chemical phenotype can then be used to detect genes of interest. A genotype is the specific sequence of the DNA and offers another means of analyzing the metagenomic DNA fragment. The sequences of the bases in DNA can be compared to the database of known DNA to get information regarding the structure and organization of the metagenomic DNA. Comparisons of these sequences can provide insight into how the gene proteins function.

4.6 Analysis of Microbial Diversity

4.6.1 Conventional and Biochemical Methods

Both conventional and biochemical methods are of high significance in the study of microbial diversity. The diversity can be described using physiological diversity measures too, which avoid the difficulties that may arise in grouping of similar bacteria into species or equivalents. These measures include various indices (tolerance, nutrition, etc.). Multivariate data analyses have also been used for extracting relevant information in the large data-sets frequently obtained in diversity studies.

a. Plate counts

The most traditional method for assessment of microbial diversity is selective and differential plating and subsequent viable counts. Being fast and inexpensive, these methods provide information about active and culturable heterotrophic segment of the microbial population. There are many limiting factors in this assessment method including the difficulties in removing bacteria or spores from soil particles or biofilms, selecting suitable growth media (Tabacchioni et al. 2000), arrangement of specific growth conditions (temperature, pH, light), inability to culture a large number of bacterial (Barnes et al. 1994) and fungal species using techniques available at present, and the potential for inhibition or spreading of colonies other than that of interest (Trevors 1998).

b. Sole carbon source utilization (SCSU)

Garland and Mills (1991) introduced biochemical identification systems (such as API and Biolog), the sole carbon source utilization (SCSU) system, also known as community level physiological profiling (CLPP) system. This was initially developed as a tool for identifying pure cultures of bacteria in the species level, based upon a broad survey of their metabolic properties. SCSU examines the functional capabilities of the microbial population, and the resulting data can be analyzed using multivariate techniques to compare metabolic capabilities of communities (Preston-Mafham et al. 2002). However, as microbial communities are composed of both fast and slow growing organisms, the slow growers may not be included in this analysis. Growth on secondary metabolites may also occur during incubation.

c. Phospholipid fatty acid (PLFA) analysis

The fatty acid composition of microorganisms has been used extensively in characterizing microorganisms. Taxonomically, fatty acids in the range C2–C24 have provided the greatest information and are present across a diverse range of microorganisms (Banowetz et al. 2006). The fatty acid composition is stable and is independent of plasmids, mutations, or damaged cells. The method is quantitative, cheap, robust and with high reproducibility. However, it is important to notice that the bacterial growth conditions are reflected in the fatty acid pattern. This method is also known as the fatty acid methyl ester (FAME) analysis.

4.6.2 Molecular Techniques for Studying Microbial Biodiversity

An effort must be made to study microbial diversity to make sure its conservation. The exact extent of microbial diversity remains unknowable. Nevertheless, fingerprinting patterns denaturing gradient gel electrophoresis (DGGE), single strand conformation polymorphism (SSCP) provide an image of a microbial ecosystem and contain diversity data (Loisel et al. 2006). An astonishingly small amount of research is devoted to bacterial diversity, as opposed to the genetics and molecular biology of select species (Ehrlich and Wilson 1991). Clearly, this must be changed, for all microorganisms, not just the prokaryotes (Fig. 4.2).

New techniques allow for environmental screening to determine the presence of nucleic acids within environmental samples. These molecular genetics techniques allow screening of

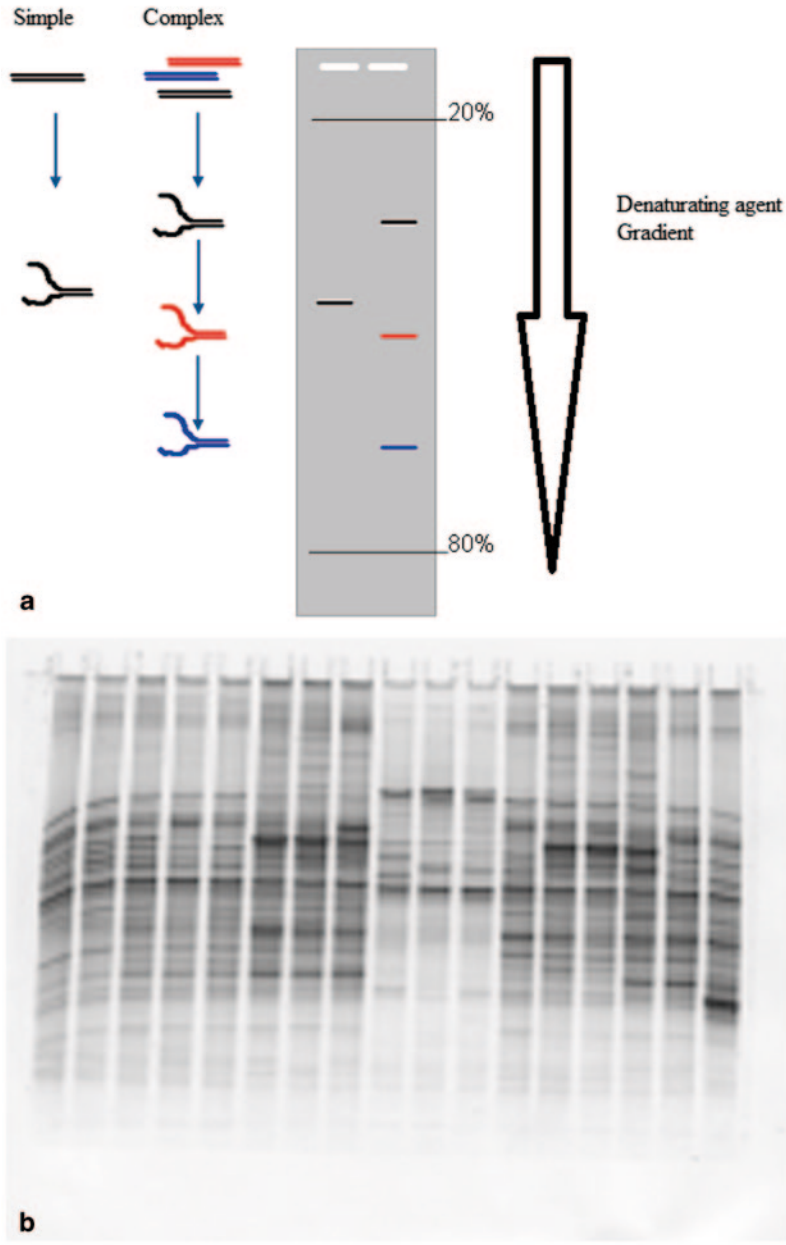

Fig. 4.2 DGGE: PCR products of mixed communities are loaded on a gel with a gradient of denaturant (typically 20–80 % formamide). Double stranded DNA will run down the gel until it melts. Melting is determined by sequence and GC content. Different sequences migrate different distances (**a**), You obtain a "barcode" of the community (**b**)

organisms that could be maintained in culture along with those that cannot be cultured. Those uncultivable microorganisms cannot be identified by standard means. Pace, for example, extracted DNA directly from samples, then used the polymerase chain reaction (PCR) to amplify small subunit ribosomal RNA (rRNA) genes, selectively amplifying those found in archaea and eukaryotes.

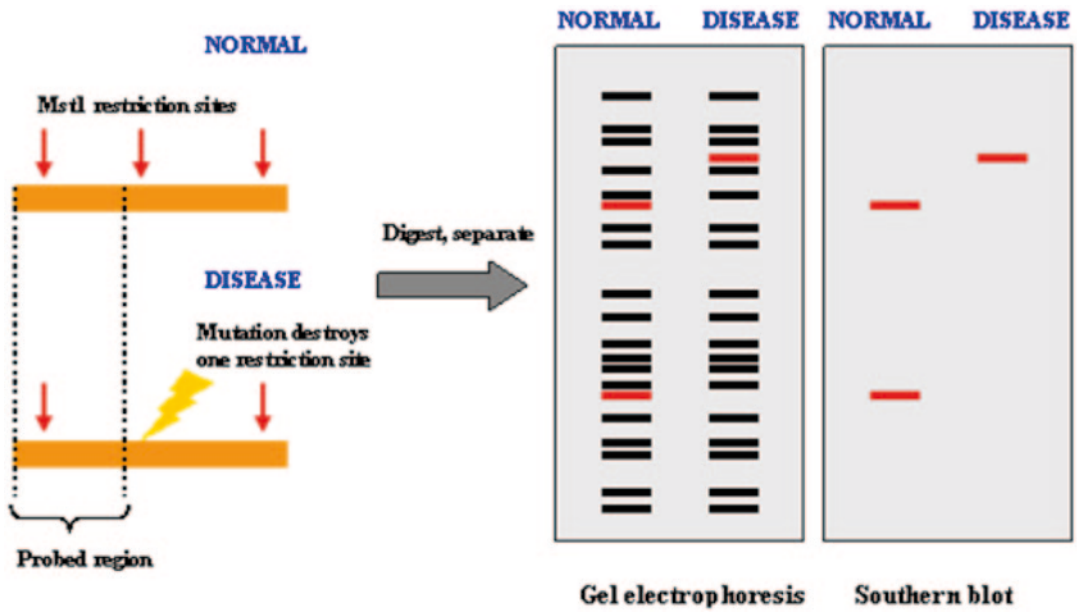

Fig. 4.3 RFLP helps us see the differences between the DNA

Then the comparison of the ribosomal DNA sequences with known rRNA sequences was done. (Muyzer and Smalla 1998; Nakatsu et al. 2000) Although this technique does not give the full description but numbers and lineages of microorganisms within environmental samples can be determined, remarkably their phylogenetic relationships and genetic similarity to sequences in known databases.

Many scientists have amplified 16S rRNA genes from environmental samples, and have produced important results from such works (Giovannoni et al. 1990; Fuhrman et al. 1993a; Mishra and Thakur 2010). This is still a time-consuming technique even after simplification of steps and is not suitable for analysis of hundreds of samples and along with requires a relatively large database (Colwell and Hawksworth 1991).

It is now possible to study microbial communities using the randomly-amplified polymorphic DNA (RAPD) method. This generates DNA fingerprinting characteristics of the community. This technique also does not allow the identification of individual microorganism.

Restriction fragment length polymorphism (RFLP) is another tool used to study microbial diversity and community structure (Moyer et al. 1996). This method relies on DNA polymorphisms. In this method, electrophoresed DNA bands are blotted from agarose gels onto nitrocellulose or nylon membranes and hybridized with appropriate probes prepared from cloned DNA segments of related organisms (Fig. 4.3). RFLP has been found to be very useful particularly in combination with DNA–DNA hybridization and enzyme electrophoresis for the differentiation of closely related strains (Rastogi and Sani 2011). RFLPs may provide a simple and powerful tool for the identification of bacterial strains at and below species level (Kauppinen et al.1994). This method is useful for detecting structural changes in microbial communities. But this technique cannot be used as an extent of diversity or for detection of specific phylogenetic groups (Liu et al. 1997). Banding patterns in diverse communities become too complex to analyze using RFLP since a single species could have four to six restriction fragments (Tiedje et al. 1999).

Terminal restriction fragment length polymorphism (T-RFLP) is a technique that talks about some of the limitations of RFLP (Zhang et al. 2008). This technique is an extension of the RFLP/amplified ribosomal DNA restriction

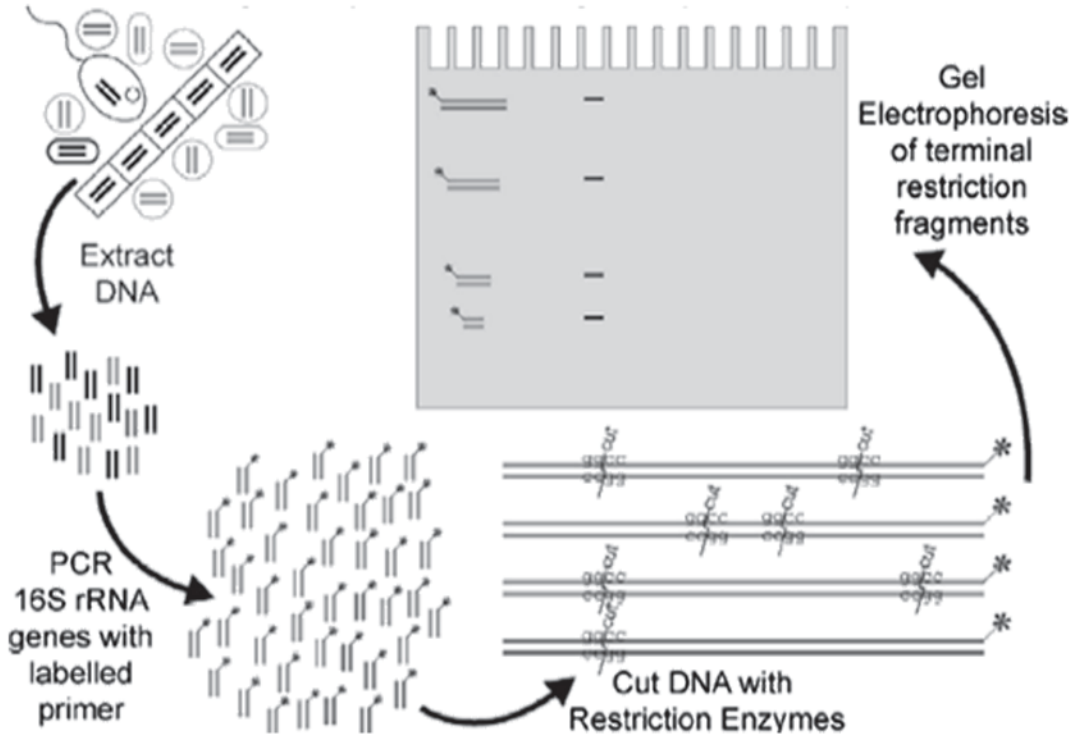

Fig. 4.4 Diagrammatic representation showing T-RFLP. Mixed population is amplified using a *16S* primer with a fluorescent tag. *PCR* product is cut with a 4 bp cutting restriction endonuclease. Different sequences will give different length fragments. Sample is injected into a capillary sequencer to sort fragments by size

analysis (ARDRA) and provides an alternative method for rapid analysis of microbial community diversity in various environments. It follows the same principle as RFLP except that one PCR primer is labeled with a fluorescent dye (Fig. 4.4).

Similar in principle to RFLP and T-RFLP, ribosomal RNA (rRNA) intergenic spacer analysis (RISA), automated ribosomal RNA (rRNA) intergenic spacer analysis (ARISA), and ARDRA provide ribosomal-based fingerprinting of the microbial community. In RISA and ARISA, the intergenic spacer region between the 16S and 23S ribosomal subunits is amplified by PCR, denatured and separated on a polyacrlyamide gel under denaturing conditions. This region may encode tRNAs and is useful for differentiating between bacterial strains and closely related species because of heterogeneity of the intergenic space length and sequence (Scheinert et al. 1996). Sequence polymorphisms are generally detected by silver staining in RISA. In ARISA, as the name suggests fluorescently labeled forward primer is detected automatically (Fisher and Triplett 1999). Both methods can provide highly reproducible bacterial community profiles. The process of RISA requires large quantities of DNA, relatively longer time requirement, insensitivity of silver staining in some cases, and low resolution which can be taken as its limitations. ARISA has better sensitivity than RISA and is less time consuming but traditional limitations of PCR also applies for ARISA (Brown and Fuhrmans 2005). RISA has been used to compare microbial diversity in soil, in the rhizosphere of plants (Borneman and Triplett 1997), and in contaminated soil (Ranjard et al. 2000).

Presently, DNA–DNA hybridization has been used along with DNA microarrays to detect and identify bacterial species or to evaluate microbial diversity (Rastogi and Sani 2011). This tool could

be valuable in bacterial diversity studies since a single array can contain thousands of DNA sequences (De Santis et al. 2007) with high specificity. Specific target genes coding for enzymes such as nitrogenase, nitrate reductase, naphthalene dioxygenase, etc., can be used in microarray to elucidate functional diversity information of a community. Sample of environmental "standards" (DNA fragments with less than 70% hybridization) representing different species likely to be found in any environment can also be used in microarray (Greene and Voordouw 2003).

There are some other molecular methods that have been used potentially to study the microbial community. Fluorescent in situ hybridization (FISH) (Dokić et al. 2010), DNA sequencing based community analysis such as pyrosequencing based community analysis (Fakruddin and Chaudhary 2012), illumina-based high throughput microbial community analysis (Degnan and Ochman, 2012) etc. are some examples for these techniques. Most of these methods are not as appropriate as previously mentioned methods.

Viruses in marine samples can be studied by nonmolecular methods: transmission electron microscopy (TEM) and epifluorescent microscopy (DAPI stain) by differential filtration. The development of improved methods for isolating and characterizing virus in the marine environment now makes it possible to study their role in ecosystem (Table 4.1).

4.7 Conservation of Microbial Diversity

The problem of biodiversity is essentially one of conflict resolution between the human kind on one side and living organisms inhabiting different environment on the other side. The UNCED (United Nations Conference on Environment and Development) process has helped place the loss of biodiversity and its conservation on global agenda. The Convention on Biological Diversity (CBD) that emerged from the UNCED or Earth Summit at Rio de Janerio in June 1992 is now a treaty.

World Conservation Monitoring Center has described 1,604,000 species at the global level. India accounts for 8% of global biodiversity existing in only 2.4% land area of the world. Microbial diversity conservation requires certain specialized techniques for applications in reclamation of a tainted habitat. Both ex situ and in situ techniques can be employed to preserve the biodiversity.

4.7.1 Ex Situ Preservation

The most effective and efficient mechanism for conserving biodiversity is to prevent the destruction or degradation of the habitat. Because of the uncertainties associated with in situ conservation of microorganisms, ex situ preservation plays a major role in microbiology and includes the gene banks, culture collections, and microbial resource centers forming the repository for microbial isolates and do away with need for costly and time consuming reisolation protocols. The CBD encourages adoption of measures for ex situ conservation of biodiversity, preferably in the country of origin. Application of this approach is supported by the World Federation for Culture Collection (WFCC) and Directory of Collection of Cultures of Microorganisms. Moreover, four other associations that directed toward this effort are Oceanic and Atmospheric Administration for marine microbial diversity, National Institute of Health for deciphering the emerging microbial pathogen diversity, American Society for Microbiology, and American Phytopathological Society.

In India, this work has been carried out by the Ministry of Environment and Forests and the Ministry of Science and Technology that includes various departments such as the Department of Agriculture Research and Education, Indian Council of Forestry Research and Education, Department of Biotechnology. The level of the microbial type culture collection section of IMTECH, Chandigarh has now been upgraded to an International Depository Authority (IDA) and it involves the culture collection and maintenance as well as distribution of pure cultures

Table 4.1 Advantages and disadvantages of some molecular-based methods to study soil microbial diversity. (Source: Fakruddin and Mannan 2013 and Kirk et al. 2004)

Method	Advantages	Disadvantages
Mole % (G+C)	Not influenced by PCR	Requires large quantities of DNA
	Includes all DNA extracted	Dependent on lysing and extraction efficiency
	Includes rare members of community	Less sensitive resolution
Nucleic acid reassociation and hybridization	Total DNA extracted	Lack of sensitivity
	Not influenced by PCR biases	Sequences need to be in high copy number for detection
	Can study DNA or RNA	Dependent on lysing and extraction efficiency
	Can be studied in situ	
DNA microarrays and DNA hybridization	Same as nucleic acid hybridization	Only detect the most abundant species
	Process thousands of genes simultaneously	Need to culture organisms
	If using genes or DNA, fragments specificity increases	Only accurate in low diversity systems
Single strand conformation polymorphism (SSCP)	Same as DGGE/TGGE	PCR biases
	No GC clamp	Some ssDNA can form more than one stable conformation
	No gradient	
Denaturing and temperature gradient gel electrophoresis (DGGE and TGGE)	Large number of samples can be analyzed simultaneously	PCR biases
	Reliable, reproducible, and rapid	Dependent on lysing and extraction efficiency
		Way of sample handling can influence community, i.e., the community can change if stored for too long before extraction
		"One band-one species" is not always true
		Cannot compare bands between gels
		Only works well with short fragments (<500 bp), thus limiting phylogenetic characterization
		Only detects dominant species
Restriction fragment length polymorphism (RFLP)	Detect structural changes in microbial community	PCR biases
		Banding patterns often too complex
Terminal restriction fragment length polymorphism (T-RFLP)	Simpler banding patterns than RFLP	Dependent on extraction and lysing efficiency
	Can be automated	PCR biases
	Can process large number of samples	Type of Taq can increase variability
	Highly reproducible	Choice of restriction enzymes will influence community fingerprint
	Ability to compare differences between microbial communities	

Table 4.1 (continued)

Method	Advantages	Disadvantages
Ribosomal intergenic spacer analysis (RISA)/automated ribosomal intergenic spacer analysis (ARISA)/amplified ribosomal dna restriction analysis (ARDRA)	Highly reproducible community profiles	Requires large quantities of DNA (for RISA) PCR biases

internationally. The ex situ collections of microorganisms form the key repositories of biodiversity and an essential resource for the future as these could be linked to the research programs and developmental aspects of the country that owns it by assimilating the microbiological aspects, molecular evolution, systematics, and microbial chemistry with genome science. Enhanced funding of stock centers and greater emphasis on education and research in microbial systematics will amplify the broad base of research into microbial diversity.

4.7.2 In Situ Preservation

Basically, in situ preservation involves on-site conservation of the microbial flora involving the conservation of the ecosystems and on-site conservation of the microbial flora involving the conservation of the ecosystems and natural habitats and the maintenance as well as recovery of viable populations of species in their natural surroundings and in case of the domesticated or cultivated species, in surroundings where they have developed their distinctive properties. Conservation of all subsets of life existing in interplaying networks will lead to preservation of microbes as well. Avoiding deforestation and planting trees (aforestation) will not allow the surface soil to be washed out by torrential rains, which contains diverse microflora. Further, avoiding pollution of water bodies such as oceans, rivers, or lakes will preserve phytoplanktons, zooplanktons (rotiferans, microalgae, diatoms, dinoflagellates), and other floating microbes such as *Vibrio parahaemolyticus*, *Bacillus sp.*, *Spirillum sp.*, *Aquaspirillum sp*. and others. Certain countries such as Italy, Canada, Brazil, Mexico, Chile, Argentina are facing new kind of natural conservation on account of widespread jungle fires. Microbial diversity in forest soils is a key factor in ecosystem function. Staddon et al. (1996) have described the role of fire and its impact on conservation of microbial diversity of forest soil. Large-scale endemic fires in Andes Mountain ranges increased the carbon content besides considerable increase in phosphorus, calcium, zinc, and other trace elements. This contributed toward increase in number and variety of microorganisms in soil after second or third rains. National Biodiversity Conservation Board has taken interest in microbial diversity and its preservation. The corporate-led globalization and economic models imposed by WTO were also discussed and were found to be the main driving force and underlying cause of biodiversity loss.

Forest conservations were reviewed with exclusion on large-scale monoculture tree plantations and time-bound action plants for stopping the convention of natural forests. The convention also issued statement that a tough and clear standpoint on the spread of genetically modified (GM) crops and genetic pollution, invasion by alien species that threaten the ecosystem as well as ban on terminator technologies is needed. An integral component of conservation biology will be of proper economic valuation. The direct value of microbes rests in their utilization in biotechnology, as single cell protein products, as biofertilizers, while indirect value involve their role as decomposers and involvement in recycling of plant and animal matter, as indicators of environmental pollution, as bioremediation agents and in other subtle functions of human life.

4.8 Consequences of Ignoring the Conservation of Microbial Diversity

In today's scenario the research is mainly focused on the profitable and producible projects where one can make his future. Due to some funding constraints, the hard core taxonomy projects are not going on in a massive scale. If this philosophy continues, the role and identification of numerous unknown microorganisms will be lost. Major ecological changes are occurring already which may result into deleterious ecosystem conditions.

4.9 Conclusion

With respect to the role of microorganisms in sustainable development, little is known about the potential contribution of microbial diversity to the national economy, to wealth creation and to improvements in the quality of life. An appreciation of these factors might be one way of changing government and public perception of microorganisms by showing that the sustainable use of microbial diversity has positive economic value. This would help justify the costs involved in conserving microbial diversity, but equally provide a useful indicator of the costs of inaction. In terms of the scientific rationale needed to underpin policy, quantification of microbial diversity has been limited. This makes it difficult to indicate what needs to be conserved in order to support the biotechnology industries and to understand fully the interactions between organisms responsible for maintaining a functional ecosystem.

Microbial diversity in natural environments is extensive. Methods for studying diversity vary and diversity can be studied at different levels, i.e., at global, community, and population levels. The molecular perspective gives us more than just a sight of the evolutionary past; it also brings a new future to the discipline of microbial ecology. Since the molecular-phylogenetic identifications are based on sequences, as opposed to metabolic properties, microbes can be identified without being cultivated. Consequently, all the sequence-based techniques of molecular biology can be applied to the study of natural microbial ecosystems. These methods characterize the microbial processes and thereby can be used to reach a better understanding of microbial diversity. In future, these techniques can be used to analyze microbial diversity quantitatively and expand our understanding of their ecological processes to make our ecosystem livelier.

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Phytoremediation: A Biotechnological Intervention

5

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Abstract

Phytoremediation, a growing sector of bioremediation, exploits the natural ability of a large variety of plants to filter chemicals through their root systems and to aerate the soil, allowing different microorganisms to grow. Phytoremediation has many advantages over other existing technologies in terms of safe and nondisturbing natural surroundings of contaminated sites. The modification in technology leads to different methods of phytoremediation, including phytotransformation, rhizoremediation, phytostabilization, phytoextraction and rhizofiltration. The application of a selected method depends on the nature and site of contaminant. To understand the mechanism of hyperaccumulation, various studies have been conducted on model (*Arabidopsis thaliana*) and commonly grown plants such as *Populus*, *Brassica*, *Hydrilla* etc. in phytoremediation. Further, based on mechanism and identified genes such as those involved in uptake, sequestration, remobilization and homeostasis, transgenic plants were designed and used efficiently to remove heavy metals and organic chemicals from the soil. However, further efforts are required for advancements in efficiency and robustness of transgenic plants and to popularize the phytoremediation technology on a commercial scale.

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5.1 Introduction

Worldwide technological advancements, uncontrolled anthropogenic activity (mining, metal extraction, fertilizers, pesticide industries, household activities and vehicles) and natural events (seepage from rocks, volcanic eruption and forest fires) cause environmental deterioration in terms of heavy and toxic metal contamination in soil, aqueous water streams and groundwater, thus posing a major community problem that needs to be addressed. The main threats to environment and human health from heavy toxic metals and

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minerals are associated with exposure to lead, mercury, chromium, cadmium, copper, arsenic and aluminium. These enter the human system mainly through contaminated water, food and air, leading to various health complications. Global environmental agencies such as United Nations Environment Programme (UNEP), European Environment Agency (EEA), World Nature Organization (WNO) and in India Ministry of Environment (MoE), Government of India, work for finding preventive and remedial solutions for management. Existing options involve expensive technology and recurring investments, thus making them difficult to be affordable to most of the developing countries like India. Therefore, considering developing countries' economic status, increasing population and malnutrition, appropriate intervention in terms of indigenous research towards mitigation and remediation needs to be pondered and designed for effective and efficient application.

In general, heavy metal toxicity can cause chronic degenerative diseases, with the symptoms of mental disorders, pain in muscles and joints, gastrointestinal disorders, vision problems, chronic fatigue and susceptibility to fungal infections. Sometimes the symptoms are vague and difficult to diagnose at early stages and lead to genotoxicity and cancers. Industrial workers and populations living near the polluting industries are more susceptible and need to be monitored regularly. Malnourished people and pregnant women are vulnerable. Crippling effects of fluoride and arsenic toxicity due to nonavailability of safe water for drinking and farming has become a major public health problem and needs to be addressed. Management strategies are being prioritized to mitigate such problems. Considering the fact that the metal once out of the rock is destined to mix in the environment, different physicochemical and bioremediation strategies are being implemented to reduce the environment load, preferably at the site of generation. However, large industries should be forced to set up their own effluent treatment plants, and smaller industries should use common effluent treatment facilities. Industries as sources of heavy metals are summarized in Table 5.1.

Although heavy metals and minerals (fluoride arsenic salts) are reported to be hazardous beyond safe limits, smaller quantities of Fe, Zn, Cu, Co, Cr, Mn and Ni are required for proper human metabolism. However, Pb, Hg, Cd, and As have no beneficial role and are absolutely toxic. Small amounts of fluoride help to prevent dental cavities, but excess is harmful. In the environment, these elements have a tendency to get stabilized in the form of organic salts and complexes and are bioaccumulative. Consequently, deriving their safe limits is very difficult. The toxicity of metals also depend on their chemical form and oxidation state which further complicate the toxicity assessment. Toxicity studies therefore required the consideration of metal speciation in terms of valency and oxidation state e.g. CrIII (non-toxic) and CrVI (toxic).

To alleviate these microbial hazards, bioremediation strategy has been emphasized, and extensive funding has been provided to research and development (R&D). Bioremediation is using microorganisms to degrade pollutants in situ. Since heavy metals and radionuclide wastes cannot be chemically degraded, application of microbial bioremediation is limited to the immobilization of heavy metals by precipitation or reduction or conversion of toxic to nontoxic forms in situ.

The use of plants for cleaning the environment has been implemented from ancient times and considered as indigenous knowledge. The physiological exploration of plants revealed ion exchange pumps and transporters that can extract

Table 5.1 Industries as sources of heavy metal contaminants

Metal	Industry
Chromium (Cr)	Mining, industrial coolants, chromium salts manufacturing, leather tanning
Lead (Pb)	Lead acid batteries, paints, e-waste, smelting operations, coal-based thermal power plants, ceramics, bangle industry
Mercury (Hg)	Chlor-alkali plants, thermal power plants, fluorescent lamps, hospital waste (damaged thermometers, barometers, sphygmomanometers), electrical appliances etc.
Arsenic (As)	Geogenic/natural processes, smelting operations, thermal power plants, fuel burning
Copper (Cu)	Mining, electroplating, smelting operations
Vanadium (Va)	Spent catalyst, sulphuric acid plants
Nickel (Ni)	Smelting operations, thermal power plants, battery industry
Cadmium (Cd)	Zinc smelting, waste batteries, e-waste, paint sludge, incinerations and fuel combustion
Molybednum (Mb)	Spent catalyst
Zinc (Zn)	Smelting, electroplating

and concentrate elements from the environment. These metals include Fe, Mn, Zn, Cu, Mg, Mo and Ni, essential for growth and development; whereas Cd, Cr, Pb, Co, Ag, Se and Hg are also reported to be accumulated in plants but have no known biological function. Thus, green plants being used to remove pollutants from the environment is referred to as phytoremediation. Under phytoremediation, plants exhibit the ability to tolerate elevated levels of heavy metals and accumulate them to unusually high concentrations either independently or in combination and have been reported for Ni, Co, Cu, Mn, Pb, Zn and Se (Brooks et al. 1978, 1979, 1981; Reeves and Brooks 1983; Banuelos and Meeks 1990).

5.2 Phytoremediation: Need or Necessity

Phytoremediation is very competitive with other treatment alternatives. It is simple to use and has high public acceptability. Due to its advantages over microbial bioremediation it is considered as a need of the present time, but the industrial revolution and increasing population manipulate this need into necessity. Efficiency and effectiveness are the two scales that we have compared and summarized in Table 5.2, in which 5-year costs are compared between phytoremediation by hybrid poplar trees, and conventional pump and treatment with a reverse osmosis system. Phytoremediation costs less than half of the pump and reverse osmosis treatment technology. According to a report by Phytotech (1997), phytoextraction provides significant cost advantages over in situ fixation, excavation and landfilling in a Resource

Table 5.2 Five-year cost comparison between phytoremediation by hybrid poplar trees, and conventional pump and treatment with reverse osmosis system

Phytotransformation	Cost in dollars ($)
Design and implementation	50,000
Monitoring equipment	
Capital	10,000
Installation	10,000
Replacement	5,000
Five-year monitoring	
Travel and administration	50,000
Data collection	50,000
Reports (annual)	25,000
Sample analysis	50,000
Total	250,000
Pump and treatment (three wells and reverse osmosis system)	
Equipment	100,000
Consulting	25,000
Installation/Construction	100,000
Five-year cost	
Maintenance	105,000
Operation (electricity)	50,000
Waste disposal	180,000
Waste disposal liability	100,000
Total	660,000

Table 5.3 Cost advantage of phytoextraction for metals (Schnoor 1997)

Type of treatment	Cost/m^3 ($)	Time required (months)	Additional factors/expense	Safety issues
Fixation	90–200	6–9	Transport/excavation long-term monitoring	Leaching
Landfilling	100–400	6–9	Long-term monitoring	Leaching
Soil extraction, leaching	250–500	8–12	5000 m^3 minimum Chemical recycle	Residue disposal
Phytoextraction	15–40	18–60	Time/land commitment	Residue disposal

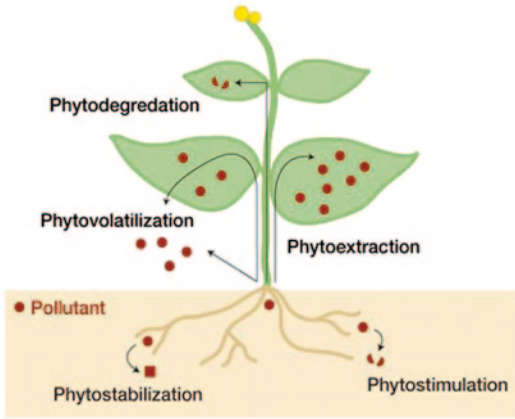

Fig. 5.1 Different methods of phytoremediation are exhibiting involved mechanism. (Source: http://www.personal.psu.edu/dgh5037/extEssay.html)

Conservation and Recovery Act (RCRA)-approved hazardous waste facility, and soil extraction. The only limitation of cheap and effective phytoremediation technology is the requirement of a long time period compared to competing technologies (Table 5.3). Phytoremediation is most comparable with in situ bioremediation and natural attenuation.

5.3 Phytoremediation Methods

Phytoremediation consists of a collection of four different plant-based technologies, each having a different mechanism of action for the remediation of metal-polluted soil, sediment and water (Fig. 5.1). The main processes that involve the treatment of environmental problems by using plants are:

5.3.1 Phytotransformation

Phytotransformation, also known as phytodegradation, is the breakdown of organic and nutrient contaminants present in soil and groundwater after sequestration by plants via metabolic processes within the plant and specific enzymes produced by the plant. The organic contaminants are degraded into simpler compounds that are integrated with plant tissue, which in turn, support plant growth. Remediation of any site by phytotransformation is dependent on the efficiency of direct uptake of contaminants from soil water and the accumulation in form of metabolites in plant tissue. The metabolites which are nontoxic or significantly less toxic should be accumulated in vegetation. Potential applications include phytotransformation of petrochemical sites and their storage areas, ammunition wastes, fuel spills, chlorinated solvents, landfill leachates and agricultural chemicals (pesticides and fertilizers). Sometimes phytoremediation is used in combination with other approaches such as ex situ treatment of highly contaminated wastes, or removal actions or polishing treatment. Plants either directly uptake contaminants from the soil water or release exudates that help to degrade organic pollutants via cometabolism in the rhizosphere.

Direct uptake of organics by plants is generally observed at shallow-depth contaminated sites with moderately hydrophobic organic chemicals, including benzene, toluene, ethylbenzene and xylene (BTEX) chemicals, chlorinated solvents and short-chain aliphatic chemicals. Hydrophobic chemicals (log Kow > 3.5) are not easily translocated within the plant due to strong bonding to the surface of roots and soils. Chemicals which are readily water-soluble (log Kow < 1.0) are not

sufficiently absorbed by roots and also not actively transported through plant membranes (Briggs et al. 1982). It was found that highly hydrophobic chemicals (log Kow > 3.5) are candidates for phytostabilization and/or rhizosphere bioremediation. The uptake efficiency consequently depends on physical–chemical properties, chemical speciation of contaminant and the type of plant.

Chemical uptake also depends on transpiration where its rate depends on the plant type, leaf area, nutrients, soil moisture, temperature, wind conditions and relative humidity. Once an organic chemical is translocated within plants, it can either be stored into new plant structures via lignification (chemical contaminant or its fragments covalently bound to lignin of the plant) or volatilized, metabolized or mineralized completely to carbon dioxide and water. Chlorinated aliphatic compounds such as trichloroethylene (TCE) have been reported to be mineralized to CO_2 and less toxic aerobic metabolites such as trichloroethanol, trichloroacetic acid and dichloroacetic acid (Newman et al. 1997). These products are consistent with those found in the human liver as a result of action of cytochrome P_{450} on TCE. Cytochrome P_{450} is an abundant enzyme in plants as well as humans, thus plants are sometimes referred as "green livers" in terms of their enzyme biochemistry.

Another form of phytotransformation is phytovolatilization, where volatile chemicals or their metabolic products are released to the atmosphere through plant transpiration. Many recalcitrant organic chemicals in the subsurface environment react rapidly in the atmosphere with hydroxyl radicals, oxidants formed in the photochemical cycle. Nitroreductase and laccase enzymes in plants can break down ammunition wastes such as TNT (2,4,6-trinitrotoluene) and might incorporate the broken ring structures into new plant material or organic detritus. Detoxification mechanisms may transform the parent chemical into nonphytotoxic metabolites that are stored in plant tissues (Schnoor et al. 1995). Typical plants used in various applications of phytoremediation are summarized in Table 5.4.

5.3.2 Rhizosphere Bioremediation

Rhizosphere bioremediation is also referred to as phytostimulation or plant-assisted bioremediation, since phytoremediation of the rhizosphere takes place by initially providing increased soil organic carbon and nitrogen content required for bacterial and mycorrhizal fungi, which altogether encourage degradation of organic chemicals in soil. Similar observations are recorded around poplar trees where numbers of beneficial bacteria increased in the root zone, including denitrifiers, *Pseudomonas* sp., BTEX-degrading organisms and general heterotrophs, relative to an unplanted reference site (Jordahl et al. 1997). Plants may release exudates in the soil environment to help microbial communities metabolize organic contaminants by inducing enzyme systems of existing bacterial populations, stimulating growth of new species that are able to degrade pollutants and/or increase soluble substrate concentrations. These exudates comprise sugars, alcohols and acids that amount to 10–20 % of plant photosynthesis products annually (Foth 1990).

The most widely used plants in rhizosphere bioremediation are grasses and *Papilionaceae,* which possess rich root systems that hold a larger soil volume compared to other plant species (Smreczak and Maliszewska-Kordybach 2005). The study conducted on hybrid poplar trees revealed distribution of short-chain organic acids, phenolics and small concentrations of high molecular weight compounds (enzymes and proteins) in exudates based on molecular weight characterization. Five plant enzyme systems: dehalogenase, nitroreductase, peroxidase, laccase and nitrilase are identified in sediments and soils released from plant exudates. Dehalogenase enzymes are important in dechlorination reactions of chlorinated hydrocarbons. Nitroreductase is needed in the first step for degradation of nitroaromatics, while laccase enzyme serves to break aromatic ring structures in organic contaminants. Peroxidase and nitrilase are important in oxidation reactions. These enzymes are active in rhizosphere soils in close proximity to the root

Table 5.4 Typical plants used in various phytoremediation applications

Application	Media	Contaminants	Key plants
Phytotransformation	Soil, groundwater, landfill leachate, land application of wastewater	Herbicides (atrazine, alachlor) Aromatics (BTEX) Chlorinated aliphatics (TCE) Nutrients (NO_3^+, NH_4^+, PO_4^{3-}) Ammunition waste (TNT, RDX)	Phreatophyte trees (poplar, willow, cottonwood aspen); grasses (rye, bermuda, sorghum, fescue); legumes (clover, alfalfa, cowpeas)
Rhizosphere bioremediation	Soil, sediments, land application of wastewater	Organic contaminants (pesticides, aromatics and polynuclear aromatic hydrocarbons (PAHs))	Phenolic releasers (mulberry, apple, orange); grasses with fibrous roots (rye, fescue, Bermuda) for contaminants 0–3 ft deep; phreatophytes trees for 0–10 ft; aquatic plants for sediments
Photostabilization	Soil, sediments	Metals (Pb, Cd, Zn, As, Cr, Cu, Se, U) Hydrophobic organics (PAHs, PCBs, dioxins, furans, PCP, DDT, dieldrin)	Phreatophyte trees to transpire large amount of water for hydraulic control; grasses with fibrous roots to prevent soil erosion; dense root system to sorb/bind contaminants
Phytoextraction	Soil, brownfields, sediments	Metals (Pb, Cd, Zn, Ni, Cu) with EDTA addition for Pb and Se (volatilization)	Sunflowers, Indian mustards, rapeseed plants, barley, crucifers, serpentine plants, dandelions
Rhizofiltration	Groundwater, water and wastewater in lagoons or created wetlands	Metals (Pb, Zn, Cu, Ni, Cd) Radionuclides (^{137}Cs, ^{90}Sr, U) Hydrophobic organics	Aquatic plants; Emergents (billrush, cattail, coontail, pondweed, arrowroot, duckweed); Submergents (algae, stonewort, parrot feather, Eurasian water milfoil, *Hydrilla*)

BTEX benzene, toluene, ethylbenzene and xylene, *TCE* trichloroethylene, *TNT* 2,4,6-trinitrotoluene, *RDX* Research Department explosive, *PCB* polychlorinated biphenyl, *PCP* pentacholorophenol, *DDT* dichlorodiphenyltrichloroethane, *EDTA* ethylenediaminetetraacetic acid

(1 mm) for transformation of organic contaminants. When plants are grown in soil or sediment slurries, pH is buffered, metals are biosorbed or chelated and enzymes remain protected inside the plant or absorbed to plant surfaces. In US Environmental Protection (EPA) studies of TNT breakdown, plants like hornwort increase soil water pH from 3 to 7 and sorb high concentrations of metals that usually inhibit bacteria, while the plants remain healthy and viable. Overall, plants and their root systems can accommodate mixed wastes (organic and metals) and other harsh conditions (Schnoor et al. 1995).

Shaw and Burns (2007) have demonstrated the importance of biodegradation in the rhizosphere. Plants are associated with microbial transformations in many ways, such as: mycorrhiza fungi associated with plant roots metabolize the organic pollutants; plant exudates stimulate bacterial transformations (enzyme induction); build-up of organic carbon increases microbial mineralization rates (substrate enhancement); plants provide habitat for increased microbial populations and activity; oxygen is pumped to roots ensuring aerobic transformations.

Narasimhan et al. (2003) have reported that flavonoids and coumarin are released by root turnover from trees like mulberry, orange and apple that selectively stimulate polychlorinated biphenyl (PCB)- and PAH-degrading organisms.

Certain organics are not solely degraded by bacteria, but instead utilize the enzymatic pathways of other plant symbionts such as fungi. In addition to soluble exudates, the rapid decay of fine root biomass can become an important addition of organic carbon to soils which serves to retard organic chemical transport. Microbial mineralization of atrazine is directly related to the fraction of organic carbon in the soil (Zablotowicz et al. 2006). Rhizofiltration is effective and economically utilized under low concentrations of contaminants and large volumes of water, therefore particularly applicable to radionuclide-contaminated water. The cationic and anionic radionuclide contaminants are substantially or completely removed from water using selective metal-accumulating plants under an optimized rhizofiltration system, although mechanism of uptake is not fully studied (Macaskie 1991).

5.3.3 Phytostabilization

Phytostabilization refers to stabilization of heavy metal contaminants in soil and sediments through revegetation with metal-tolerant plant species. Generally heavy metal-polluted soils lack vegetation cover due to the toxic effects of pollutants, which makes such soil prone to erosion and leaching, leading to the spread of pollutants in the environment (Salt et al.1995). The rooted vegetation established at contaminated sites prevents windblown dust, thus preventing human exposure of hazardous waste. Migration of leachate can be prevented through transpiration-mediated hydraulic control of groundwater or receiving waters. Phytostabilization is preferred for metal contaminants at waste sites where confinement of contaminants is required at a localized place. Since metals do not ultimately degrade, the best alternative is capturing them in situ at sites with low contamination levels (below risk thresholds) or vast contaminated areas where a large-scale removal action. For phytostabilization, vigorously growing plants are necessary to exert hydraulic control and immobilization at the site where plants cannot die or removed during the phytostabilization design period. Low-level radionuclide contaminants can also be confined in place by phytostabilization, and result in significant risk reduction under small half-lived contaminants. Soil amendments such as phosphate, lime and organic matter are sometimes needed to immobilize toxic metals such as lead, cadmium, zinc and arsenic. Cadmium is readily translocated to leaves in many plants, which represents a risk to the food chain, and this pathway may be the limiting consideration in applying phytostabilization at some metal-contaminated sites.

5.3.4 Phytoextraction

Phytoextraction refers to the use of metal-accumulating plants that translocate and concentrate metals from the soil to roots and shoots or leaves. It has been used effectively at brownfield sites with relatively low-level lead and cadmium contamination and proposed for extraction of radionuclides from sites with mixed wastes (Mulligana et al. 2001). Phytoextraction offers significant cost advantages over alternative schemes of soil excavation and treatment or disposal. The issue that needs to be considered in phytoextraction is whether the metals can be recovered economically from the plant tissue or directly disposed as waste. Design considerations include the accumulation factor (ratio of metal in the plant tissue to that in the soil) and the plant productivity (kilogram of dry matter that is harvestable each season). In order to use phytoextraction as regular practice, one needs a vigorously growing plant (>3 tons dry matter/ha year) accumulating large concentrations of metal in the harvestable portion (>1000 mg/kg metal) which is easy to harvest. Metals like cadmium, nickel, zinc, arsenic, selenium and copper are generally considered to be readily bioavailable for phytoextraction. Moderately bioavailable metals are cobalt, manganese and iron; while lead, chromium and uranium are not readily bioavailable. Bioavailability of lead can be enhanced by greatly adding ethylenediaminetetraacetic acid (EDTA) to soils. The disadvantage with this technology is longer time requirement than other technologies; thus many crops are usually required to reduce all the contaminants to the desired levels.

5.3.5 Rhizofiltration

In rhizofiltration, plant roots are used to absorb, concentrate and precipitate metal contaminants from the surface or groundwater. Prior to growing at the contamination site, suitable plants with stable root systems are supplied with contaminated water to acclimate the plants, and then these plants are transferred to the site of contamination for maximum absorption. Further saturated roots are harvested and processed. Rhizofiltration allows in situ treatment, a process that does not disturb the environment (Salt et al. 1995). Rhizofiltration has been employed by Phytotech using sunflowers at a US Department of Energy (DOE) pilot project with uranium wastes at Ashtabula, OH and on water from a pond near the Chernobyl nuclear plant in Ukraine. Shallow lagoons have been engineered as wetlands and maintained as facultative microbial systems with low dissolved oxygen in the sediment. Groundwater or wastewater is pumped through the system for the removal of contaminants by rhizofiltration.

Usually this technology is intended for metals or mixed wastes but also suited for ammunition wastes. TNT is an organic contaminant that gets strongly absorbed by roots but is not efficiently translocated to other parts, and is confined to roots. An engineered wetland technology has been used at the Milan, TN, and Volunteer Army Ammunition Plants with bulrush. Rhizofiltration is also used in large-scale treatment of a Comprehensive Environmental Response, Compensation , and Liability Act (CERCLA) site at the Iowa Army Ammunition Plant at Middletown, IA, for TNT and RDX polishing of soil and groundwater after removal actions. Long-term utilization of wetland plants and sulphate-reducing conditions resulted in increased pH and decreased toxic metal concentrations after treatment of acid mine drainage (Adams et al. 2014). Root systems and sediments in wetlands are facultative (aerobic and anaerobic zones), which facilitates sorption and precipitation of toxic metals.

5.4 Plant Systematics of Heavy Metal Accumulation

The study of molecular mechanism in plants that are capable of hyperaccumulation will provide further insights towards engineering plants for phytoremediation in the future. Heavy metals are the main group of pollutants where the molecular mechanism of plant stress response against them has been under progress, especially in herbaceous plants such as *Arabidopsis thaliana*, *Arabidopsis halleri* and *Thlaspi caerulescens* (Verbruggen et al. 2009; Thapa et al. 2012). High-throughput technologies, such as microarray and next generation sequencing technologies, have allowed the complexity of plant stress response to be tackled. Much work has been reported recently in this field and presented in different plants. The genes involved in heavy metal uptake, accumulation, remobilization, vacuolar sequestration and homeostasis have been summarized in Table 5.5.

5.4.1 *Arabidopsis thaliana*

A. thaliana with its completely sequenced genome played a very important role in uncovering the molecular mechanism of plant response to pollutants since the genes involved can be easily identified through mutation studies. Three *Arabidopsis* genes, oxophytodienoate reductases 1 (*OPR1*), *OPR2*, and *OPR3* were found to be upregulated by exposure to TNT where biochemical characterization revealed that two of the three OPR1 lines and all of the OPR2-overexpressing lines exhibited enhanced tolerance to TNT (Beynon et al. 2009). Rao et al. (2009) identified the potential target gene in *A. thaliana* for phytoremediation and phytosensing of the chemical contaminants RDX and TNT by microarray analysis. Genes that were differentially expressed included oxidoreductases, cytochrome P_{450}s, transferases, transporters and several unknown expressed proteins. Two transcription factors, bZIP19 and

Table 5.5 Potential genes involved in uptake, vacuolar sequestration, remobilization and homeostasis of heavy metals in plants

Function	Genes involved	Annotation
Metal uptake into cells	*ZIP4, ZIP6, ZIP9, ZIP10*	ZIP family of metal transporters
	IRT1, IRT3, ZIP7	ZIP family of metal transporters
Metal vacuolar sequestration	*MTP1, MTP8, MTP11*	Cation diffusion facilitator
	CAX2 Ca^{2+}	cation antiporter
	AtHMA3	P-type metal ATPase
Metal remobilization from the vacuole	*NRAMP1, NRAMP3, NRAMP5*	Natural resistance-associated macrophage
Xylem loading/unloading of metal/ligands/ metal–ligand complexes	*HMA4*	P-type metal ATPase
	FRD3	Multidrug and toxin efflux family transporter
	YSL3, YSL6, YSL7	Yellow-stripe-like transporter
Synthesis of metal ligands	*NAS1, NAS2, NAS3, NAS4*	Nicotinamine synthase
	SAMS1, SAMS2, SAMS3	S-adenosyl-methionine synthetase
	AOSA2	Cysteine synthase
Other roles in iron homeostasis	*FER1, FER2*	Ferritin Fe(III) binding
	IREG2	Iron regulated transporter 2
	At4g35830	Cytoplasmic aconitase
Stress protection/response	*PDI1, PDI2*	Protein disulfide isomerise 1
	At1g45145	H-type thioredoxin
Homeostasis of macronutrient	*PHT1-4* Phosphate	H1 symporter family

bZIP23, were reported to regulate the adaptation to zinc deficiency and zinc homeostasis in plants (Assunção et al. 2010). Based on microarray analysis on the seedlings grown on toluene-containing media, potential genes related to the sensing mechanism and metabolisms of toluene are detected; Among them 202 are induced and 67 are suppressed in response to toluene, mostly including genes encoding cytochrome P_{450}s, glucosyl transferases and transporters (Gao et al. 2012). Pineau et al. (2012) revealed crosstalk between Fe homeostasis and Zn tolerance in *A. thaliana* by analysing natural variation at the FRD3 MATE transporter locus.

5.4.2 *Populus*

In recent years *Populus* tree has been used either in natural form or as a transgenic for phytoremediation. Its genes have been identified and characterized as playing a role in heavy metal uptake, but prior selection of potential hyperaccumulating genotype is required. Gaudet et al. (2011) selected two *Populus nigra* L. genotypes originating from contrasting environments in northern (genotype 58-861) and southern (genotype Poli) Italy for studying the physiological and molecular response to cadmium stress, and found that Poli was more tolerant to cadmium stress. The study also revealed that the glutathione pathway was also involved in the differential cadmium tolerance of the two genotypes. He et al. (2013) conducted the transcript analysis of *Populus* × *canescens* response to cadmium and found 48 % of the differentially regulated transcripts involved in coregulation networks; among them, 43 hub genes played a significant role in crosstalk during distinct biological processes. These include a putative wall-associated kinase, a GDP dissociation inhibitor family protein/Rab GTPase activator family protein, and a chloroplast sensor kinase actively involved in signalling; the study enhanced our understanding about the molecular mechanism of woody plant response to heavy metal.

Quantitative trait loci (QTL) analysis in response to cadmium revealed a total of 16 QTL where whole-genome microarray analysis showed 9 cadmium-responsive genes, including

NHL repeat membrane-spanning protein, a metal transporter and a putative transcription factor providing cadmium tolerance. Additional candidates in the QTL intervals include a putative homolog of a glutamate cysteine ligase, and a glutathione-S-transferase. Functional characterization of these candidate genes further enhances our understanding of cadmium metabolism and transport and phytoremediation capabilities of *Populus* (Induri et al. 2012).

5.4.3 *Brassica juncea*

Brassica juncea is another promising plant species that can be used for phytoremediation of heavy metals. *B. juncea* root proteome was analysed in response to cadmium exposure, and it was found that enzymes such as peptide methionine sulfoxide reductase and 2-nitropropane dioxygenase play a role in alternative redox regulation mechanisms, whereas O-acetylserine sulfhydrylase, glutathione-S-transferase and glutathione-conjugate membrane transporter were involved in the Cd hyperaccumation and tolerance of *B. juncea* (Alvarez et al. 2009). Another study reported that under stress condition of Zn, Cd, NaCl or Polyethylene glycol (PEG), the transcript levels of two *B. juncea* cation-efflux family proteins, BjCET3 and BjCET4, substantially increased, suggesting their roles in stress resistance (Lang et al. 2011).

5.4.4 *Crambe abyssinica*

*Crambe abyssinica*is a member of Brassicaceae and an ideal candidate for phytoremediation due to nonfood, fast-growing, high biomass crop. Thirty-eight genes encoding glutathione-S-transferases, antioxidants, sulphur metabolism, heat shock proteins, metal transporters and enzymes in the ubiquitination pathway of protein degradation as well as several unknown novel proteins involved in arsenic metabolism and detoxification were analysed and isolated successfully (Paulose et al. 2010). Zulfiqar et al. (2011) reported 72 differentially expressed transcripts after comparative Cr exposure analysis using PCR-based suppression subtraction hybridization (SSH) and found 43 genes specifically involved in Cr detoxification (Zulfiqar et al. 2011).

5.4.5 Other Plants

Elsholtzia splendens is a Cu-tolerant and high metal-accumulating plant species, thus a likely candidate for phytoremediation of Cu-contaminated soils. Li et al. (2009) conducted proteomic analysis of copper stress response in *E. splendens* roots and leaves by two-dimensional gel electrophoresis and found that 45 protein spots were significantly changed in roots, but only 6 were changed in leaves. The identified root proteins were involved in various cellular processes such as signal transduction, regulation of transcription and translation, energy metabolism, regulation of redox homeostasis and cell defence, while the leaf proteins were mainly degraded fragments of RuBisCo and antioxidative protein. Lyubenova et al. (2009) showed that when tobacco (*Nicotiana tabacum*) plants originating from different mutants were grown under field conditions with varying fertilizer application, the uptake of cadmium and zinc from soil increased with increasing biomass. Depending on Cd and Zn uptake, several antioxidant enzymes showed significantly different activities. Among them SOD and CAT were usually elevated; however, isoforms of GST and several other enzymes were strongly inhibited.

Zhou et al. (2009) revealed the broccoli (*Brassica oleracea*var. *italica*) COQ5 methyltransferase (*BoCOQ5-2*) gene's involvement in the ubiquinone biosynthetic pathway. It was found to promote Se volatilization in both bacteria and transgenic *Arabidopsis* (*A. thaliana*) plants. Bacteria expressing *BoCOQ5-2* showed an over 160-fold increase in volatile Se compounds under selenate exposure and exhibited enhanced tolerance to selenate. Transgenic *Arabidopsis* expressing *BoCOQ5-2* volatilized three times more Se than the vector-only control plants when treated with selenite and exhibited an increased tolerance to Se. Ding et al. (2011) showed As concentration in different tissues of maize using a set of re-

combinant inbred line (RIL) populations derived from an elite hybrid, Nongda108, and found 11 QTLs for arsenic accumulation in maize (*Zea mays*L.). In *Portulaca oleracea*, the peroxidase 2a (PoPRX2a) is potentially useful in the remediation of phenolic pollutants (Matsui et al. 2011).

In Se hyperaccumulator *Astragalus racemosus*, 125 Se-responsive candidate genes were identified, among which 6 responded to both selenate and selenite treatments. In the same study, a novel gene *CEJ367* was reported to be highly induced by both selenate (1920-fold) and selenite (579-fold) and to provide lead to generate Se-enriched transgenic plants (Hung et al. 2012). HvHMA2, a P (1B)-ATPase from barley, is highly conserved among cereals and functions in Zn and Cd transport (Mills et al. 2012). *Solanum nigrum* is a cadmium (Cd) accumulator, whereas *Solanum torvum* is a low Cd-accumulating plant. Their comparative transcriptome analyses revealed that increased Cd loading into the root xylem was responsible for the differential Cd accumulation in the two *Solanum* species. The higher expression of genes encoding several metal transporters as well as antioxidant-related genes, and several organic and amino acid biosynthesis/metabolism-related genes in Cd-treated *S. nigrum*, indicate different responsive mechanisms of the transporter genes, which under different metal deficiency (Fe), might be responsible for differential uptake and redistribution of the metals in the two *Solanum* species (Xu et al. 2012). A major latex-like protein is a key factor in *Cucurbitaceae* family crop contamination by persistent organic pollutants (Inui et al. 2013). TaHMA2 is another gene from wheat (*Triticum aestivum* L.), which belongs to heavy metal ATPase 2 (HMA2; Tan et al. 2013).

5.5 Plants Used in Phytoremediation

5.5.1 For Heavy Metals

Hydrilla verticillata (L.f.) Royle, a submerged macrophyte widely distributed throughout the world, has the ability to accumulate arsenic (As). The phytofiltration studies revealed that shoots of this plant possess high potential for As accumulation (Xue and Yan 2011). To provide some insight on the possibility of using serpentine adapted plants for phytoextraction of Cd, Barzanti et al. (2011) investigated variations in cadmium tolerance, accumulation and translocation in three *Alyssum* plants, and the results indicated that the serpentine adapted population of *Alyssum montanum* showed significantly higher cadmium tolerance and accumulation than *Alyssum bertolonii* and the ones not adapted to serpentine soil. Plants of two aquatic macrophytes, *Ceratophyllum demersum*and *Lemna gibba* showed potential for removing two toxic heavy metals Pb and Cr (Abdallah 2012). The study also revealed that *L. gibba*was more efficient at the removal of selected heavy metals than *C. demersum*. *L. gibba* was reported to accumulate heavy metals without the production of toxins.

Brachiaria mutica (Forssk) Stapf was found to have luxuriant growth with massive fibrous roots when grown in Cr-contaminated soils (11,170 mg/kg dry soil). These results indicated that para grass could be used to remediate chromium-contaminated soils in situ as it showed rapid growth even with a high concentration of Cr present (Mohanty and Patra 2012). Meeinkuirt et al. (2012) analysed six tree species for phytoremediation abilities of Pb in sand tailings and found that *Acacia mangium* with the addition of organic fertilizer gives the best results. Adki et al. (2013) performed various studies taking *Nopalea cochenillifera* and revealed its potential as a chromium (VI) hyperaccumulator plant.

Amer et al. (2013) assessed the potential for phytoremediation of heavy metals (Ni, Pb and Zn) in three endemic Mediterranean plant species—*Atriplex halimus, Portulaca oleracea* and *Medicago lupulina*—and found that *A. halimus*and *M. lupulina* had the potential to be used in phytoremediation and phytostabilization. The potential of kenaf (*Hibiscus cannabinus* L.) and corn (*Z. mays* L.) for phytoremediation of dredging sludge contaminated with trace metals was tested by Arbaoui et al. (2013), and after tolerance and bioaccumulation studies, it was found that both species could be used in phytoremediation. Pratas et al. (2013) assessed the

phytoremediation potential of flora that are tolerant to heavy metal in the contaminated soils of an abandoned Pb mine in Central Portugal and found several plants exhibiting high uptake of metals, including *Cistus salvifolius* (Pb 548 mg/kg), *Digitalis purpurea*(Zn 1017 mg/kg and Fe 4450 mg/kg), *Mentha suavolens* (Ag 1.9 mg/kg) and *Ruscus ulmifolius* (Ag 1 mg/kg). Ruiz et al. (2011) reported development of a transplastomic approach showing expression of mouse metallothionein gene (*mt1*) in a plant chloroplast resulted in high accumulation of mercury within plant cells. Recently, *Wolffia globosa* was found to be a strong Cd accumulator and has great potential for Cd phytoremediation (Xie et al. 2013).

5.5.2 For Other Pollutants

Besides heavy metals, plants are also screened for the phytoremediation of other pollutants such as PCB, benzo[a]pyrene (B[a] P) and tetracycline (TC). Ficko et al. (2011) investigated the effects of plant age, contaminant characteristics and species-specific properties on PCB uptake and accumulation patterns in plant tissues of the three perennial weed species (L. (*ox-eye daisy*), L. (*curly dock*), and L. (*Canada goldenrod*)) and correlated that shoot contaminant concentrations and total biomass are dependent on plant age and life cycle (vegetative and reproductive stages).

Sun et al. (2011) showed that the French marigold (*Tagetes patula*) might be useful for phytoremediation of B[a] P and B[a] P–Cd contaminated sites. The presence of veterinary and human antibiotics in soil and surface water is also an emerging environmental concern. Datta et al. (2013) evaluated the potential of vetiver grass (*Chrysopogon zizanioides* L.) for removing TC from aqueous media and provide a base for the development of a cost-effective, in situ phytoremediation technique to remove antibiotics consisting of TC groups from wastewater. Ma et al. (2013) reported the use of legume (alfalfa, *Medicago sativa* L.) grass (perennial ryegrass, *Lolium perenne* L. and tall fescue, *Festuca arundinacea*) for removing phthalic acid esters (PAEs) by intercropping in e-waste contaminated agricultural soils in China. Their study revealed that alfalfa was effective in both monoculture and intercropping for removing PAEs from contaminated soils.

Saiyood et al. (2013) found that an evergreen mangrove tree, *Bruguiera gymnorhiza gymnorhiza,* is tolerant to bisphenol A (BPA) and has the capability to remove BPA. Souza et al. (2013) showed that *Myriophyllum aquaticum* can reduce oxygen demand (COD), biochemical oxygen demand (BOD), and total phosphorus (TP) in 15 days, and ammoniacal nitrogen (AN) as well as total Kjeldahl nitrogen (TKN) in 30 days, indicating potential as a candidate for phytoremediation of polluted water.

5.6 Genetic Engineering in Phytoremediation

Phytoremediation, although having potential for cleaning up the environment, alone cannot successfully detoxify or interconvert the metals, PCBs and other contaminants to more benign forms. But biotechnological approaches, especially the application of genetic engineering, may prove to be a potential technique through which the gene from other organisms can be integrated to enhance phytoremediation capabilities in plants.

In case of mercury pollution the plants have been genetically engineered both via nuclear genome and chloroplast genome (Ruiz and Daniel 2009). The cloning of *merA* (mercuric ion reductase) and *merB* (organomercurial lyase) genes from bacteria for the remediation of Hg (Bizly et al. 2003; Che et al. 2003; Heaton et al. 2003; Lyyra et al. 2007) is a well-understood protocol. The chloroplast has been the main target for mercury poisoning (Bernier and Carpentier 1995; Sinha et al. 1996; Sabat 1996). The protection of essential metabolic reactions occurring within plastids has been working area for expressing *merA* and *merB* genes within plant chloroplasts (Ruiz et al. 2003).

For iron phytoremediation, the *FRO2* gene, which encodes ferric chelate reductase was found to restore ferric chelate reductase activity in an *Arabidopsis* mutant deficient in this enzyme

(Robinson et al. 1999). *FRE1* and *FRE2* genes isolated from *Saccharomyces cerevisae* (Dancis et al. 1990; Georgatsou and Alexandra 1994) were cloned in tobacco (Samuelsen et al. 1998) and the double mutants (*FRE1*+*FRE2*) were found to be more tolerant, having high Fe concentration in leaves than the control and *FRE1* plants. Ferritin gene from soybean increased Fe accumulation in *Nicotiana* and rice (Goto et al. 1998, 1999). The gene for the phytoremediation of arsenic, γ-glutamyl cysteine synthetase (*g-ECS*), was isolated from *Escherichia coli* and cloned in *Arabidopsis* with an *actin* promoter. The plant had moderate tolerance for arsenic. Selenium is another major environmental hazard and is lethal if amounts go larger than required dose. The oxidized selenium (selenate or selenite) is less hazardous than the inorganic ones because the inorganic selenium (selenide or elemental Se) is insoluble, and therefore, available in low quantities for degradation (Eapen and D'Souza 2005). Genes such as ATP sulphurylase activates the assimilation of sulphate and selenium and converts it into adenosine phosphoselenite, which gets converted to selenite (DeSouza et al. 2000). The APS transgenics are more tolerant to Se and grow at a faster pace than the wild type (Pilon-Smits et al. 1999). Various metallothionein (MT) genes such as *MT2* gene from humans, *MT1* gene from mice and *MTA* gene from pea has been transferred to *Nicotiana sp.* and *Arbidopsis sp.* (Misra and Gedamu 1989; Evans et al. 1992; Pan et al. 1994) for Cd tolerance. MTA gene in *Arabidopsis* augmented copper (Cu) accumulation. The *CUP1* gene from yeast provided Cd tolerance in *Nicotiana* and *B. oleracea* (Hasegawa et al. 1997; Thomas et al. 2003). *YCF1* gene from yeast provided Cd and Pb tolerance in *Arabidopsis*. *NtCBP4* gene in *Nicotiana* showed Ni tolerance and Pb accumulation (Arazi et al. 1999).

Besides metal, genetic engineering also aided in the phytoremediation of PCBs. Pioneering work was done by Francova et al. (2003), but the plants were not tested for their capability to metabolize PCB. But then the *bPh* gene from *Burkholderia xenovorans* LB400 was transformed into *Nicotiana sp*., and the purified enzymes showed that it was capable of oxidizing 4-chlorobiphenyl into 2,3-dihydro-2,3-dihydroxy-4′-chlorobiphenyl (Mohammadi et al. 2007). *bphC* gene from *Pseudomonas testosteroni* B-356 when transferred in *Nicotiana sp*. and grown in the presence of 2,3-dihydroxybiphenyl (0.5 mM), then one of the transgenic lines exhibited greater toxic resistance than the wild type (Novakova et al. 2009).

More development is required for the successful application of this innovative strategy such as improvement of metal and PCB-degrading enzymes through genetic engineering and studies on molecular level to bring success regarding the coordinated expression of different genes responsible for degrading different contaminants.

5.7 Future Research Prospects and Impact

Presently, trends for phytoremediation technology are approaching commercialization. Concurrently, short-term advances in phytoremediation are likely to occur through selection of more efficient plant varieties and soil amendments and from optimizing agronomic practices used for plant cultivation. Major long-term improvements achieved through identification of potential candidate genes from plants and microorganisms and through understanding of hyperaccumulation mechanisms in plants, leading to biotransformation or biodegradation of organics. Additionally, genetically modified rhizospheric bacteria for bioremediation and symbiotic association with plants are required to increase the efficiency of the future phytoremediation efforts. Transgenic plants also represent the candidates for the most efficient and cost-effective phtoremediation. Transgenic events include modifications in specificity of trnasporters, overexpression of transporters resulted in increased number of transporters, intracellular ligand production directing metal targeting into vacuoles without disturbing cellular processes and biochemical transformation of metal volatile forms. The biology alone cannot make phytoremediation work. Multidisciplinary research efforts are required that integrate plant biologists, microbiologists, soil chemists

and environmental engineers. The acceptance of phytoremediation technology will depend on its socioeconomical impact. Removing heavy metals and hazardous contaminants from the environment using plants prevent industrial-level cleaning efforts involving costly machines and chemicals. Plants used in phytoremediation offer greenery on contaminated, dusty, barren lands, and also help in minimizing the greenhouse effect and global warming. Thus, initiatives are required for increasing awareness of this green technology and encouraging basic and applied research to improve existing technology.

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Ameliorative Approaches for Management of Chromium Phytotoxicity: Current Promises and Future Directions

6

Punesh Sangwan, Prabhjot Kaur Gill, Dharmendra Singh and Vinod Kumar

Abstract

Chromium is a heavy metal of serious environmental implications in excess amounts as it poses a threat to human health as well as plant growth and development. Considering limited agricultural land for such a large population and increasing heavy metal pollution in soil, there is an immense need of strategies for alleviation of phytotoxic effects of chromium and its removal from soil. Several studies have been carried out in relation to the aforementioned problems with emphasis on application of plant growth regulators (kinetin, gibberellic acid, brassinosteroids, salicylic acid) and metal chelators (EDTA), bioremediation using microbial inoculants, and reduction of the toxic form of chromium to a nontoxic form. These approaches have been shown to be promising in one or more soil and environmental conditions and plant types. In this chapter, these strategies are discussed considering their chromium amelioration potential from supporting evidences and challenges ahead for successful implementation of any strategy as a universal approach.

Keywords

Alleviation · Amelioration · Bioremediation · Chromium phytotoxicity

6.1 Introduction

Since the beginning of the industrial revolution, pollution of the biosphere with toxic metals has accelerated dramatically (Swaminathan 2003). Chromium (Cr) is a heavy metal that causes serious environmental contamination in soil, sediments, and groundwater (Shanker et al. 2005). Cr contamination is rising due to use of wastewater and industrial effluents as irrigation sources for crop production, mostly in the urban lands

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(Mushtaq and Khan 2010). Cr exists in several oxidation states and the two most stable forms present in soils are Cr(VI) and Cr(III). Among these two, Cr(III) is considered as less toxic in comparison to bioavailable Cr(VI) compounds in the form of chromate (CrO_4^{-2}) and dichromate ($Cr_2O_7^{-2}$) (Messer et al. 2006). Cr (VI) can be toxic to plants up to concentrations of 0.5 mg L^{-1} in solution and 5 mg kg^{-1} in soil (Turner and Rust 1971). Cr(VI) is a very toxic, powerful epithelial irritant and an established human carcinogen by International Agency for Research on Cancer (IARC 1980), the Environmental Protection Agency (EPA 1984), and the World Health Organization (WHO 1988). Toxicity of Cr has been studied in many plants and its excess amount causes inhibition of chlorophyll biosynthesis in terrestrial plants (Vajpayee et al. 2000), affected germination process, plant growth, yield and total dry matter production; causes deleterious effects on plant physiological processes such as photosynthesis, water relations, and mineral nutrition (Shanker et al. 2005); generates reactive oxygen species (ROS) and alters metabolic enzymes (Yadav 2010); and leads to nutrient imbalance, wilting of tops, and root injury (Scoccianti et al. 2006; Yadav 2010). Cr phytotoxicity also affects fodder nutritive value (Sangwan et al. 2014a); activities of nitrogen metabolism enzymes (Sangwan et al. 2014b) and carbohydrate, protein, and guar gum content of cluster bean (Sangwan et al. 2013). The activities of antioxidant enzymes, viz., superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase are also significantly affected by Cr(VI) treatment in wheat (Subrahmanyam 2008), and no seed formation was observed even at 1.0 mM Cr(VI) (Sharma et al. 1995). It has been reported that the toxic property of Cr(VI) originates from the formation of ROS, i.e., superoxide radical, hydrogen peroxide and hydroxyl radical, and in higher concentrations, these ROS produce cytotoxic effects due to their ability to oxidize lipids, proteins, and nucleic acids (Shanker et al. 2004; Panda 2007; Pandey et al. 2009). In order to mitigate deleterious effects of ROS, plants possess complex defense mechanisms that involve both enzymatic and nonenzymatic antioxidants (Panda 2007). The simultaneous action of various antioxidant enzymes is essential for regulation of ROS levels within the cell (Shanker et al. 2004; Panda 2007). Nonenzymatic antioxidants such as ascorbate and glutathione (GSH) also play an important role in preventing oxidative stress (Noctor and Foyer 1998). Considering the negative effects of Cr(VI), the development of efficient, cost-effective, and environmentally sound methods for removing Cr(VI) from contaminated sites or alleviation of its phytotoxic effects is important to safeguard the quality of drinking water, agricultural products, and the environment (Diwan et al. 2008). In India, Cr(VI) contamination is a big problem around various industries using Cr compounds, which causes considerable negative impact on crop production. This problem further gets exacerbated due to the use of Cr-contaminated water by farmers in irrigation. Thus, the cleanliness of the environment for safer food production is a major concern. Therefore, methods are needed to alleviate Cr toxicity, and also to decrease the Cr content in crops, which may be helpful to minimize health risks (Tripathi et al. 2012).

6.2 Promising Approaches for Amelioration of Chromium Phytotoxicity and Its Removal from Contaminated Sites

In this section of the chapter, we have discussed several studies in support of alleviation of toxic effects of Cr(VI) to understand the best possible way to avoid the phytotoxic effects of Cr(VI). Most studies have been focused on application of plant growth regulators (PGRs), metal chelators, modification of soil nutrients, and Silicon (Si) application. Further, a variety of other methods have also been developed for remediation of contaminated soil to protect plants from phytotoxic effects of Cr. Some commonly used soil remediation methods are chemical immobilization (Kumpiene et al. 2008), phytoremediation (Memon and Schroder 2009), and soil washing (Davezza et al. 2011). Among them, chemical immobilization is a cost-effective and promising

soil remediation technique, and has been extensively used in immobilization of heavy metals in contaminated soils (Kumpiene et al. 2008). The application of silicon, H_2O_2, and iron are also discussed.

6.2.1 Phytohormones Application for Amelioration of Chromium Phytotoxicity

1. Salicylic acid application

Several reports were published in the last decade demonstrating the role of salicylic acid (SA) applied as a seed soaking treatment on various physiological processes. In several recent studies, application of SA through different modes was found to be beneficial for the growth of *Arabidopsis thaliana* under arsenic stress (Odjegba 2012), wheat under salinity stress (Shakirova 2007), tomato and amaranth under water stress (Umebese et al. 2009), garlic under drought stress (Bideshki and Arvin 2010), and common bean under water stress (Sadeghipour and Aghaei 2012). Hayat et al. (2010) reported that presoaking of pea seeds in SA had a beneficial effect on growth and photosynthesis with decreased oxidative injuries caused by heavy metal stress. Similarly, Fariduddin et al. (2003) investigated that dry matter accumulation was significantly increased in *Brassica juncea* with spray of SA. Furthermore, Farooq et al. (2010) demonstrated application of some other chemicals such as glycine betaine (GB), nitric oxide, brassinosteroid (BR), and spermine along with SA to improve growth of rice under drought stress with a possible effect on improved carbon assimilation, enhanced synthesis of metabolites, and maintenance of tissue water status. Addition of SA could induce activity of H^+-ATPase (Gordon et al. 2004), which plays an important role in the transport of multiple ions through plasma membrane (Shi and Zhu 2008). Application of SA was also reported to increase the Zn concentration, which is required for the synthesis of indole-3-acetic acid (IAA) and has the ability to inhibit nicotinamide adenine dinucleotide phosphate (NADPH) oxidation and centered free radical generation (Cakmak 2000).

Aly and Soliman (1998) studied the effect of SA on iron uptake in soybean genotypes. They found that SA was effective in correcting iron chlorosis in soybean genotypes grown in calcareous soils. Al-Hakimi and Hamada (2001) also observed similar effects of SA in the Na, K, Ca, and Mg content of wheat plants grown under salinity, whereas in maize, exogenous SA applications inhibited Na^+ accumulation, but stimulated N, P, K, Mg, Fe, Mn, and Cu uptake (Gunes et al. 2007). According to El-Tayeb (2005), an increase in concentrations of K and Ca in plants under salt stress could ameliorate the deleterious effects of salinity on growth and yield. Therefore, alteration of mineral uptake from SA applications may be one mechanism for the alleviation of salt/metal stress (Karlidag et al. 2009).

Recently, Singh and Chaturvedi (2012) observed that SA at concentration of 10 μM was inducing nitrate reductase (NR) activity. On the contrary, Fariduddin et al. (2003) reported low concentrations of SA increased NR activity, while higher concentrations were inhibitory in *B. juncea*. The concentration of SA might play an active role in such a regulation, where the lower concentration favored an increase in the NR protein and higher quantity of SA decreased it by affecting the balance between its synthesis/activation and degradation/inactivation. The increase in the content of nitrates and thereby activity of NR due to exogenous SA treatment under normal growth conditions was also reported by Hayat et al. (2012). Incorporation of NH_4^+ into glutamate is brought about by successive and highly regulated actions of nitrogen metabolism enzymes (glutamine synthetase, GS; glutamate synthase, GOGAT; and glutamate dehydrogenase, GDH). SA, due to its action at transcriptional and/or translational levels, might have accelerated the synthesis and thereby activity of GDH, GS, and GOGAT (Hayat et al. 2010). Hence, it might be possible that lower concentration of SA might enhance the release of auxins and increase the activity of GS, GOGAT, and GDH enzymes (Hayat et al. 2012).

2. Brassinosteroid Application

Recent research advances have shown the promising effects of PGRs like auxins, abscisic acid (ABA), cytokinins, gibberellins, BRs, and polyamines (PAs) in abiotic stress mitigation (Choudhary et al. 2012). BRs and PAs are well-established growth regulators playing key roles in stress management among plants. BRs are a steroidal sixth group of phytohormones with significant growth-promoting effects and are essential for many processes in plant growth and development (Anuradha and Rao 2007). Besides growth stimulation they have an ability to confer resistance to plants against various abiotic stresses (Priti 2003). BRs are able to regulate the uptake of ions into plant cells and can be used to reduce the accumulation of heavy metals (Sharma and Bhardwaj 2007), because they can reduce the metal uptake by roots and can also stimulate the synthesis of some ligands such as the phytochelatins (PCs), which are combined with metal ions (Choudhary et al. 2010; Vázquez et al. 2013). Epibrassinolide was found to increase drought tolerance in wheat (Nilovskaya et al. 2001). Among PGRs, BRs form a group of steroidal lactones with a wide array of roles in physiological activities, such as stem elongation, xylem differentiation, leaf bending, epinasty, pollen tube growth, fruit development, ethylene biosynthesis, photosynthesis, and proton pump activation (Xia et al. 2009). Their ability to improve antioxidant system by elevating the activities and levels of enzymatic and nonenzymatic antioxidants have made them a favorite tool to increase resistance potential of important agricultural crops against various abiotic stresses such as heavy metal excess (Vázquez et al. 2013). PAs are small aliphatic nitrogenous compounds with ubiquitous distribution and implication of PAs in amelioration of various abiotic and biotic stresses has made them an essential component of plant defense mechanism (Hussain et al. 2011). Enhanced expression of spermidine (Spd) synthase (SPDS) and Spd titers has been associated with improved heavy metal and salinity tolerance in three transgenic European pears (Wen et al. 2011).

Choudhary et al. (2012) evaluated the effects of 24-epibrassinolide (EBL), an active BR, and Spd, an active PA, on the contents of endogenous PAs, auxins, and ABA, as well as on the antioxidant systems, stress markers, and growth parameters in seedlings of radish grown under Cr(VI) stress. They demonstrate that coapplication of BRs and PAs is more effective in alleviation of Cr-stress than individual treatments and provided a unique, ecofriendly strategy to overcome heavy metal stress mitigation, and abiotic stress in general, in radish. The significant influence of EBL on the synthesis of IAA, ABA and PAs of radish seedlings under Cr(VI) metal stress was demonstrated by Choudhary et al. (2010). On the one hand, EBL could enhance the synthesis of IAA in order to promote normal seedling growth under Cr(VI) metal stress. On the other hand, it also slightly improved the production of ABA to increase Cr(VI) stress tolerance. Altered synthesis of PAs observed under the influence of EBL may be helpful in protecting the seedlings against Cr(VI) stress by enhancing one pool of PAs (putrescine and Spd) and decreasing the other pool (cadaverine). Increased levels of antioxidants and antioxidant enzymes activities upon EBL application with Cr(VI) metal stress also indicate its significant effect on the antioxidant system of radish plants. Similarly, reduced membrane damage; enhanced proline, photosynthetic pigments, sugars; and radical scavenging activities also show a major impact of EBL on radish seedling metabolism under Cr(VI) metal stress. Earlier, Arora et al. (2010) had reported the effect of EBL treatment to regulate the diminution of Cr metal toxicity in mustard plants.

Concomitantly, Sharma et al. (2011) evaluated effect of another BR, 28-homobrassinolide (28-HBL), on the seeds of *Raphanus sativus* L. (Pusa Chetaki) which were pretreated with different concentrations of 28-HBL and raised under various concentrations of Cr(VI). Upon analysis of morphological and biochemical parameters of 7-day-old radish seedlings, the 28-HBL treatment considerably reduced the impact of Cr stress on seedlings. The toxic effects of Cr in terms of reduced growth; lowered contents of chlorophyll,

protein, and proline; increased malondialdehyde (MDA) content; and elevated metal uptake were ameliorated by applications of 28-HBL. In addition, the activities of all the antioxidant enzymes except guaiacol peroxidase (POD), increased significantly when subjected to Cr stress in combination with 28-HBL. Overall, seed presoaking treatment of 28-HBL at 10^{-7} M was most effective in ameliorating Cr stress.

3. Kinetin application

Heavy metals have been reported to reduce the contents of cytokinins probably as a result of hormone breakdown or by enhancing the activity of cytokinin oxidase (Kaminek et al. 1997). Hence, exogenous application of kinetin to alleviate the deleterious effects of heavy metal toxicity in plants is gaining importance (Hussain et al. 2007). Hussain et al. further studied the role of kinetin in alleviating the toxic effects of Pb and Cr on four black gram cultivars commonly cultivated in Pakistan. In the roots of one line (Mash ES1), they reported relatively higher concentration of both metals as compared to its shoot, suggesting its importance for phytoremediation, while in another line (Mash 80), heavy metal content was lower in both shoot and roots suggesting its utilization in future breeding programs and cultivation as a fodder crop in the riverine areas of Pakistan and other places which are prone to excessive heavy metal contamination, particularly Pb and Cr (Hussain et al. 2007).

4. Gibberellic acid application

Gibberellic acid (GA) is one of the key plant hormones influencing seed germination, stem elongation, leaf expansion, and reproductive development (Hooley 1994; Matsuoka 2003). Studies have shown that exogenous application of GA provides protection to plants against abiotic stresses and increases crop yield (Tuna et al. 2008; Wen et al. 2010). However, excess applications of GA have shown to increase ethylene production, ROS generation, and alterations in defense mechanisms of plants, causing tissue damage and retarded growth (Celik et al. 2007; Gangwar et al. 2011). Furthermore, Gangwar et al. (2011) also studied effects of exogenous GA (10 and 100 μM) application on growth, protein and nitrogen contents, ammonium (NH_{4+}) content, enzymes of nitrogen assimilation, and antioxidant system in pea seedlings under Cr(VI) phytotoxicity. They showed that exogenous application of GA led to different changes in pea seedlings, that Cr and 100 μM GA alone as well as in combination decreased growth and altered nitrogen assimilation in pea seedlings compared to control, which was attributed to decreased levels of antioxidants. In contrast, application of 10 μM GA together with Cr was able to alleviate Cr phytotoxicity appreciably. This 10 μM GA-mediated amelioration of Cr phytotoxicity was assigned to the better antioxidant system and sustained activities of enzymes of nitrogen assimilation. Therefore, it is suggested that GA may play different roles based on its exogenous concentrations and plant species used under specific developmental and environmental conditions.

6.2.2 Glutathione Application for Amelioration of Chromium Phytotoxicity

GSH is a tripeptide detected virtually in all cell compartments such as cytosol, chloroplast, endoplasmic reticulum, vacuole, and mitochondria. The chemical reactivity of the thiol group of GSH makes it particularly suitable to serve a broad range of biochemical functions in all organisms, and it is one of the major sources of nonprotein thiols in most plant cells. The nucleophilic nature of the thiol group is also important in the formation of mercaptide bonds with metals and for reacting with selected electrophiles. This reactivity along with the relative stability and high water solubility of GSH makes it an ideal biochemical to protect plants against stresses including oxidative stress, heavy metals, and certain exogenous and endogenous organic chemicals (Millar et al. 2003; Foyer and Noctor 2005; Rausch et al. 2007; Yadav 2010). Reduced GSH acts as an antioxidant and is involved directly in the reduction of most ROS generated during stress (Millar

et al. 2003; Foyer and Noctor 2005; Shao et al. 2008). In addition to the above, GSH also acts as a precursor for the synthesis of PCs. These are a set of novel heavy metal-binding peptides also found in higher plants (Gekeler et al. 1989). PCs are synthesized inductively by exposure not only to Cd but also to other heavy metals. Thereafter, numerous physiological studies have indicated their role in heavy metal detoxification as well as in the maintenance of ionic homeostasis (Zenk 1996; Hirata et al. 2005). A survey of the plant kingdom has provided evidence for the occurrence of PCs in angiosperms, gymnosperms, and bryophytes (Gekeler et al. 1989; Yadav 2010). Recently, the role of reduced GSH in maintaining cellular oxidation balance and protection against drought, salinity, and heavy metals has been proven (Chen et al. 2010; Cai et al. 2011; Zeng et al. 2012). In one such study, a hydroponic experiment was conducted to determine the possible effect of exogenous GSH in alleviating Cr stress through examining plant growth, chlorophyll contents, antioxidant enzyme activity, and lipid peroxidation in rice seedlings exposed to Cr toxicity (Zeng et al. 2012). The results showed that plant growth and chlorophyll content were dramatically reduced when rice plants were exposed to 100 μM Cr. Addition of GSH in the culture solution alleviated the reduction of plant growth and chlorophyll content. It also enhanced antioxidant capacity in Cr-stressed plants as antioxidant enzymes like SOD, CAT, glutathione reductase, and glutathione peroxidase showed increased activities under Cr stress in both leaves and roots. Furthermore, exogenous GSH also caused significant decrease of Cr uptake and root-to-shoot transport in the Cr-stressed rice plants, assuming that GSH was involved in Cr compartmentalization in root cells (Zeng et al. 2012). The effects of exogenous reduced GSH on alleviation of Cr(VI) toxicity to rice seedlings and its physiological mechanisms were also investigated in a series of experiments by Qiu et al. (2013). The addition of GSH alleviates negative effects due to Cr-induced toxicity. It was concluded that the alleviation of Cr(VI) toxicity by exogenous GSH is directly attributed to its regulation on forms of Cr ions in the rhizosphere and their distribution at subcellular levels. In addition to reduced GSH, Cao et al. (2013) conducted a series of experiments to determine the alleviating effects of GSH, Se, and Zn under combined contamination of Cd and Cr in rice. GSH and GSH + Zn application significantly alleviated growth inhibition induced by combined stress of Cd and Cr in rice plants. Exogenous GSH and GSH + Zn effectively decreased Cr accumulation.

6.2.3 Iron Application for Amelioration of Chromium Phytotoxicity

Iron (Fe) is a cofactor for approximately 140 enzymes that catalyze unique biochemical reactions (Brittenham 1994). It is also required at several steps in the biosynthetic pathways and fills many essential roles in plant growth and development, including chlorophyll synthesis, thylakoid synthesis, and chloroplast development (Miller et al. 1995). Fe is also required by both legume and root nodule bacteria for many metabolic functions at several key stages in the symbiotic N_2 fixation process and is critical for N_2 fixation due to its role in the activity of both leghemoglobin and nitrogenase (Kaiser et al. 2003). Symbiotic N_2 fixation was shown to have a high requirement for iron in lupine (Tang et al. 2006) because Fe is an essential component of nitrogenase, leghemoglobin, and ferrodoxins (Evans and Rossel 1971). Several theories have been proposed to explain the underlying protection mechanism of Fe to heavy metal toxicity. Supplemental Fe on roots was suggested to act as: (a) a shield that protects roots by coprecipitation of other heavy metals, (b) a nutrient reservoir, and (c) a reservoir for active ferrous (Fe^{2+})–Fe inside cells that could compete with heavy metals for metabolically sensitive sites inside plants (Sinha et al. 2005). Improvement in growth characters as a result of application of micronutrients might also be due to the enhanced photosynthetic and other metabolic activity, which leads to an increase in various plant metabolites responsible for cell

division and elongation (Hatwar et al. 2003). Adverse effect of Cr was found to be nullified by the supply of suitable amount of Fe and Zn in moong, gram, and pea plants, possibly due to the importance of these two essential nutrients in growth and metabolism of plants (Vazquez et al. 1987). Among the factors which may limit NO_3^- assimilation, Fe plays a crucial role, being a metal cofactor of enzymes of the reductive assimilatory pathway (NR, nitrite reductase (NiR), and GOGAT, all requiring Fe as Fe–heme group or Fe–S cluster (Borlotti et al. 2012). Fe is also suggested to induce NR activity and/or prevent degradation of the enzyme. It might induce NR synthesis by mobilization of intracellular NO_3^- and provide protection to in vivo NR degradation in absence of NO_3^- (Singh et al. 1997). Yadav et al. (2007) observed that the fresh weight, shoot length, and chlorophyll in leaves of 2 mM Cr + 0.2 mM Fe-treated maize plants was higher than that in 2 mM Cr-treated plants, both after 45 and 90 days. To overcome the toxic effects of Cr in *R. sativus*, Nath et al. (2009) used Fe in the recovery treatments and reported a significant recovery in most of the studied plant growth parameters. Hasegawa et al. (2012) also observed that foliar spray of Fe or increased Fe supply to roots also ameliorated the chlorosis in rice plants under exposure to high Ni concentrations. Sinha et al. (2005) conducted an experiment to determine whether the ill effects of excess Cr can be ameliorated by Fe application in spinach by withdrawal of Cr by iron application through different modes. After 14 days of metal supply, the pots of spinach with excess Cr were divided into five lots and different recovery treatments were given with a separate lot of control pots without Cr. With all these various treatments, recovery from ill effects of Cr was observed, and most conspicuously when Fe was supplied through roots (250 μM) and through spray (250 μM) together. This resulted in changes in the Biomass, concentration of chlorophylls, ferrous content, Hill reaction activity, relative water content; and recovery in activity of CAT, peroxidase, ribonuclease, and starch phosphorylase; along with lowered Cr concentration.

6.2.4 Application Potential of Chelating Agent EDTA for Amelioration

Chelation is simply defined as a process by which a molecule encircles and binds to the metal. Chelating agents such as low molecular weight organic acids (LMWOAs), e.g., citric acid, oxalic acid, tartaric acid, etc., and synthetic chelators (ethylenediaminetetraacetic acid, EDTA and diethylene triamine pentaacetic acid, DTPA) are the amendments most commonly applied for chemically assisted phytoextraction of metals from soils (Nascimento et al. 2006). Such substances are capable of forming complexes with metal ions, thereby increasing the bioavailability of heavy metals in soils. Synthetic chelators like EDTA form chemically and microbiologically stable complexes with heavy metals, which otherwise contaminate groundwater (Satroutdinov et al. 2000). LMWOAs provide alternative chelators by being easily biodegradable and more environmentally compatible (Meers et al. 2005; Nowack et al. 2006; Bala and Thukral 2011). Synthetic chelating agents have considerable effects on the fraction and solubility of heavy metals in soils. EDTA is a synthetic chelating agent commonly used in various fields (pulp and paper industry, detergents industry, food industry, medicine, biomedical labs) in order to remove harmful metal ions from many processes and products (Iranshahi et al. 2011). It also reflects a promising alternative in plant protection under heavy metal toxicity. In a supporting evidence, Mohanty and Patra (2011) observed that total chlorophyll content in the rice (*Oryza sativa* L.) seedlings treated with Cr(VI)–EDTA (10 μM) solution was more as compared to the untreated. The alfalfa (*Medicago sativa* cv. *Trifolium alexandrinum*) is a sensitive plant to heavy metal (e.g., Co and Cr) stress. Zeid et al. (2013) investigated the effect of different concentrations of Co and Cr on alfalfa growth, photosynthesis, antioxidant enzymes, carbohydrate, protein, and mineral ion content with an aim to overcome the toxic effects of these heavy metals. There was a gradual reduction in growth, metabolic activities, and the antioxidant enzyme activity with increasing concentrations

of Co and Cr, while the precipitation and EDTA treatments reduced and alleviated the inhibitory effects of the high concentrations of Co and Cr, and returned all the measured parameters to become around the control values. So the application of these treatments can be recommended, considering that it is cheap, simple, and easy to apply and also safe on the plant, soil, and environment; this will in turn help maintain soil fertility, then plant, animal, and human health.

6.2.5 Silicon Application for Amelioration of Chromium Phytotoxicity

Silicon (Si) is the second most abundant element both on the earth's surface and in the soil (Gong et al. 2006). Majority of Si in soil is insoluble and combined with other elements to form oxides or silicates, therefore, not available for plants (Richmond and Sussman 2003). Si concentrations vary greatly with plant species and tissues ranging from 0.1 to 10% of dry weight (Liang et al. 2007). Rice plants are typical Si accumulators and are able to accumulate Si up to 10% of dry weight (Ma et al. 2006). Although Si has not been evidenced as an essential element for higher plants, it is generally considered as a beneficial element for higher plants, especially for those grown under abiotic stressed environments, in particular for gramineous plants, including rice (Richmond and Sussman 2003; Liang et al. 2007). Many evidences demonstrated that Si can reduce the toxicity of heavy metals to plants such as Cd (Liang et al. 2005; Shi et al. 2010), Mn (Shi 2005), and Zn (Kaya et al. 2009). Previous studies suggested that Si-mediated increase in metal tolerance is based on several possibilities such as decrease in metal accumulation and transportation, improved mineral elements status, decreased oxidative stress, increased antioxidant capacity, and maintained ultrastructure. Therefore, a better understanding of these mechanisms associated with exogenous Si addition in plants could shed light on mechanisms related to Cr tolerance (Tripathi et al. 2012). In an attempt to analyze the effect of Si application on growth, photosynthesis and ultrastructure of barley under Cr stress, Ali et al. (2013) carried out a hydroponic experiment. The treatments consisted of three Si (0, 1, and 2 mM) and two Cr (0 and 100 μM) levels. The study revealed that Si application at both levels enhanced plant growth relative to the control, and alleviated Cr toxicity by significant increase in growth and photosynthetic parameters and also alleviated the ultrastructural disorders both in roots and leaves, with 2 mM Si having greater effect than 1 mM Si. Exogenous Si, apparently behaved antagonistically to Cr, suggesting Si as a candidate for Cr detoxification in crops under Cr-contaminated soil. In another study using rice plants, Zeng et al. (2011) investigated the alleviatory effect of Si on Cr toxicity using a hydroponic experiment with two Cr levels (0 and 100 μmol L^{-1}), three Si levels (0, 1.25, and 2.5 mmol L^{-1}), and two rice genotypes, differing in grain Cr accumulation. The results showed that toxic effects of 100 μmol L^{-1} Cr treatments on antioxidant enzymes (SOD and APX in leaves; CAT and APX in roots) and other parameters were greatly alleviated due to Si addition to the culture solution. Compared with the plants treated with Cr alone, Si addition markedly reduced Cr uptake and translocation in rice plants. No significant differences were observed between the two Si treatments (1.25 and 2.5 mmol L^{-1}) in this case. It was concluded that Si alleviated Cr toxicity mainly through inhibiting the uptake and translocation of Cr and enhancing the capacity of defense against oxidative stress induced by Cr toxicity (Zeng et al. 2011). Tripathi et al. (2012) have also observed the role of exogenous Si addition in increasing Cr(VI) tolerance in rice seedlings, where Si addition alleviated Cr toxicity and promoted growth of rice by decreasing Cr accumulation, root-to-shoot Cr transport, and MDA level. It is significant in reduction of Cr content in edible parts as Si addition in Cr-contaminated soils can help to reduce Cr contamination of grains by inhibiting Cr accumulation, and therefore, its transport into the edible parts.

6.2.6 Hydrogen Peroxide in Amelioration of Cr(VI) Phytotoxic Effects

The exogenous application of hydrogen peroxide (H_2O_2) has been found to counter toxic effects of several abiotic stresses (Yıldız et al. 2013). There are evidences from several studies that H_2O_2 increases tolerance of plants to salinity, drought, heavy metal, and heat stress (Uchida et al. 2002; Xu et al. 2008; Hu et al. 2009). To evaluate the ameliorating effects of H_2O_2 (200 μM) on Cr(VI) toxicity in canola (*Brassica napus* L.), Yıldız et al. (2013) observed plant growth, chlorophyll content, thiol contents, lipid peroxidation, antioxidant enzymes, and the expression of metallothionein protein (BnMP1) mRNA. Cr(VI) at 50 μM significantly decreased the plant growth (fresh and dry weights) accompanied by increased lipid peroxidation and decreased chlorophyll content in leaves. H_2O_2 pretreatment, however, enhanced plant growth parameters and led to the reduced levels of lipid peroxidation and higher levels of pigment. In addition, H_2O_2 pretreatment increased Cr accumulation in aerial parts of seedlings. The tendency of increase in thiol content under Cr(VI) stress was further increased with H_2O_2 pretreatment. The activities of antioxidant enzymes such as SOD, APX, POD, and CAT were differentially altered. SOD and POD activities increased under Cr(VI) stress, whereas APX and CAT activities decreased. This study suggested that H_2O_2 may act as a signal that triggers defense mechanisms which in turn protects canola seedlings from Cr(VI)-induced oxidative damage.

6.3 Soil Amendments for Enhanced Cr Tolerance in Plants

6.3.1 Potential of Micro- and Macronutrient Amendments in Soil

Many plant enzymes require Zn ions for their activity and for chlorophyll biosynthesis, while Zn deficiency is associated with an important carbohydrate metabolism and protein synthesis (Taiz and Zeiger 2002). Similarly, potassium (K) also plays an important role in regulation in osmotic potential in plant cells and also activates many enzymes in respiration and photosynthesis (Bassi et al. 1990). In protein synthesis, K is probably involved in several steps of the translation processes, including the binding of tRNA to ribosomes (Evans and Wildes 1971). The high K concentrations in the sieve tubes are probably related to the mechanism of phloem loading of sucrose. The adverse effect of Cr was found to be nullified by the supply of suitable amounts of Fe and Zn in moong, gram, and pea plants, possibly due to importance of these two essential nutrients in growth and metabolism of plants (Vazquez et al. 1987). In addition, Fe also plays an important role as a component of enzymes involved in the transfer of electron redox reaction, like with cytochromes, and it is reversibly oxidized from Fe^{2+} to Fe^{3+} during electron transfer. Under condition of Fe deficiency, the activity of both types of enzyme declines. Nath et al. (2009) have shown that lower levels of tannery effluent can be used for irrigation of *R. sativus* L. plants in combination with Zn, K, and Fe sulfate. The application of Zn in combination with tannery effluent has been shown to reduce the toxicity of Cr, leading to increased growth.

6.3.2 Amendment in Phosphorus Levels and Addition of Glucose in Soil

Phosphorus (P) is well known as an essential and limiting nutrient for plant growth and development. P and Cr compete with each other during the plant uptake process. Sayantan (2013) studied the role of P in moderating the Cr toxicity in *R. sativus* L. The toxic effects of Cr and the moderation of toxicity due to P amendment were determined as accumulation of Cr, nitrogen, and P in root tissues, and their effects were also examined in the changes in biomass, chlorophyll, and antioxidant enzyme levels. Cr and N accumulation were almost doubled at the highest concentration of Cr supply, without any P amendment, whereas

at the highest P concentration, the accumulation was reduced to almost half. Therefore, P amendment moderates the toxicity caused by the supplied Cr in *R. sativus*. This finding can be utilized to develop a novel technology for the amelioration of Cr stressed fields. Generally, Cr(VI) negatively affected both the size and activity of soil microbial biomass. Recently, Leita et al. (2011) reported that with the addition of glucose increased the reduction rate of Cr(VI) as it induced soil microbial biomass size and activity with an indirect role in the increased rate of Cr(VI) reduction, by promoting growth of indigenous microbial biomass.

6.3.3 Effect of Biochar Application on Soil Properties and Plant Nutrient Uptake Under Cr Toxicity

The addition of biochar increased soil pH, electrical conductivity (EC), organic carbon, total nitrogen, available P, cation exchange capacity (CEC), and exchangeable cations of Cr-polluted and Cr-unpolluted soils (Topoliantz et al. 2002). Uptake of nitrogen, P, and K were also increased by addition of biochar. The presence of plant nutrients and ash in the biochar, high surface area and porous nature of the biochar, and the capacity of biochar to act as a medium for microorganisms are identified as the main reasons for the increase in soil properties and highest nutrient uptake at biochar-treated soils (Verheijen et al. 2009). The increase in the availability of major plant nutrients due to application of biochar was also reported by Glaser et al. (2002) and Lehman et al. (2003). Application of biochar on Cr-polluted and Cr-unpolluted soils significantly ($p<0.01$) increased the mean values of soil organic C and total N (Nigussie et al. 2012). The increases in organic carbon were observed in soils treated and total nitrogen upon addition of biochar due to presence of high amounts of carbon and nitrogen in the maize stalk. High organic carbon in soils treated with biochar has been also reported by Lehmann (2007).

6.4 Application of Biosludge as Metal Chelator

Recently, National Environmental Engineering Research Institute (NEERI), India has started to develop cost-effective and ecofriendly technologies that use microorganisms and industrial wastes for cultivation of petro-crops in lands degraded due to metals (Juwarkar et al. 2006). Juwarkar et al. (2008) evaluated the effect of different concentrations of As-, Cr-, and Zn-contaminated soils, amended with biosludge and biofertilizer on the growth of *Jatropha curcas,* which is a biodiesel crop. The study revealed that biosludge alone and in combination with biofertilizer significantly improved survival rates and enhanced the growth of the plant. With the amendments, the plant was able to grow and survive up to 250 mg kg^{-1} of Cr-contaminated soil. In absence of biosludge, heavy metal accumulation in the plant increased with increasing concentrations of heavy metals in soil, whereas in soils amended with biosludge, a significant reduction in the metal uptake in the plant was observed. It was assumed that the organic matter present in the biosludge acted as a metal chelator, thereby reducing the toxicity of metals to the plant. Findings suggest that plantation of *J. curcas* may be promoted in metal-contaminated soils, degraded soils, or wastelands suitably after amending with organic waste. Role of other organic amendments, such as fermented compost, has also been reported in the reestablishment of vegetation on contaminated sites by decreasing the bioavailability of heavy metals in soil (Tordoff et al. 2000; Walker et al. 2004).

6.5 Bioremediation of Cr Using Microbial Inoculants

The bioremediation is an approach to exploit the naturally occurring biodegradative processes to clean up contaminated sites. In situ, ex situ, and intrinsic bioremediation are receiving increasing attention as viable remediation alternatives. The application of the bioremediation approach depends on the type of pollutant or pollutant

mixtures present and the type of microorganisms present as well as environmental conditions and nutrient availability (Abdel-Sabour 2007). For instance, the microbes resistant to high Cr(VI) are promising source for the Cr(VI) bioremediation. Further, the use of plant growth-promoting rhizobacteria is one of the inexpensive and environment-friendly ways to alleviate the Cr toxicity in plants (Khan et al. 2012, 2013; Kang et al. 2012). Rhizobacteria are the root-colonizing bacteria that exert beneficial effects on plant development via direct or indirect mechanisms (Nelson 2004) and have potential to decrease the toxic effects of heavy metals (Bertrand et al. 2000). Rhizobacteria having 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase) enzyme could improve the plant growth under stress conditions (Nadeem et al. 2006). Harms of Cr on plants could be minimized by rhizobacteria via different mechanisms like biosorption and bioaccumulation, bioreduction to a less toxic state, and chromate efflux (Nazir et al. 2011; Khan et al. 2012). Cr(VI)-resistant rhizobacterial isolates might cause changes in plant growth and development due to involvement of single or multiple possible mechanisms of action, i.e., solubilization of insoluble phosphate (Yasmin and Bano 2011); production of siderophore (Meyer 2000); production of phytohormones (Humphry et al. 2007); indirect mechanisms of action, i.e., reduction of Cr(VI) to Cr(III) by which it decreases the harmful effects of Cr(VI) to the plants (Salunkhe et al. 1998); biocontrol (Chandra et al. 2007); or induction of systemic resistance in plants against phytotoxicity of Cr(VI) (Mishra et al. 2006). Turick et al. (1996) investigated several bacteria from various soils for Cr(VI) resistance and reducing potential. Microbes selected from both Cr(VI)-contaminated and Cr(VI)-noncontaminated soils and sediments were capable of catalyzing the reduction of Cr(VI) to Cr(III) a less toxic, less water-soluble form of Cr. Cr reduction capacity of these isolates was compared with that of *Pseudomonas aeruginosa* and *Bacillus circulans*. *Bacillus coagulans*, isolated and identified from Cr-polluted soil, gave maximum reduction potential among all organisms studied. Morales et al. (2007) isolated *Streptomyces* sp. CG252, which was highly tolerant to Cr(VI) and has the ability to reduce Cr(VI) into Cr(III). Similarly, Mistry et al. (2009) reported that Cr-resistant bacterial strain *Pseudomonas olevorans* had the ability to reduce the Cr(VI) into Cr(III) and to bioremediate Cr(VI)-containing waste. Recently, Datta et al. (2011) reported Cr(VI) tolerance capability of different varieties of wheat and several other studies showed that rhizosphere bacteria stimulate plant growth and development under stress conditions. Kumar et al. (2009) suggested that plant growth-promoting bacteria (*Enterobacter aerogenes* and *Rahnella aquatilis*) reduce the toxicity of Ni and Cr in *B. juncea* (Indian mustard) and promoted plant growth.

6.6 Phytoremediation of Cr Using Potential Plants

A new research area of using plants for the bioremediation (phytoremediation) of contaminated soil and water was reviewed by Brown (1995). Reduction of heavy metals in situ by plants may be a useful detoxification mechanism for phytoremediation. The key role is played by plant roots, and they are a significant metal sink. Metal uptake by plants can be passive, facilitated, or active. The regulation of metal uptake by both soil–root and root–shoot interfaces varies within plant species and cultivars. Plants are effective at removing metals because they require certain trace elements to survive. Some plant species, known as hyperaccumulate toxic metal and they accumulate upto 5 % of their dry weight and as on to date about 400 plants that hyperaccumulate metals are reported. One of the earliest examples of a hyperaccumulator was the Italian serpentine plant *Alyssum bertolonii* and another more recently identified is the Alpine pennycress *Thlaspi caerulescens*. Abdel-Sabour et al. (2002) studied the use of hyperaccumulator plant species to extract Cr from contaminated soils. They investigated three soils (A, B, and C) and four plant species, i.e., sorghum (*Sorghum vulgare* L.), clover (*Trifolium pratense* L.), panikum (*Panicum antidotal*), and canola (*Brassica napus*), and concluded that canola accumulated the highest Cr

quantity among the four plant species, irrespective of the clipping or soil type. Calculation of recovery percentage based on Cr removed from the soil after cultivation ranged between 3.7 and 40.6 % of total initial Cr. The highest values were noticed in case of clover and canola. Diwan et al. (2008) also reported the ability of Pusa Jai Kisan genotype of Indian mustard to grow in the presence of high Cr(VI) levels in the hydroponic as well as natural environmental conditions, and the amounts of Cr concentrated in the aerial part of this plant indicate that there is great potential for its use in the remediation of Cr-contaminated sites. On the other hand *Azolla* (an aquatic water fern) biosystem has already been proven to be a potent tool for biofiltration of various toxic metals. In a report by Rai (2008), high increase of the metal content in the biomass suggests that *Azolla pinnata* has tremendous potential to take up Cr(III) and Cr(VI) (70–88 %) and may be used as a bioaccumulator to polish heavy metals in ash slurry, coal mines, and tannery effluent. The concentration of metals in the *A. pinnata* biomass was directly related to that of the solution. Aquatic macrophytes can also be a good remediation option, and duckweeds have proven to be promising prospective scavengers of heavy metals from polluted waters (Zurayk et al. 2001; Zhang et al. 2007). *Lemna gibba* and *Lemna minor* L. are the most studied species for phytoremediation (Mkandawire and Dudel 2005). They exhibit relatively high tolerance to Cr toxicity and are capable of active uptake and accumulation of this element against the concentration gradient (Staves and Knaus 1985). Chandra and Kulshreshta (2004) revealed that *Spirodela polyrrhiza* is a potential accumulator of Cr(VI). Since phytoremediation generally removes only a small percentage of heavy metals from contaminated sites and can only be used in situations with low-level contamination, for extremely contaminated sites other approaches must be applied (Lasat 2002). Peterson and Girling (1981) reported other plants for phytoextraction, such as *Sutera fodina*, *Dicoma niccolifera* and *Leptospermum scoparium,* which accumulate Cr to high concentrations in their tissues. Hyperaccumulators are generally metal-specific and yield a low annual biomass production, thus limiting the overall amount of heavy metals that can be extracted per harvest (Meers et al. 2004). In most cases, limited translocation of Cr following uptake by the roots is the bottleneck limiting the overall efficiency of phytoextraction from the environment.

6.7 Other Techniques for Cr Remediation

Overall, the cleanup goals discussed so far are based on the Cr(VI) concentration in the soils and the volume and physical–chemical properties of the Cr-containing soils. Therefore, most of the available treatment technologies consist of following these three mechanistic approaches, i.e., (1) removing the Cr(VI)-containing soils from the site; (2) immobilizing the Cr so that it will not leach after treatment under field conditions; or (3) reducing the Cr(VI) in the soils to the Cr(III) state (Abdel-Sabour 2007). As an example of first technology, "*soil washing and in situ flushing*" involves the addition of water with or without additives, including organic and inorganic acids, sodium hydroxide, methanol, EDTA, acids in combination with complexation agents or oxidizing/reducing agents as well as biosurfactants, which enhance removal of metals from contaminated soils and sediments (Mulligan et al. 2001). According to United States Environmental Protection Agency (USEPA), efficiency of metal removal by soil washing ranges from 75 to 99 % (USEPA 1992), depending on a number of factors, including the length of time the soil has been exposed to the metals of concern, the amount of fines in the soil, and the affinity of the contaminants for the washing solution. It is believed that if a soil has greater than 20–30 % fines (with particle sizes less than 0.06 mm in diameter), soil washing may not be the most effective technology (Oravetz et al. 1992).

The second technology for alleviation includes "*in situ immobilization*" of the pollutants has the advantage of minimizing the exposure of site works and local residents to airborne pollutants as well as minimizing disruption to or demolition

of existing structures. Mobility is strongly related to the physicochemical state and the location of pollutants. If elements or organic compounds (pesticides) become trapped within the structure of minerals or humic substances, they are neither mobile nor bioavailable and, particularly in the case of organics, they are physically protected and not accessible to microorganisms that might be able to transform them (Abdel-Sabour 2007). "Clay and clay minerals," a mixture of clay minerals and natural zeolites with calcium compounds can also be used as liner material at solid waste disposal sites (particularly soils polluted by Cr(VI)) as their impermeability and sorption properties prevent migration of toxic metals from waste sites (Minato and Shibue 1998). "Organic matter" content and bioactivity are considered as important factors in reducing almost 96% of the added Cr(VI) under aerobic, field moist conditions (Losi et al. 1994). Similarly, Cifuentes et al. (1996) added easily degradable organic substances of a very narrow C:N ratio and found marked Cr(VI) reduction.

The third technology, "*chemical reduction,*" can also be used to convert Cr(VI) to the trivalent valence state, which is generally less toxic and less soluble (Patterson 1985). Reducing agents (such as ferrous sulfate, ferrous ammonium sulfate) can be delivered to the soil subsurface by injection wells or in situ soil mixing equipment. James et al. (1997) stated that effective remediation of Cr(VI)-contaminated soils by reduction depends on: (1) reduction of Cr(VI) to Cr(III) which is inert toward reoxidation; (2) absence of undesirable reaction products; and (3) establishment or maintenance of soil pH and Eh conditions that favor the reduction of Cr(VI) and disfavor oxidation of Cr(III). The extent of oxidation of Cr(III) in soils amended with wastes is based on four interacting parameters: (1) solubility and form of Cr(III) related to oxidation waste oxidation potential; (2) reactive soil Mn(I, IV) hydroxide levels (soil oxidation potential for Cr(III); (3) soil potential for Cr(VI) reduction (soil reduction potential); and (4) soil waste pH as a modifier of the first three parameters (pH modification value). Each of these four parameters can be quantified with laboratory tests and ranked numerically; the sum of which is the potential Cr oxidation score (PCOS) for assessing the relative hazard of a waste–soil combination as proposed by James et al. (1995). Patterson and Fendorf (1998) reported that the reduction of Cr(VI) to Cr(III) decreases the toxicity and mobility of Cr contaminants in soils and water. In addition, the formation of a highly insoluble Cr(III) product would decrease the likelihood of future Cr(III) reoxidation. They noticed that amorphous iron sulfide minerals like mackinawite (FeS1-x) have the potential to reduce large quantities of Cr(VI) and in the process form very stable [Cr, Fe]$(OH)_3$ solids. In their study the effectiveness of amorphous FeS as a reductant of Cr(VI) was assessed by identifying the solution and solid phase products of the reaction between FeS suspensions and chromate. Results showed that iron sulfide removed all of the added Cr(VI) from solution for the reaction conditions studied and reduced between 85 and 100% of the Cr(VI) to Cr(III). Chromate reduction occurred dominantly at the FeS surface and resulted in $[Cr_{0.75},Fe_{0.25}](OH)_3$; while less extensive, reduction of Cr(VI) by Fe(II) (aq) was noted and produced a solid with the opposite Cr:Fe ratio, $[Cr_{0.25},Fe_{0.75}](OH)_3$ (Abdel-Sabour 2007).

6.8 Current Challenges and Future Directions

Heavy metal stress is one of the major problems affecting agricultural productivity of plants. Natural flora show relative differences in their heavy metal tolerance capacity. Some plants grow well in a soil enriched with toxic levels of heavy metals while others cannot. The roles of several effectors in amelioration of Cr have been discussed above. Additionally, several natural plant species have been identified showing heavy metal accumulator behaviors. These natural heavy metal accumulators could be a potential source for genetic manipulation of other important agricultural crop plants. However, this needs a further detailed account of experimental validation. Metal chelation proves to be of high importance as a generalized means of heavy metal removal from soil and water. However, in situ application of chelat-

ing agents can also cause groundwater pollution by uncontrolled metal dissolution and leaching. Therefore, the risk assessment must be carried out thoroughly in the use of EDTA or other chelators for phytoextraction before taking further steps towards development and commercialization of this remediation technology. There is a need for further experimental validation of such promises under different soil and environmental conditions with major crops. Further, complications due to soil and water contamination by multiple heavy metals demands integrative approaches and trials to get conclusive results. This would lead to development of more generalized and effective strategies for amelioration of phytotoxic effects. Another important aspect that must be considered during the study of any approach is "maintenance of natural beneficial microbial population" under such experimental conditions. This would be important in case of bioremediation using microbial inoculants. There are no doubts about symbiotic and beneficial relationship between plants and microbes. Therefore, use of microbes with dual characteristics of Cr tolerance and plant growth promotion will be of high significance for amelioration as well as increased crop productivity for increased population. Apart from bioremediation and metal chelation, PGRs are also being studied extensively for amelioration of different heavy metals, and SA has been shown to be highly effective in several such studies. BRs are again of high importance considering the supporting relevant studies for Cr. Integrative use of such growth regulators and inclusion of major crops under further experiments with multifield and multisite trials will lead to development of consensus among outcomes and final recommendations. Again, combined effect of heavy metals and their effective amelioration will require in-depth research on molecular mechanisms behind such effects. Current research trends toward understanding of key genes under different abiotic stresses and transcriptomics studies would be a benchmark for development of these strategies. The actual success of any of the above strategies will be governed by a thorough understanding of the molecular basis of their action by extensive research, its cost effectiveness, user-friendliness, and environmental impact of course.

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Management of Environmental Phosphorus Pollution Using Phytases: Current Challenges and Future Prospects

7

Vinod Kumar, Dharmendra Singh, Punesh Sangwan and Prabhjot Kaur Gill

Abstract

Phosphorus is an important element for plant and animal nutrition considering its diverse roles in their growth and development. It is derived from different organic and inorganic sources rich in phosphorus. Inorganic sources are most commonly used for development of phosphorus fertilisers while organic sources like phytic acid phosphorus of plant origin is a major source of phosphorus in animal nutrition. Excessive application of phosphorus fertilisers without proper analysis of its soil concentration results in high phosphorus and associated heavy metals deposition in agricultural soils. This has multiple environmental consequences like loss of biological diversity in aquatic system due to phosphate runoff from soil by rain water. Further, inability of monogastric animals to hydrolyse phytate phosphorus and utilise it makes it necessary to supplement external phosphorus in animal feed. This leads to increased phosphorus load and release of excess phosphorus in faecal material at intensive livestock production area, which contributes to environmental phosphorus pollution. The supplementation of animal feeds with microbial phytases increases the bioavailability of phosphorus and minerals besides reducing the aquatic phosphorus pollution in the areas of intensive livestock production. Phytases are of significant value in effectively combating environmental phosphorus pollution. This chapter describes different application of phosphorus, its pollution consequences and use of phytases for strategic management of this problem phosphorus pollution and various promises and challenges therein.

Keywords

Animal feed · Eutrophication · Phosphorus · Phytase · Phytic acid

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7.1 Introduction

Phosphorus (P) is a macronutrient for plants and plays important roles in the biosynthesis of nucleic acids, cell membranes and regulation of many enzymes. It is available in soil either as inorganic or organic fractions in significant amounts and not readily accessible to plants, leading to P deficiency in soil as a major problem for agricultural production. The major storage form of organic P in soil is phytate (salts of phytic acid), which is not readily provide P to plants because of its complex with cations or adsorption to various soil components. This form of P is also the principal storage form in plants. Phytic acid P constitutes 1–5% weight in cereals, legumes, oils, seeds and nuts (Sapna et al. 2013). The phytate P is generally unavailable to monogastric animals (chicken, swine, fish, humans) due to either absence or insufficient secretion of enzymes essential for phytate hydrolysis in digestive tract (Kumar et al. 2014). Consequently, P remains unabsorbed in the digestive tract and gets excreted along with faeces as such in the environment leading to P pollution. To supplement P requirement, animal feeds are commonly supplemented with inorganic P. Further, the excretion of undigested phytate along with inorganic P imposes global ecological problems of P eutrophication at the sites of intensive livestock production. This excessive P in soil runs off under different climatic cycles to different water sources such as ponds and rivers causing rapid growth of phytoplanktons, algae, creating dense population of cyanobacterial blooms, hypoxia and death of marine animals. These blooms also make plants unable to photosynthesise and produce food for their survival (Vats and Banerjee 2005).

Adopting a suitable approach for phytic acid P hydrolysis may lead to decreased environmental pollution and feed cost. Several interventions have been suggested for phytic acid hydrolysis comprising physical methods, e.g. autoclaving, cooking; and chemical methods, e.g. ion exchange and acid hydrolysis, but reported to compromise the nutritional value of the food (Singh et al. 2011). Therefore as an alternative, enzymatic hydrolysis is preferred for the reduction of phytic acid content in food and feed in several studies (Vohra and Satyanarayana 2003; Vats and Banerjee 2004; Greiner and Konietzny 2006; Rao et al. 2009; Singh and Satyanarayana 2011; Singh et al. 2011; Kumar et al. 2014). These studies showed considerable interest of food and feed industries towards phytate-degrading enzymes, mainly phytases (myo-inositol hexakisphosphate phosphohydrolase), which catalyse the hydrolysis of the phosphate moieties in phytic acid (Fig. 7.1). Since

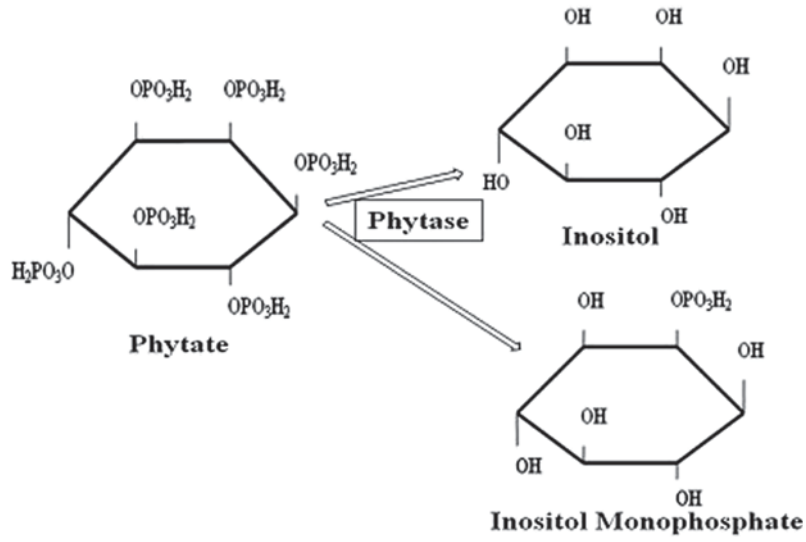

Fig. 7.1 Phytic acid hydrolysis by phytase enzyme

last 20 years, interest in phytases has increased remarkably, not only because of its wide range of applications in animal and human nutrition, but also in response to heightened concerns over phosphorous pollution in the environment (Lei and Porres 2003). Suzuki et al. first detected phytase activity in rice bran in 1907, but it was not until 1991 that the first phytase feed enzyme became commercially available (Haefner et al. 2005; Cao et al. 2007). Phytase can be found in plants, bacteria, fungi, yeast and animals. However, among microorganisms phytase activity has been observed most commonly in fungi, particularly in *Aspergillus* species (Kim et al. 1998). Until now, a number of phytase producing organisms have been reported, but the search for a thermostable and acid-stable phytase with broad substrate specificity and high specific activity has been under progress for animal nutrition purposes. The aforementioned parameters were considered as key factors in the use of phytase for animal nutrition. Additionally, the low yield and high cost of enzyme production are major limiting factors in use of phytase enzyme in animal diet.

In this chapter, importance of P and phytic acid in agriculture and animal nutrition leading to P pollution is outlined. The consequences of P pollution are discussed. Further, an insight in phytases from different sources and various considerations during their uses in phytic acid removal and environmental pollution management are discussed for better understanding and implementation of future strategies.

7.2 Importance and Need of P in Animal and Plant Nutrition

7.2.1 Phosphorous: A Vital Source of Animal Nutrition

Phosphorus is the 11th most abundant element on earth. It exists in soil either in dissolved (i.e. solution) or solid form (particulate P), the solid form being dominant. In solid form, P is classified as inorganic P (P bound to Al, Fe, Ca, Mg etc. as complex salts) and organic P (P bound to organic material such as dead and living plant material and micro-organisms, soil organic matter etc.). Both forms of P are interconvertible with the aid of soil bacteria and growing plants (Magette and Carton 1996). Mineral soil contains 33–90% of total P in inorganic form. In common with other major elements, the concentration of total P in soils is relatively higher considering the crop requirements and available P fraction. The typical range for total P content of agricultural soils is estimated between 0.20 to 2.0 g/kg. Dissolved P is typically less than 0.1% of the total soil P and usually exists as orthophosphate ions, inorganic polyphosphates and organic P (Magette and Carton 1996).

In animal nutrition, P plays a key metabolic role with more physiological functions than any other mineral. These functions include P as a major constituent of nucleic acids and cell membranes, major constituent of the structural components of skeletal tissues (80% P found in the bones and teeth), and is directly involved in all energy-producing cellular reactions, maintenance of osmotic pressure and acid–base balance, protein synthesis, transport of fatty acids, amino acid exchange, growth and cell differentiation, appetite control, efficiency of feed utilisation and fertility (NRC 1993; Dobrota 2004). The P nutritional requirements for most farm animals are well documented (dairy cattle 85–95 g/day, beef cattle 35–40 g/day). The variation in P content of natural feed has been observed in plants at the species level. For example, the P content of barley, maize and oats is very low compared to rape seed meal. The P present in animal diet is digested and metabolised differently by ruminant and monogastric animals (Bomans et al. 2005). P deficiency in animal diet can affect the animal's physical well-being including compromise of the immune system, bone breakage, loss of appetite, reduction in fertility and loss in live weight gain due to low feed efficiency (Aehle 2007). Diets with low P content can be considerably improved by the use of P feed supplement in the form of compound feed or as separate mineral supplements. P supplements are manufactured in many chemical and physical forms to suit different feeding and handling practices (http://www.nhm.ac.uk/mineralogy/phos/) (Bomans et al. 2005).

7.2.2 Role and Behaviour of P in Plants

In plant nutrition, P is usually the critical limiting element for plant production, and throughout the history of natural production and human agriculture, P has been short in supply. P is absorbed by plants from the soil as monovalent (H_2PO_4) and divalent (HPO_4) orthophosphate anions, where the percent composition of each varies with respect to soil pH. For instance, H_2PO_4 and HPO_4 represent 50% of total P at pH 6–7 while at pH 8, H_2PO_4 represents 20% and HPO_4 represents 80% of total P. H_2PO_4 is about 100% of total P in soil solution at pH 4–6 (Black 1968). The seeds and grains must store ample P so that the seedling has enough to develop its first roots and shoots. In the natural environment, P is supplied through the weathering and dissolution of rocks and minerals with very slow solubility. Maximum P absorption occurs during the vegetative growth of plants, and thereafter, most of it is retranslocated into fruits and seeds during reproductive stages. After absorption into the plant, much of the phosphate reacts very quickly to form organic compounds (Wild 1988). Less absorption of P through situations that inhibit root growth, such as soil compaction or cold soil temperature may lead to its deficiency. Deficiency of P often appears early in plant growth as stunting, with purple or reddish tints in the leaf and vegetative tissues. It affects not only plant growth, development and crop yield but also the quality of the fruit and the formation of seeds. Therefore, increases in productivity require external nutrient inputs where external P inputs are available on a large scale from the mining of P deposits (Bomans et al. 2005).

7.2.3 Phytic Acid Phosphorus: Significance in Nutrition and Agriculture

Phytic acid or *myo*-inositol 1,2,3,4,5,6-hexa*kis* (dihydrogen phosphate), IP_6 is a naturally occurring compound that can significantly influence the functional and nutritional properties of foods. Phytic acid is a simple ringed carbohydrate with one phosphate group per carbon. Its molecular formula is $C_6H_{18}O_{24}P_6$ and its molecular weight is 660.035 g/mol. Three terms, namely phytate, phytin and phytic acid, are used in the literature to describe it. Phytate, the most commonly used term, refers to the mixed salt of IP_6. Phytin specifically refers to the deposited complex of IP_6 with potassium (K), magnesium (Mg) and calcium (Ca) as it occurs in plants, whereas phytic acid is the free form of IP_6 (Selle and Ravindran 2007). Phytic acid is a strong chelator and is the principal storage form of P in the plant seeds and can account for up to 80% of the total P in the seed (Lopez et al. 2002). Phytic acid is found within legumes, cereals, oil seeds, pollens as well as in the hulls of nuts, constituting about 1–5% of their weight (Table 7.1) (Vats and Banerjee 2004; Singh et al. 2011). It accumulates in seeds and grains during ripening along with other storage substances such as starch and lipids, and plays an

Table 7.1 Total P, phytate P content and phytase activity of plant origin feedstuffs. (Source: Paik 2001)

Ingredients	Phytate P	Total P	Phytate P	Phytase activity
	mg/100 mg	mg/100 mg	% of Total P	U/kg
Corn	60	182	32.7	0.2
Lupin	55	307	17.8	3.2
Tropica	7	59	11.9	18.8
Wheat	199	295	67.5	1120
Sesame meal	542	816	66.4	3.0
Soybean meal	286	577	49.6	7.5
Cotton seed meal	303	678	44.7	2.4
Rape seed meal	532	1.16	52.7	103
Rice bran	12.1	1886	63.7	–
Wheat bran	742	893	83.1	2935

important role in P storage, as an energy store, as a source of cations and as a source of *myo*-inositol and also helps in initiating dormancy (Singh et al. 2011). In cereals and legumes, phytic acid accumulates in the aleurone particles and globoid crystals, respectively (Reddy et al. 1982; Tyagi and Verma 1998). Graf et al. (1987) suggested that phytic acid in seeds acts as a natural antioxidant during dormancy. The unique phytate ion structure, with 12 replaceable protons and high density of negatively charged phosphate groups (responsible for its characteristic properties), allows it to form very stable complexes with multivalent cations (Dost and Tokul 2006). A considerable number of researchers have reported the chelating ability of the phytate ion with several mineral elements including, Cu^{2+}, Zn^{2+}, Co^{2+}, Cd^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, Fe^{2+}, Ni^{2+} and Ca^{2+} to form phytate–mineral and/or other protein–mineral–phytate complexes (Sapna et al. 2013). Intestinal absorption is a key and complex stage for maintaining normal mineral homeostasis and requires that minerals remain in the ionic state for absorption (Lopez et al. 2002). Due to the inability of monogastric animals to hydrolyse the phytate–mineral complex, minerals are not absorbed in the intestine and are excreted out. Also, binding or interaction of phytic acid with dietary proteins reduces their digestibility due to steric hindrance to proteases through changes in protein solubility or by altering the protein structure (Cowieson et al. 2006). According to Liu et al. (2010), phytate can also interfere with lipid metabolism and consequently energy regulation in chickens. The inhibition of activity of important digestive enzymes such as α-amylase, trypsin, lipase, acid phosphatase and pepsin was also reported to be affected by phytic acid (Harland and Morris 1995; El-Batal et al. 2001).

Apart from some antinutritional effects given above, phytic acid has been shown to exert an antineoplastic effect in animal models of both colon and breast carcinomas (Iqbal et al. 1994). The inositol phosphate intermediates synthesised from phytic acid hydrolysis play a role in the cellular transport, whereas inositol triphosphates play a role in signal transduction and regulation of cell functions in plant and animal cells (Vohra and Satyanarayana 2003; Greiner and Konietzny 2006; Rao et al. 2009; Singh and Satyanarayana 2011; Sapna et al. 2013).

7.3 Consequences of Phosphorus Pollution

7.3.1 Phosphorus Loss, Buildup and Environmental Impacts

Phosphorus remains in short supply over large parts of the globe, still some developed countries with a long history of P fertiliser application and intensive animal farming despite having small lands are facing problems of P pollution. There are two major aspects related to P deposition and pollution. First, elaborated systems of fertility management and prolonged use of P fertiliser in some developed countries have built up an extensive level of soil P up to the extent that further limited or no addition of P is required for crop production. However, continued use of P fertilisers and use of P-supplemented animal feed is leading to the deposition/production of P waste resulting in P pollution (Howarth et al. 2000; Bomans et al. 2005). The problem is further perplexed by variation in plant capability for P uptake with crop type, crop yield and soil type. The low uptake of P by crops can allow P to accumulate in soils, which can eventually create P runoff and contaminate nearby surface water (Garikipati 2004).

Second, intensive animal production on farms with little land produces manure in excess of the nutrient requirements of crops and pasture lands. Generally, the application this manure to field is determined by measuring N content of the manure and the N requirement of the crop. Because animal manures are typically rich in P (P concentrations range from 4 to 7 mg/g dry weight of dairy manure, compared to 0.08–1.56 mg/g dry weight of benchmark soils and 0.486–2.439 mg/g dry weight of surface soils), its use leads to accumulation of excess P to the soil (Garikipati 2004). According to NRC (2001), among all dietary mineral elements for dairy animals, P represents the greatest potential risk if

an excess amount is released into the environment, contaminating surface waters and causing eutrophication. The waste/unutilised P from the animal manure accumulates in the surface soils and, thereafter, leads to increased leaching and erosion losses of that P to aquatic environments (Bohn et al. 2008). This leaching as "run-off" is more pronounced in P saturated soils and even more easy from soils with low retention capacity, e.g. highly organic (especially peat) soils and sandy soils. P transferred in this way can be either in soluble (dissolved) or "attached" (sorbed to or part of soil inorganic and organic materials) forms. These losses of P that may not regarded as significant in agronomic terms can be significant in environmental terms due to the fact that a very small concentration (ca. 20 μg/L) in susceptible surface waters can lead to eutrophic conditions. It has possible consequences in water use for fisheries, recreation, industry or drinking due to the increased growth of algae and aquatic weeds and oxygen shortages (Bomans et al. 2005). Manure-borne P is a serious environmental hazard that has been reviewed by several researchers (Centner 2004; Shigaki et al. 2006; Powers and Angel 2008).

In a survey, Bertrand et al. (1999) found that dairy diets in the USA were formulated with 20% more P than the recommended limit released by NRC (2001). This P oversupplementation (~20%) to the national dairy herd was reported to cost $ 100 million p.a. and contributed to undesirable high manure P levels (Satter and Wu 2001). According to Wu et al. (2000), the extra P was not needed as no difference in animal performance parameters was reported. A reduction of 25–30% in manure P and a saving of $ 10–15/year-cow could occur with reduction in this P over supplementation.

7.3.2 Eutrophication and Loss of Biodiversity

Eutrophication of aquatic systems is further considered as a result of external loading (nutrient inputs from outside the aquatic system) and internal loading (nutrient recycling within the water column and sediments). External loading of P in streams and rivers is usually contributed by anthropogenic processes through nutrient inputs from the fertilisation of soils, soil erosion, animal farming waste and disposal of municipal or industrial effluents, and atmospheric deposition of P enhanced by emissions (Bomans et al. 2005). Internal loading, which is particularly important in shallow lakes, estuaries and near-shore seas while less significant in deep lakes and ocean basins, results from seasonal or annual return to the water column of nutrients that have sunk and accumulated in sediments. In general, the effects of anthropogenic eutrophication are negative, and the beneficial effects are rare or accidental (Bomans et al. 2005). The eutrophication of P in water bodies may result in a number of consequences in aquatic ecosystems. The potential P eutrophication of fresh water streams, lakes and near-coastal areas can cause algal blooms, hypoxia and death of aquatic animals followed by production of nitrous oxide, a potential greenhouse gas (Mallin and Cahoon 2003). The population explosions of algal species are called red tides or algal blooms and their proliferation and occasional dominance by particular species is a result of the combination of physical, chemical and biological mechanisms and interactions. Algal species have wide differences in their tolerance and requirements of nutrients; where some are tolerant to high levels of P, others are adapted to low P conditions. The change in nutrients, light conditions due to high algal growth and oxygen availability favour growth of some species over others and cause shifts in the structure of phytoplankton and zooplankton, thus altering community structure. It may trigger reduced growth and recruitment of fish species and death of fishes, causing low fishery production. Moreover, moderate nutrient enrichment can sometimes also lead to an increase in population of economically valuable fishes in some ecosystems.

Another consequence of eutrophication is decreased availability of silica, which diatoms require to form their glasslike shells. This can also alter the phytoplankton community by limiting growth of diatoms or causing a shift in other types of diatoms. Studies off the German coast

lasting more than two decades revealed a striking change in the composition of the phytoplankton community, as diatoms decreased and flagellates increased more than tenfold because of fourfold increase in the ratio of available N and P to silica. Further, eutrophication also results in higher levels of dissolved organic matter (DOM) and affects the availability of biologically useable forms of iron and other essential metals. Since iron must be in soluble form to be bioavailable and higher DOM leads to higher soluble iron, this leads to changes in the species composition of the phytoplankton community and poses enormous consequences for animal grazers and predators. The degradation or complete loss of seagrass beds in an aquatic ecosystem is another consequence of nutrient eutrophication, because plant growth in these beds is often light-limited and lower light availability by nutrient-stimulated growth of phytoplankton and algal bloom. It brings marked changes in the associated animal life. Coral reefs, one of the most diverse ecosystems in the world and sensitive to nutrient pollution, are found in naturally nutrient-poor surface waters in the tropics and subtropics; however, a high nutrient level is generally detrimental to reef health and lead to shifts away from corals towards algal turfs or seaweeds that overgrow or cover the reefs (Bomans et al. 2005).

7.3.3 Heavy Metal Pollution

Phosphorus is a major limiting factor to crop productivity on many types of soils, including acidic and infertile soils. Therefore, they require applications of fertilisers for better productivity in the form of organic and inorganic P fertilisers. During the production process of inorganic P fertilisers from phosphate rock (PR), varying amounts of heavy metals (minor constituents in the PR ores) are transferred to P fertilisers. These heavy metals may accumulate in the soil with repeated fertiliser applications. The main organic fertilisers, animal manure and sewage sludge (biosolids) are also applied for better crop production and may also contain heavy metal contaminants. Their repeated application will cause pollution of constituent heavy metals in agricultural soil and may also be hazardous for human health. Several heavy metals that are present as contaminants include cadmium (Cd), arsenic (As), chromium (Cr), lead (Pb), mercury (Hg), nickel (Ni) and vanadium (V). However, the metal that is of the most concern is Cd because of its maximum bioavailability on acid soils, while the rest are not as readily absorbed by plants on P-fertilised soils (Mortvedt and Beaton 1995; Bomans et al. 2005).

7.4 Phytases: An Introduction to Their Use in Phosporous Pollution Management

Considering the aforementioned consequences of P pollution and eutrophication, several alternative approaches have been suggested for reduction of phytic acid levels in animal feed and are categorised as chemical methods (e.g. extraction and precipitation), feed processing and enzymatic method (phytase application). Chemical methods affect the nutritional quality of the products and are generally expensive (Pandey et al. 2001); however, phytase application for P management is considered as the most promising alternative. Numerous animal trials have shown that adding phytase to feed at 500–1000 phytase units/kg may replace inorganic P supplements for pigs and poultry and reduce their P excretion by approximately 50% (Lei et al. 1993; Augspurger et al. 2003).

Phytases are acid phosphohydrolases that catalyse the hydrolysis of phosphate from phytic acid to inorganic phosphate and *myo*-inositol phosphate derivatives (Roopesh et al. 2006). Phytases can be classified into three classes depending on the position of the first dephosphorylation of phytate, namely, 3-phytases, 4/6-phytases and 5-phytases. Within each class, there are not only structural differences but also different mechanisms for the hydrolysis of phytic acid. The 3-phytase (*myo*inositol hexakisphosphate-3-phosphohydrolase, E.C.3.1.38) removes the phosphate from the 3-position of phytate and is found typically in microorganisms. The 4/6-phytase (*myo*-inositol-hexakisphosphate-4/6-phos-

Table 7.2 Different microbial sources with reported phytase production

Source organism	Reference
Fungi	
Aspergillus terreus	Mitchell et al. (1997)
Aspergillus carneus	Ghareib (1989)
Aspergillus oryzae	Shimizu (1993)
Aspergillus niger	Vats and Banerjee (2005)
Aspergillus fumigatus	Mullaney et al. (2000)
Aspergillus ficuum	Ullah and Gibson (1987)
Aspergillus heteromorphus	Lata et al. (2013)
Rhizopus oligosporus	Casey and Walsh (2004)
Rhizopus oryzae	Ramachandran et al. (2005)
Myceliophthora thermophila	Mitchell et al. (1997)
Penicillium simplicissimum	Tseng et al. (2000)
Mucor racemosus	Bogar et al. (2003)
Sporotrichum thermophile	Singh and Satyanarayana (2008)
Mucor hiemalis	Boyce et al. (2007)
Rhizomucor pusillus	Chadha et al. (2004)
Yeast	
Saccharomyces cerevisiae	Haraldsson et al. (2005)
Pichia anomala	Vohra and Satyanarayana (2001)
Pichia spartinae, Pichia rhodanensis	Nakamura et al. (2000)
Hanseniaspora guilliermondii	Hellstrom et al. (2010)
Pichia stipitis, Candida tropicalis	Jeffries et al. (2007)
Debaryomyces castellii	Ragon et al. (2008)
Kodamaea ohmeri	Li et al. (2009)
Hansenula fabianii	Watanabe et al. (2008)
Bacteria	
Lactobacillus sanfranciscensis	Angelis et al. (2003)
Lactobacillus amylovorus, Lactobacillus rhamnosus	Raghavendra and Halami (2009)
Bacillus subtilis	Powar and Jagannathan (1982)
Bacillus amyloliquefaciens	Idriss et al. (2002)
Bacillus licheniformis	Kumar et al. (2014)
Bacillus sp.	Kumar et al. (2013)
Advenella sp.	Singh et al. (2014)
Escherichia coli	Greiner et al. (1993)
Serratia sp.	Zhang et al. (2011)
Actinomycetes sp.	Ghobarbani-Nasrabadi et al. (2012)

phohydrolase; E.C.3.1.3.26) hydrolyses the phosphate ester at the L-6 (or D-4) position of phytic acid and is generally present in seeds of higher plants. The 5-phytase (*myo*-inositolhexakisphosphate 5-phosphohydrolase, E.C.3.1.3.72) was identified by Barrientos et al. (1994) in pollen from the lily flower. The initial hydrolysis of the phosphate ester occurs at the D-5 position of phytic acid. Phytase was first discovered by Suzuki et al. (1907) in the course of rice bran hydrolysing studies. The enzyme was identified in rice bran and reported to catalyse the hydrolysis of phytic acid to inositol and orthophosphoric acid. These resultant products can be found in plants, certain animal tissues and microorganisms like fungi, bacteria and yeast. The phytase activity of microorganisms has been comprehensively studied (Table 7.2). Shieh and Ware (1968) screened more than 2000 cultures of microorganisms isolated from 68 soil samples and identified *Aspergillus niger* as the most active group producing phytases. In 1982, Powar and Jagan-

nathan showed that an enzyme that hydrolysed only phytate was present in culture filtrates of *Bacillus subtilis* (Powar and Jagannathan 1982). Nayini and Markakis (1984) first reported the extraction of phytase from baker's yeast, *Saccharomyces cerevisiae,* and performed characterisation and purification studies. The first commercial phytase was prepared by fermentation of a genetically modified *A. niger* strain in 1991 by Gist-Brocades and marketed by BASF in Europe under the brand name Natuphos™ (Haefner et al. 2005). Ever since, the commercial application and the research on phytase developed a symbiotic relationship and became an increasingly important area of interest. To date, only a handful of commercial phytase products are available (Haefner et al. 2005).

7.4.1 Sources of Phytase

Phytases occur widely among plants, animals and microorganisms. Microbial sources of phytase are widespread and can be found in soils, aquatic systems and animals. In the last 15 years, research has indicated that several strains of bacteria, yeast and fungi can produce high yields of phytase with application at the industrial scale. With this objective in mind, scientists started to purify and express phytase in a wide range of hosts using various biochemical methods. Depending on the source and/or expression host, phytases can present different biophysical and biochemical properties (Rao et al. 2009).

1. **Fungal phytases**

One of the first systematic studies on fungal phytase was reported by Shieh and Ware (1968), where various microorganisms where tested for extracellular phytase production and a strain of *A. niger* known as *Aspergillus ficuum* NRRL 3135 was identified as the most efficient. This strain exhibited highest phytase activity without sporulation which is a prerequisite in large scale production. Wodzinski and Ullah (1996) reviewed production and activity of *A. ficuum* NRRL 3135 and observed that the selected strain produces more phytase activity in liquid culture than any other naturally occurring organism. Numerous studies have documented on other phytase producing fungi, however, they yielded lower phytase activity. The genus *Aspergillus* (*A. niger* in particular) continues to be preferred for production of phytase, other enzymes and organic acids. The reason behind this preference is generally recognised as safe (GRAS) status, its great secretory potential and the in-depth knowledge with respect to growth cultivation (Shivanna and Govindarajulu 2009). Two pH optima, at 2.5 and 5.0–5.5, can be observed for the *A. niger* NRRL 3135 phytase, phy-A (Wodzinski and Ullah 1996; Dvoráková 1998). Only one pH optimum has been noted for the pH 2.5 optimum acid phosphatase, which has been referred to as phy-B phytase (Ehrlich et al. 1993).

Phytases from *Aspergillus* species usually exhibit optimum temperature between 50 and 65 °C (Vats and Banerjee 2004). *A. niger* phytase (EC 3.1.3.8) has been well characterised by Ullah and Gibson (1987) and reported as an extracellular glycoprotein with the mass of 85 kDa. *A. niger* phy-B phytase has also received attention from enzymologists and protein chemists because of its high catalytic activity and enhanced thermal stability (Ullah et al. 2008). However, restrictive and narrow pH optima limit its use in animal feed industries or enzyme producers (Ullah et al. 2008). The production of phytase from this fungus has been achieved by three different cultivation methods, i.e. solid state (Ebune et al. 1995), semisolid (Han et al. 1987) and submerged fermentation (Howson and Davis 1983; Vats and Banerjee 2004). Due to acid tolerance and high yield (Kim et al. 1998) fungal phytases are widely used as an animal feed additive in comparison with bacterial phytases (Soni and Khire 2007).

2. **Yeast phytases**

Yeasts are ideal candidates for phytase and phosphatase research due to their mostly nonpathogenic and GRAS status; however, they have not been utilised to their full potential (Satyanarayana and Kunze 2009). To date, only a few studies have been published on yeast phytase, such as

S. cerevisiae (Howson and Davis 1983; Greiner et al. 2001), *Saccharomyces castellii* (Segueilha et al. 1993) and *Arxula adeninivorans* (Sano et al. 1999). Nakamura et al. (2000), identified among numerous yeast species that *Pichia spartinae* and *Pichia rhodanensis* exhibited the highest levels of extracellular phytase with optimal temperatures at 75–80 °C and 70–75 °C and optimum pH at 3.6–5.5 and 4.5–5.0, respectively. The presence of intracellular phytase was also verified in *S. cerevisiae*. In a recent work, Olstorpe et al. (2009) developed a reliable, fast and easy-to-use screening method that clarifies the ability of different yeast strains to utilise phytic acid as the sole phosphorous source. After measuring the specific phytase they established that *A. adeninivorans* displayed the highest intra- and extracellular specific activities and that the extracellular phytase activity detected in *Pichia anomala* was strain-specific. The authors also concluded that there were large differences in both extra- and intracellular phytase activities amongst the screened species. Recently yeasts present in the gut of aquatic species have also been studied for phytase activity. Hirimuthugoda et al. (2007), isolated and identified two phytase-producing strains, *Yarrowia lipolitica* and *Candida tropicalis* in the intestine of sea cucumber. These strains produced high amounts of extracellular and cell-bound phytase. Li et al. (2008) isolated a marine yeast strain *Kodamea ohmeri* BG3 in the gut of a marine fish that produced phytase and showed its highest activity at pH 5.0 and temperature 65 °C. Yeasts have been reported to be a rich genetic resource for heat-resistant phytase; however, the possibility of applying these phytases in the industry has not been extensively investigated (Kaur and Satyanarayana 2009).

3. Bacterial phytases

Phytases have been detected in several types of bacteria, such as *bacilli*, *enterobacteria*, anaerobic ruminal bacteria and *Pseudomonas* (Jorquera et al. 2008; Kumar et al. 2013). Although it was only after 1980s that several bacterial strains (wild or genetically modified) such as *Lactobacillus amylovorus*, *Escherichia coli*, *B. subtilis*, *Bacillus amyloliquefaciens* and *Klebsiella sp*., have been applied for phytase synthesis (Pandey et al. 2001). Gram-negative bacteria are known to produce phytase intracellularly, while Gram-positive bacteria and fungi produce it extracellularly (Greiner et al. 1993). An enzyme which liberated phosphate from phytic acid was shown to be present in culture filtrates of *B. subtilis*. This enzyme differed from other previously known phytases in its metal requirement and in its specificity for phytate. It required Ca^{2+} specifically for its activity (Powar and Jagannathan 1982). Greiner et al. (1993), purified two periplasmatic phytases, named P1 and P2 from *E. coli*. The P2 enzyme was characterised as a 6-phytase based on its hydrolysis of phytate. Sreeramulu et al. (1996), identified that *L. amylovorus* could have the potential to improve the nutritional qualities of cereal and pulse-based food fermentations. After the screen of a range of strains of lactic acid-producing bacteria, for the synthesis of extracellular phytase, they verified that *L. amylovorus* B4552 under submerged cultivation conditions was the highest producer. The strain *Bacillus sp*. DS11A was isolated by Kim et al. (1998), as a producer of a thermostable phytase (DS11 phytase), which could improve the value of some grains, rice flour in particular. In their work, Sajidan et al. (2004) showed that a *Klebsiella sp.* strain ASR1 hydrolysed phytate. A recombinant version of this enzyme was identified as a 3-phytase and was different from other general phosphatases and phytases. These researchers proposed the *phyK* gene product as an interesting candidate for industrial and agricultural applications. In general, the phytases from bacteria have a pH optimum between neutral and alkaline (Vats and Banerjee 2004) and have temperature optima from 40 up to 70 °C (Kim et al. 1998; Cho et al. 2003). According to Igbasan et al. (2000) within bacterial phytases, an enzyme with high thermal stability (*Bacillus* phytase) or high proteolytic stability (*E. coli* phytase**)** does exist. The future of bacterial phytases will depend on them being developed for their favourable properties as feed additives.

4. **Plant phytases**

Many plant seeds contain significant amounts of phytic acid that is degraded during germination by one or more phytases. Seeds contain both constitutive phytase activity and phytases that are synthesised again during germination; however, this last mechanism is not well understood. The activity of phytase has been well reported from *Arabidopsis thaliana* AtPAP15 (Li et al. 2012), *Glycine max* GmPhy (Hegeman and Grabau 2001), and *Medicago truncatula* MtPHY1 (Xiao et al. 2005). The optimum temperature and pH measured for most plant phytases ranges from 45 to 60 °C and from 4.0 to 7.2 pH, respectively. Alkaline phytases with unique catalytic properties have been identified in plants. Garchow et al. (2006) purified alkaline phytase from pollen grains of *Lilium longiflorum*. These investigators suggested that the unique properties of this alkaline phytase attributed to it the potential to be useful as a feed and food supplement.

5. **Animal phytases**

The existence of the first animal phytase was demonstrated in the blood and liver of calves in 1908 by McCollum and Hart. Since then, controversy has persisted regarding the existence of phytases in animals in the digestive tract of animals (especially monogastric animals). According to Rapoport et al. (1941), other investigators failed to find phytase in the extracts of intestine, pancreas, kidney, bone, liver and blood of several species of animals. Preliminary work on the activity of phytase produced by rumen microorganisms was initiated by Raun et al. (1956) and undertaken again by Yanke et al. (1998). They further examined the presence of phytase activity in species of obligatory anaerobic ruminal bacteria and concluded that the most highly active strain was *Selenomonas ruminantium*. With the objective of outlining the complete system of phytate degradation in the gut of humans and the enzymes involved, Schlemmer et al. (2001), carried out a study using pigs as model for humans and concluded that negligent amounts of endogenous phytase activity were found in stomach chyme and small intestine, though in the colon the phytate hydrolysis was of an endogenous origin. Intestinal bacteria with endogenous phytase activity were discovered in several species of fish. Huang et al. (2009) screened the intestinal contents of grass carp and found the phytate-degrading isolates, *Pseudomonas*, *Bacillus* and *Shewanella* species.

7.4.2 Consideration in Use of Phytases in Animal Feeds

Over the past 20 years, animal producers raised the issues and interventions demanding a more efficient, economical and environmentally friendly approach to the industry. Phytase supplementation to animal feed is an effective way of increasing the availability of P to animals, thus improving their performance and reducing manure-borne P pollution. In addition to its major application in animal nutrition, phytase is also used for processing of human food. Wodzinski and Ullah (1996), recognised that the addition of phytase to the diet of every monogastric animal reared in the USA would not only diminish the P released into the environment by 8.23×10^7 kg, but also would save the animal producers $\$ 1.68 \times 10^8$/year in its supplementation. Since then, the use of phytase as a feed additive has become widely accepted and several commercial phytase preparations (e.g. Natuphos™, Ronozyme P, Phyzyme XP) are used in Europe and the USA (Selle and Ravindran 2007). From their commercialisation in the early 1990s, the sales value for phytase was estimated at $ 50 million within the decade (Sheppy 2001), where today it represents more than half of all feed enzyme sales (< $ 250 million) (Wyatt et al. 2008).

Microbial phytase is one of the most commonly used enzymes in monogastric animal diets (Shim et al. 2004). Paik (2003) conducted a series of experiments in broilers and layers to evaluate the effects of microbial phytase on several minerals such as N, P, Cu, Zn and K and showed that the dietary treatment could reduce P excretion enor-

mously. Further, based on such studies, the use of selected brands of wheat bran as a source of phytase in broiler feeding has been recommended. Supplementation of phytase in low nonphytate P diets improved growth performance, relative retention of nutrients and minerals in blood and bone of broilers (Singh et al. 2003). Selle et al. (2003) demonstrated the feasibility of reducing protein, amino acids, energy and P levels in broiler diets with an appropriate level of phytase supplementation to formulate least-cost rations. The contents of crude ash, Ca, P, Mg and Zn were adversely affected by lowering nonphytate P levels in the diet of broiler chickens, but partially recovered by enzyme supplementation from *A. ficcum* (Paik et al. 2000). Further, supplementation of diets for growing–finishing pigs had improved growth performance and nutrient availability with 33% reduction in manure P (Hong et al. 2001; Abioye et al. 2010). In addition to this, Forsberg et al. (2013) reported the development of transgenic pigs producing phytase in salivary glands to reduce the impact of P on the environment and pigs' faecal P. In another study, Rosu et al. (2012), reported that phytase supplementation of combined fodder recipes (NC) composed of corn/soybean for laying hens do not require the addition of fodder phosphates in food. Excess of P supplements excreted over nutritional needs of laying hens at the national level is 313.43 t per year which is equal to 68.95 t monocalcic phosphate which is unjustified wasted.

Different studies have used different phytase doses in actuality (500–12,500 U phytase/kg) and reported a reduction in P excretion. These studies concluded that the maximum effective concentration of phytase yet remains unknown. These studies should be considered while planning phytase addition for management of P excretion and its pollution management. According to Golovan et al. (2001), addition of 250–1000 U phytase/kg diet can fully replace P supplementation in poultry feed. Also, its supplementation significantly increased serum concentrations of Ca, P, Mg, Zn, Fe and Cu. Harper et al. (1997) and Oryschak et al. (2002) observed a 27–28% reduction in P excretion when phytase was supplemented to the diets of growing–finishing pigs while Lei et al. (1993) reported a larger reduction of 35–45% for weanling pigs. However, Angel et al. (2005) found no statistical difference in total P excretion in pigs that were fed diets supplemented with 515 U phytase/kg accompanied by 0.1% unit reduction in available P. Further reduction of 0.2% units in available P at the same level of phytase inclusion reduced poultry litter P concentration (Angel et al. 2005). Thus, there is a need to investigate the effect of further reduction in dietary available P on the forms of P in the manure. Supplementation of diet with higher levels of phytase has been considered as a good replacement for inorganic P addition in pig diets. Rosen (2002) reported that microbial phytase at 2500 U phytase/kg of low P diet could triple the improvement in feed efficiency of broiler chicks compared to the industry level of 634 U phytase/kg of diet. Veum et al. (2006) supplemented low P diet fed to growing pigs by up to 12,500 U phytase/kg and observed improved apparent absorption of P, Ca and Mg. According to a study by Aiboye et al. (2010), phytase enzyme can wholly substitute for inorganic P in pig diet when added at high levels (2000 U/kg).

Phytase has also been used in combination with other enzymes to improve growth performance and nutrient digestibility in pigs fed corn/soybean meal based diets (Shim et al. 2004). Addition of phytase in isolation or in combination with xylanase replacing 0.08% dietary inorganic P increased body weight and feed utilisation efficiency of broilers fed with wheat-based diets and decreased overall mortality (Peng et al. 2003). Simultaneous addition of phytase and carbohydrases improved feed efficiency ratio, nutrient digestibility and nutritional value of soybean meal, rape seed meal and cotton seed meal by improving amino acid digestibilities in growing pigs (Shim et al. 2003). Phytase and xylanase were reported to have a synergistic effect for enhancing amino acid digestibility in broilers, which was attributed to their complementary modes of action (Selle et al. 2003). However, combination of phytase and glucanase had no positive effects on laying performance of Leghorn hens and excretion of nitrogen and P (Jacob et al. 2000).

7.5 Current Challenges and Future Prospects

The growth of the market for P to supplement animal feed has been critical for the commercial development of phytase enzyme. At present, phytase constitutes about 20% of the total enzyme use in the livestock or allied sector, which is expected to increase many fold in the future due to increasing productive research leads. Recent trends in the market have clearly shown phytase as an important enzyme and feed supplement. Due to serious concerns about environmental pollution, 22 countries have adopted the use of phyA, produced from *A. niger* NRRL 3135, as a feed additive. Industrial production of phytase currently utilises the soil fungus *Aspergillus*, on which considerable research has been conducted. Phytases are being recognised for their beneficial environmental role in reducing the P levels in manure and minimising the need to supplement P in diets. Increasing the use of phytase in aquaculture offers a tremendous opportunity in order to allow the use of low cost plant meals. Further, continued research on lowering the production cost and expanding its utilisation to other applications also suggests its importance in the immediate future. With so many beneficial effects reported so far, the actual usefulness of such enzymes are limited due to high variation in activity of phytase production, lack of farmer awareness regarding their uses, cost factor, availability, nonexistence of a single phytase with applicability in all kind of feeds or applications, storage stability, narrow pH and substrate specificity, dose response variation, high processing temperature susceptibility and low enzyme production. Phytase with broad substrate specificity is better suited for animal nutrition purposes as it will readily liberate all equatorial phosphate groups of phytic acid. Several studies are being carried out for construction of commercially viable phytase through enzyme engineering for high specific activity and broad substrate specificity along with thermostability. Similarly, phytases of broad pH optima, with suitable effectiveness for all types of fish, should be developed. Moreover, as stated above, there is a lack of consensus about the optimum dose of phytase addition to animal feed. A lot of research data are required to decide the optimum dose of dietary phytase for different species. Considering the eutrophication of aquatic system with excess P, development and use of a suitable phytase (preferably a beta propeller phytase with high activity at neutral pH and 37 °C temperature) in herbivorous fish diet is very encouraging. Intensification of livestock or aquaculture farming without considering the P discharge may threaten the environment in long run. Therefore, development of novel ideal phytase with required industrial properties is desirable.

In addition to development of commercially viable ideal phytase, some recent studies also focus on expression of microbial phytase into animal (e.g. pig) and plant roots (e.g. soybean). It is therefore considered that development of transgenic monogastric animals which are able to produce phytase and hydrolyse phytate would be of immense benefit to livestock and fish farmers. Production of canola seeds with improved phytase activity has paved the way for future research to develop transgenic soybean, cotton, sunflower and other grain and cereal plants, which have potential uses in fish feed. These plants with improved phytase production from roots might have better P utilisation efficiency. Development of genetically modified grains and cereals with reduced phytic acid content can also serve the purpose.

It is also important here to mention about possible roles of microbes present in soil in P management. Among various macronutrients for plants, the poorly available insoluble inorganic and organic forms of soil P to plants is reported to be converted to a plant accessible form by P-solubilising bacteria (PSB) in the rhizosphere mainly by means of organic acid production. In this regard, a few studies on addition of phytase or phytase-producing microbes to the soil also revealed increased P uptake by plants. Further studies with specified target for maintaining soil P level and decreasing fertiliser application might be highly promising considering environmental P pollution and increasing cost of fertilisers. In this context, bacteria with activities like production of organic acids to solubilise inorganic P and

production of phytase to mineralise phytate, will have potential to be used in soil with high content of organic P.

Considering the facts and problems, an integrated approach from a combined agricultural and environmental perspective is recommended in order to achieve better P management with reduced environmental pollution. The following four recommendations would contribute to a more equilibrated use of the available P resources. *First*, consistent and regular quality analysis of soils and fertilisers for optimum P concentration is required for determining actual P concentration and the dose of fertiliser to be applied. Possible changes in management practice such as better soil conservation; better precision in applications of fertiliser and extensification of agricultural systems such as livestock reductions may be useful in tackling the problem. *Second*, because many farmers are not familiar with the use of manure products, its proper use, disposal and related P content, the methodology and advantages in the use of phytases as a new approach should be made known to them through awareness campaigns. *Third*, cost monitoring of phytase-supplemented animal feed is also required for its maximum utilisation and reach to farmers. *Fourth*, development of biofertilisers that aid in better P solubility and its availability to plants will certainly be useful in the long term control of P pollution.

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Sustainable Production of Biofuels from Microalgae Using a Biorefinary Approach

8

Bhaskar Singh, Abhishek Guldhe, Poonam Singh, Anupama Singh, Ismail Rawat and Faizal Bux

Abstract

Biorefinery has emerged as a new concept to derive more than one utility product from biomass. The products from biorefinery include one or more biofuels (biodiesel, bioethanol, biomethane, and biohydrogen) along with other energy sources (syngas and bio-oil), pharmaceutical products, and commercially important chemicals. Biorefineries, thus could simultaneously produce biofuels, bio-based chemicals, heat, and power. The biomass production and its utilization as biofuel has a higher water footprint (WF) than fossil derived fuel. The biorefinery approach has the potential to bring down the WF. Similarly, biorefinery approach has the potential to bring down the carbon footprint. The value added product derived from biorefinery basket includes pigments, nutraceuticals, and bioactive compounds. The use of industrial refusals for biomass production includes wastewater as nutrient medium and utilization of flue gases (CO_2) as the carbon source for culture of microalgae. These processes have the potential to reduce fresh WF and carbon footprint.

Keywords

Biorefinery · Biofuel · Biomass · Carbon footprint · Water footprint

8.1 Introduction

A biofuel is one that contains energy from geologically recent fixed carbon. The source of biofuel is biogenic viz., derived from plant or microalgae. As the carbon content in biofuel is recent, it belongs to the category of renewable resource. The first generation biofuels are those that are produced from the raw materials in completion with food and feed. These included cereals, sugar cane, and oil seeds mostly from edible oil. Later, the usage of fuel crops instead of agricultural crops was debated that led to exploration of the

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second generation feedstock for biofuel. The second generation feedstock included nonfood biomass of lignocellulosic materials viz., bagasse, stover from sugar, forest and crop residues, municipal solid wastes, vegetative grass, and short rotation forest crops. The second generation feedstock provided oil from the plant seeds and the lignocellulosic material present in the residue of the seeds after oil extraction was used for the synthesis of bioethanol. Microalgae have been considered as a third generation feedstock for biofuels as they grow in water and thus do not compete with the land based food crops. Biohydrogen and bioelectricity has recently been explored as a fourth generation feedstock (Sharma et al. 2011). At present, the common biofuels produced in the world today are biodiesel, bioethanol, and biogas (comprising methane) (Cherubini and Ulgiati 2010). International Energy Agency (IEA) estimates that biofuels will fulfill 27% of global energy demand in the transport sector in 2050 due to its growing interest and popularity (Fornell et al. 2013).

To mitigate the climate change and to enhance the energy security along with maintaining sustainability has led to exploration of biorefinery concept (Cherubini and Ulgiati 2010). A biorefinery approach integrates multifunctional process, as various material products of utility and energy are coproduced simultaneously (Cherubini et al. 2011). As per the definition of IEA Bioenergy Task 42 "*Biorefining is the sustainable processing of biomass into a spectrum of marketable products and energy*." A wide range of technologies separate biomass (viz., wood, grasses, corn) from useful products (protein, carbohydrate, and lipids) which, depending on their suitability, can thereafter be converted to value added products, biofuels, and chemicals. The biobased products that are already in the market include starch, oil, cellulose, and chemicals (lactic acid, amino acids). A variety of other compounds that are also derived from biomass include adhesives, cleaning compounds, detergents, dielectric fluids, dyes, hydraulic fluids, inks, lubricants, packaging materials, paints and coatings, paper and box board, plastic fillers, polymers, solvents, and sorbents. However, the biofuels and biochemicals are usually produced independently as a single chain product that results in their competition with the food and feed industry. A biorefinery based on lignocellulosic feedstock can produce large biomass as the whole crop is available as compared to only a part with the conventional crops (Cherubini 2010). In a biorefinery, the consumption of nonrenewable energy is minimized and the complete and efficient use of biomass gets maximized (Cherubini 2010). The advantages of biofuels over the conventional fossil fuels are: renewability, CO_2 sequestration, environmental friendly and biodegradability, and sustainability. According to the National Renewable Energy Laboratory, a biorefinery is defined as "*a facility that integrates conversion processes and equipment to produce fuels, power, and chemicals from biomass*." The integrated biorefinery approach could produce fuels as well as platform chemicals, and thus could complement the petroleum industry and refineries.

Cherubini (2010) stated that bio-industries can combine their material flows for a complete utilization of all biomass components. This residue from one bioindustry can be utilized as an input for the other bio-industry. As an example, lignin from a lignocellulosic ethanol production plant becomes an input for other industries, giving rise to integrated bio-industrial systems. As biomass resources are locally available, their use may contribute to reduce dependence on fossil fuels. In a biorefinery approach, a continuous supply of feedstock is maintained as the feedstock comes from various sources of crops viz., agriculture, forestry, and industrial activities. The biomass feedstock is grouped in the following category: carbohydrates and lignin, triglycerides, and mixed organic residues (Cherubini 2010).

As microalgae comprises a variety of constituents (lipids, proteins, and carbohydrates), these substrates has the potential to serve products for different markets (Koopmans et al. 2013). Among the various constituents, lipids and proteins are the largest fraction present in the microalgae. While, lipids can be utilized for the production of biofuel, the proteins may be purified and utilized as food, feed, health, and bulk chemical market. The carbohydrates (starch and

glycogen present in cytoplasm; and cellulose in the inner cell wall) of microalgae can be utilized in the production of ethanol and chemicals. The cultivation of microalgae only for production of biofuel is cost intensive. The cost incurred during cultivation is due to colossal water requirement. Also, the downstream processing requires substantial production cost. Thus, various products can be derived from a single source of feedstock (Koopmans et al. 2013). The carbohydrate from microalgae could be utilized for the production of ethanol and chemicals. However, the techniques that could be applied for a biorefinery will vary according to the species selected. Some of the microalgae species do not have a cell wall which makes the cell disruption technique less energy intensive. The microalgal cell usually consists of lysosomes, mitochondria, and endoplasmic reticulum. It is more practical to disrupt the cells to release lipids, proteins, and carbohydrates from the cytoplasm and later fractionates the larger cell compartments (organelles) to obtain specific compounds (Koopmans et al. 2013).

Though there exists a vast reserve of oil amounting 90 billion (bn) barrels in Arctic region, environmental issues will be a major constraint for their accessibility. Nevertheless, the fossil fuel reserves are finite and nonrenewable. National Renewable Energy Laboratory defines "biorefinery" as a facility that can integrate biomass conversion process and equipment for the production of a variety of utility products from biomass viz., fuels, power, and chemicals (Charlton et al. 2009). Charlton et al. 2009 reports that in order to make a biorefinery sustainable, the whole plant along with its fibrous fraction should be utilized as feedstock. In the first generation process, microbes are used to transform readily fermentable sugars and starch. In the second generation process, the "locked-up" sugar and other molecules in the lignocellulosic fraction are also utilized. The novel technologies include steam, acid, alkali, and enzymatic processes to produce fermentable sugars which can then further be utilized as substrate for production of various chemicals. The integrated approach in a biorefinery, thus, could provide a range of useful end products viz., biofuels (e.g., biodiesel, bioethanol), other energy sources (syngas and bio-oil), pharmaceutical products (e.g., cancer drugs), and commercially important platform chemicals (organic acids) (Charlton et al. 2009). Among the feedstocks, grass can be utilized as a low cost material to obtain high value products. Hence, the biorefinery extracts the maximum value from the biomass by the production of a variety of valuable products and make the overall process sustainable as well as economically viable. Combined production of biofuel and generation of heat makes biorefinery an attractive operation. The biorefinery market utilizing the entire biomass is estimated to reach US$ 295 billion by 2020 (Hernandez et al. 2013). The products from the biorefinery has the potential to replace compounds that are chemically identical (ethylene from bioethanol could replace ethylene obtained from natural gas) and those with similar functionality (Hernandez et al. 2013). In biorefineries, high value products (biofuels, specialty chemicals, pharmaceuticals) could be derived. An integrated processes viz., digestion, fermentation, pyrolysis, gasification, results in enhancement of energy efficiency and material recovery (Ng 2010).

8.2 A Biorefinery Approach for Production of Biofuels

The term biofuel is referred to soild, liquid, or gaseous fuel that is obtained from biorenewable feedstocks. The biorefinery concept is applied to biomass in a similar way as that is adopted in refining of petroleum where a variety of products are obtained. Hence, biorefineries simultaneously produce biofuels, bio-based chemicals, heat, and power.

8.2.1 Biodiesel

Biodiesel that is derived from the oil from crops, waste cooking oil, or animal fats is unable to fulfill the demand for the transport fuel as it is required in bulk amount. It is envisaged that microalgae has the potential to fulfill the demand of

feedstock for biodiesel (Chisti 2007). In addition to lipids and oils, microalgae also contain proteins, carbohydrates, and other nutrients. Hence, after extraction of the oil from microalgae, the residual biomass can be used for other utilities viz., animal feed. A part of the residual biomass can also be utilized for the production of methane by anaerobic digestion. The energy generated from the methane then could further be used in biodiesel production facility which could lower the overall production cost of biodiesel (Chisti 2007). Ekman and Börjesson (2011) reported production of propionic acid by fermentation of glycerol that was obtained as a by-product from biodiesel production. A simultaneous production of biodiesel and bioethanol has been suggested by Gutierrez et al. 2009. The palm shell can be utilized for the production of bioethanol; whereas, oil from palm seed can be used for production of biodiesel.

8.2.2 Biomethane

Charlton et al. 2009 proposed a green biorefinary approach utilizing grass as a biomass. The advantages with grass as feedstock are its high digestibility, high sugar, and low lignin. Grass comprises cellular material (45 %) and cell wall components (55 %). The grass juice has been proposed to be separated from the lignocellulosic portion of the grass. Once the liquid from the grass gets separated, the transport of the fiber will be easier. The sugar rice juice has been proposed to be fermented on the farm for production of methane by anaerobic digestion, or for bulk chemical such as lactic acid. The fiber could be converted to fermentable sugar using specific enzymes or be used as animal feed owing to its protein content.

8.2.3 Biohydrogen

Ferreira et al. (2013) reported production of biohydrogen from the dark fermentation of leftover biomass of microalga, *Nannochloropsis sp.* In the biorefinery approach, biodiesel was produced as the main product from the oil of *Nannochloropsis sp.*; whereas, pigments and biohydrogen were produced as the coproduct. Nobre et al. (2013) proposed a biorefinery approach for the feedstock, *Nannochloropsis sp.* wherein, the oil and pigment was extracted from the microalgae. While, oil was utilized for the synthesis of biodiesel, the left over biomass was utilized for the production of high value compounds (carotenoids) and biohydrogen. High lipid content 33 % (w/w) of dried biomass was reported from the microalgae using supercritical CO_2 extraction under operating conditions of 40 °C, 300 bar, and CO_2 flow rate of 0.62 g/min. The lipid content was enhanced to 45 % (w/w) of dried biomass with 70 % recovery of pigments when 20 % (w/w) ethanol was doped along with supercritical CO_2. Biohydrogen was produced by dark fermentation using *Enterobacter aerogenes* with H_2 yield of 60.6 mL/g dry biomass.

8.2.4 Bioethanol

Fornell et al. (2013) reported simultaneous production of bioethanol and dimethyl ether from kraft pulp-mill-based biomass as a biorefinery approach. It is reported that cost of CO_2 capture and storage by adopting this method will be low. Goh and Lee (2010) reported that carbohydrate derived from seaweed that contains hexose sugar could be fermented to produce bioethanol. Lignocellulosic feedstock is used for the production of ethanol and dimethyl ester (DME). The conversion efficiency of the raw material to ethanol of fuel grade is in the range of 30–50 % as estimated by taking in account the lower heating value. The low conversion efficiency is attributed to heat loss and the side reaction that occurs at various processing steps (Fornell et al. 2013). Rosenberg et al. (2011) proposed growing microalgae in combination with an ethanol biorefinery. The excess heat from the ethanol biorefinery could be used to maintain the algal culture at constant temperature in winter seasons. Sequestration of CO_2 released from ethanol biorefinery by microalgae will lead to reduction in the operating cost by up to 20 %. Jung et al. (2013) reported that the carbohydrate content in the macroalgae (seaweed)

could be diverted to utilization in a biorefinery. As macroalgae contain low amount of lignin, they can be a better substrate in the production of various utility products in a biorefinery. Unlike microalgae, the macroalgae has a low protein content (7–15 % dry wt.) and lipid content (1–5 % dry wt.). The microalgae possess a comparatively high content of protein and lipid of 40–60 % dry wt. and 10–20 % dry wt. respectively. This is due to high water and alkali metal content of 70–90 % fresh wt. and 10–50 % dry wt. respectively in macroalgae (Jung et al. 2013). Goh and Lee (2010) utilized a macroalgae (seaweed) for the production of bioethanol. The carbohydrate content in the seaweed present as hexose sugars was utilized for the production of bioethanol.

Luo et al. (2011) proposed complete utilization of rapeseed plant (seed and straw) for the production of biofuels (biodiesel and bioethanol) as a biorefinery concept. Using straw as the feedstock, a bioethanol yield of 0.15 g ethanol/g dry straw was obtained with pretreatment with alkaline peroxide and stream. The coproducts and by-products obtained as rapeseed cake, glycerol, hydrolysate, and stillage were utilized for production of methane and mixture of hydrogen and methane. The energy recovery process that was only 20 % with the production of conventional biodiesel increased to 60 % by adopting the biorefinery approach that produced bioethanol, biohydrogen, and methane along with biodiesel. Lohrasbi et al. (2010) described the economic feasibility of a biorefinery from citrus waste. On hydrolysis by dilute sulfuric acid, the citrus waste can be converted to limonene, ethanol, and biogas. The total cost estimated for the production of ethanol was 0.91 USD/L with the production capacity of 100,000 tons/year inclusive of the transportation and handling cost. The production of limestone and biogas (methane) along with enhancement of plant capacity could reduce the production cost of ethanol to 0.46 USD/L which makes the process economical and sustainable.

The lignin present in the lignocellulosic biomass is not easily degraded, hence needs expensive pretreatment. Plant genetic engineering technology can lead to lower the cost of production of biofuel from lignocellulosic materials. Recent advancements in research have led to new opportunities in manipulation of lignin for development of biofuel. The cell degrading enzymes that include cellulases and hemicellulases could be produced in the crop biomass itself (Menon and Rao 2012).

8.3 Environmental Impact of Microalgal Biorefinery

8.3.1 Water Footprint (WF)

The sustainability of a biofuel can be measured in terms of its ecological footprint. Water, as a scarce commodity, can be a limiting factor for cultivation of energy crops. WF measures the water use intensity of a nation. It is estimated that the water requirement for the production of primary energy from biomass is two to threefold greater than that required from the fossil fuel (Tan et al. 2009). WF is an indicator of direct as well as indirect usage of freshwater. Gerbens-Leenes et al. (2012) reports that by 2030, the global blue biofuel WF would have grown to 5.5 % of the total blue water available for human consumption thus creating pressure on freshwater resources. A significant amount of global WF (86 %) is attributed to agriculture. Any increase in diversion of the biomass for development of energy will require additional load on the water that may lead to water shortages. The WF can be reduced by utilizing multiple feedstocks for production of biofuel. IEA estimated that the energy use attributed to biomass will increase to 71 EJ in 2030. The production of biocrops for bioethanol and biodiesel will require large amount of freshwater that includes both green water (precipitation water) and blue water (irrigation water from ground and surface water) (Gerbens-Leenes et al. 2012). Hence, biorefinery approach is expected to minimize the WF of biofuels.

Batan et al. (2013) estimated the WF of a closed photo-bioreactor based biofuel and assessed the WF on the basis of blue, green, and lifecycle WF. Blue WF comprised of the water directly used for cultivation and process needs for both consumptive and nonconsumptive use.

Green water comprised of difference between water obtained as precipitation and that lost through soil moisture evaporation and evapotranspiration. The microalgae-based biofuel had a blue WF varied as a function of pathway involved in the production of fuel and location and ranged from 23–85 $m^3 \cdot GJ^{-1}$. It has been reported that the process water uses during cultivation, harvesting, and extraction accounts for majority of the blue WF (97.6%) which was followed by fuel conversion (2.4%) and transportation and distribution (0.002%). The green WF from microalgae based biofuel has been reported to be negative thus showing water gain in the basin. The green WF has been reported to vary among the geographic locations from 1.3–17 $m^3 \cdot GJ^{-1}$. The total WF comprising both blue and green water ranged from 18–82 $m^3 \cdot GJ^{-1}$. The sustainability of production of biofuel from microalgae in terms of WF lies in the usability of wastewater. Utilization of domestic and industrial wastewater for the culture of microalgae thus has the potential to lower the total WF in synthesis of biodiesel.

8.3.2 Carbon Footprint

With the increasing environmental concerns, the usage of biofuels has increased considerably. Hence, just like the carbon footprint is accounted for fossil fuels, the biofuels also should be measured in terms of carbon footprints (Johnson and Tschudi 2012). Hammond and Seth (2013) estimated the global carbon footprint of biofuels to be 0.248 bn global hectares (gha) in 2010 and may reach to 0.449 bn gha by 2019. It was also estimated that the total environmental footprint of the global biofuel produced was 0.720 bn gha for 2010 and could reach 1.242 bn gha by 2019. Fahd et al. (2012) reported the carbon footprint during the production of biodiesel. It was reported that the emission of CO_2 and other green house gases were relatively low during the production of seeds that amounted to 0.53 $g_{CO2\text{-}equiv}$ per g dry seed. In the processing steps, i.e., oil extraction and biodiesel production, the emissions reported is 0.99 and 1.72 $g_{CO2\text{-}equiv}$ per g of oil and biodiesel respectively. This has been attributed to high demand for energy in the production process. With biorefinery approach, the carbon footprint is expected to get lower owing to production of more than one utility product.

8.4 Sustainable Production of Biofuels

8.4.1 Value Added Product Basket: Pigments, Nutraceuticals and Bioactive Compounds

Microalgae are a very diverse group of organisms that are the key components of ecosystems and produce a variety of valuable compounds as secondary metabolites in different phases of life cycle (Pulz and Gross 2004; Skjanes et al. 2013). Microalgal biomass cultivation is regarded as a potential way to overcome our current dependence on fossil fuels, as microalgal biomass can be utilized for synthesis of number of biofuels like biodiesel, bioethanol, biohydrogen (Guldhe et al. 2014; Singh et al. 2014). High production cost has become a major bottleneck for biofuels production from microalgae (Spolaore et al. 2006). However, integrated biorefinary approach can make microalgal biofuel production economically viable (Vanthoor-Koopmans et al. 2013). Commercial realization of the microalgae as a source of renewable biofuel is feasible when constituents of the algal biomass are exploited for biofuels as well as for value added coproducts (Yen et al. 2013). Thus, the development and focus was changed toward the potential of growing microalgae commercially for its applications in field of health food for human consumption, aquaculture and animal feed, coloring agents, cosmetics, and other commercial products. Carbohydrate, protein, and lipids are the major constituents of microalgae which can be exploited commercially for different markets (Borowitzka 2013). Other compounds viz., fatty acids and steroids, carotenoids, phycocolloids, lectins, mycosporine-like amino acids, halogenated compounds, polyketides, and toxins, synthesized by

microalgae makes it an attractive platform for biorefinary concept (Skjanes et al. 2013).

a. Pigments

Colorful appearance of the microalgae is because of the pigments which capture light and initiate photosynthesis (Spolaore et al. 2006). Algae can be classified on the basis of pigmentation, as particular class of algae contains specific pigments such as chlorophyll in green algae, phycobilin pigments phycocyanin in blue-green algae, phycoerthirine in red algae, and fucoxanthin in brown algae (Borowitzka 2013). Chlorophyll a is the most abundant pigment in all photosynthetic organisms, and it absorbs energy from the light, and then serves as a primary electron donor in the electron transport chain (Spolaore et al. 2006). Chlorophyll can be used as an anti-inflammatory and wound healing additive to pharmaceuticals (Yen et al. 2013). Pheophorbide a is a chlorophyll derivative compound which is used in photodynamic therapy (PDT) used for the treatment of cancer (Yen et al. 2013). Carotenoids are pigments present in all classes of algae and serve as photoprotectors against the photooxidative damage resulting from excess energy captured by light-harvesting antenna (Cardozo et al. 2007; Skjanes et al. 2013). Some of the carotenoids isolated from microalgae like β-carotenoids, astaxanthin, cantaxanthin, zeaxanthin, and lutein have commercial importance (Skjanes et al. 2013). These pigments found their application in food, pharma, and cosmetic industry and attract high cost in commercial market (Lamers et al. 2012; Vanthoor-Koopmans et al. 2013). In the biofuel production process, coproduction of these high value pigments greatly improves the economics (Draaisma et al. 2013). Astaxanthin is an oxidized from of carotenoid with high oxidation capacity. Astaxanthin has many applications in healthcare industry as it can be used for prevention and treatment of various conditions, such as chronic inflammatory diseases, eye diseases, skin diseases, cardiovascular diseases, cancers, neurodegenerative diseases, liver diseases, metabolic syndrome, diabetes, diabetic nephropathy, and gastrointestinal diseases (Wayama et al. 2013). β-carotenoids has high antioxidant properties and are used in food industry. β-carotenoids from *Dunaliella salina* (Cowan et al. 1995) and astxanthin from *Haematococcus pluvialis* (Guerin et al. 2003) are the most successful commercial products (Lamers et al. 2012; Skjanes et al. 2013). Astaxanthin has been approved by the US Food and Drug Administration (US FDA) as a food additive for use in the aquaculture as well as for use as a dietary supplement (Guerin et al. 2003). The annual worldwide market for astaxanthin was estimated at US$ 200 million in 2004, with estimations of the global astaxanthin market rising to US$ 257 million in 2009. Synthetic astaxanthin is valued at US$ 2500/kg, while the natural product is sold for over US$ 7000/kg (Yen et al. 2013). Phycobillins are another category of algal pigments which exhibit high fluorescence, and have wide absorption spectrum. These properties of phycobillins make them useful in diagnostic industry (Spolaore et al. 2006). Spirullina *sp.* produces blue phycobiliproteins (APC) (Sarada et al. 1999) and red algae such as *Porphyridium* produces red phycobillins (R-PE) (Munier et al. 2014). The global phycobillins market was estimated to be approximately US$ 50 million in 1997, with prices varying from US$ 3/mg to US$ 25/mg (Yen et al. 2013). Green microalgae produce some other pigments such as, *Scenedesmus obliquus* and *Botrycococcus sp.* are the producers of lutein, *Dunaliella Salina* and *Chlorella pyrenoidosa* are the producers of zeaxanthin, and *Chlorella vulgaris* and *Chlorococcum sp.* are the producers of canaxanthin (Table 8.1).

b. Nutraceuticals

Nutraceuticals are the group of compounds which can be used for consumption as food with medicinal benefits. Humans and animals are dependent on plants for many nutrient supplements as they cannot be synthesized in their body. Microalgae can synthesize numerous compounds that have nutraceutical values. Microalgae have become a ubiquitous source of nutraceuticals due to the capability of producing necessary vitamins including A (Retinol), B1 (Thiamine), B2 (Riboflavin), B3 (Niacin), B6 (Pyridoxine), B9

Table 8.1 Different value added products from microalgae and their application

Product	Microalgal species	Application	Reference
Pigments			
β-carotene	*Dunaliella salina, Botrycococcus sp.*	Pigment (food), provitamin A	(Jayappriyan et al. 2013)
Astaxanthin	*Heamatococcous pluvialis, Chlorella zofingiensis, Chlamydomonas nivalis, Scenedesmus obliquus, Chlorococcum sp.*	Nutraceuticals, pharmaceuticals, food and feed industries	(Guerin et al. 2003; Jian-Ping Yuan et al. 2002; Qin et al. 2008; Rezanka et al. 2013; Sun et al. 2008)
Canthaxanthin	*Chlorella vulgaris, Chlorococcum sp.*	Synthetic pigment	(Gouveia et al. 2007; Jian-Ping Yuan et al. 2002)
Phycobilins (Phycocyanin, allophycocyanin, phycoerythrin)	Spirulina *sp.*, Porphyridium *sp.*	Fluorescent dye, antioxidant	(Munier et al. 2014; R. Sarada et al. 1999)
Zeaxanthin	*Chlorella pyrenoidosa, Dunaliella salina*	Antioxidant	(Cowan et al. 1995; Inbaraj et al. 2006)
Lutein	*Scenedesmus obliquus, Botryococcus braunii*	Nutraceuticals, pharmaceuticals, food and feed industries	(Ho et al. 2014; Yen et al. 2013)
Nutraceuticals			
Protein	*Spirulina platensis, Chlorella sp.*	Human dietary supplement	(Coca et al. 2014; Szabo et al. 2013)
Animal food	*Chlorella, Scenedesmus, Monoraphidium*	aquaculture	(Isik et al. 1999; Skjanes et al. 2013)
EPA	*Ankistrodesmus sp.*, *Nannochloris sp.*, *Chlamydomonas*	Dietary supplement	(Wijffels et al. 2013; Xie et al. 2013)
Bioactive compounds			
Phycolectins	*Chlamydomonas sp.*, *Chlorella sp.*	Hemagglutinin activity	(Plaza et al. 2010)
Polysaccharides	Chlorella *pyrenoidosa*	Anticancer activity	(Cardozo et al. 2007; Ermakova et al. 2013)
Halogenated compounds	*Laurencia sp.*	Taxonomical marker	(Faulkner 2001)

(Folic acid), B12 (Cobalamin), C (L-Ascorbic acid), D, E (Tocopherol), and H (Biotin). Microalgae contains essential elements including: Potassium, Zinc, Iodine, Selenium, Iron, Manganese, Copper, Phosphorus, Sodium, Nitrogen, Magnesium, Cobalt, Molybdenum, Sulfur, and Calcium and also produces essential amino acids and Omega-6 (Arachidonic acid) and Omega-3 (Docosahexaenoic acid, eicosapentaenoic acid) fatty acids (Spolaore et al. 2006; Yen et al. 2013).

Chlorella sp. is the most successfully commercialized green algae with an annual production of 2000 metric t of dry powder. Chlorella and Spirullina are consumed in Taiwan and Japan as health food. *Chlorella* (lutein, vitamin B12), *Spirullina* (single-cell protein) *Haematococcus* (antioxidant) and *Dunaliella* (β- carotene) are the most popular nutraceuticals sources. Human and animal lack essential enzymes for synthesis of polyunsaturated fatty acids which is essential for growth. Docosahexaenoic acid (DHA) is a major structural fatty acid in the grey matter of the brain, heart tissue, and in the retina of the eye while eicosapentaenoic acid is a precursor for hormone like substances which play crucial role in regulatory physiology (Cardozo et al. 2007). Use of microalgae as animal feed is more popular nowadays. Some microalgae play an important role during the life cycle of fish, shrimps, and molluscs during their larval stage. The most commonly used microalgae as animal and fish feed are *Chlorella, Arthospira, Tetraselmis, Isochrysis, Pavlova, Phaeodactylum, Chaetoceros, Nannochloropsis, Skeletonema,* and *Thalassiosira*. In

the biodiesel production process, lipid extracted algae (LEA) has great potential to be used as animal and fish feed as it is still rich in carbohydrates and proteins (Table 8.1).

c. Bioactive compounds

Bioactive compounds are the group of active chemical products synthesized as secondary metabolite in microalgae. Most of them are having antimicrobial, antiviral, and antioxidant properties which are important for microalgae as they act as protective mechanism during stress conditions. Number of bioactive compounds such as indoles, terpenes, acetogenins phenols, polysaccharides, toxins can be obtained from microalgae. Marine red algae *Laurencia sp.* is reported as the most prominent producer of these bioactive compounds, especially halogenated compounds (Faulkner 2001). These bioactive compounds can be used as pharmaceuticals due to its antimicrobial, antitubercular, and anticancer activity. A high-weight polysaccharide from *Chlorella pyrenoidosa* has very high immunostimulatory and antitumor effect with potential use in cancer therapy (Shi et al. 2007). *Chlorella vulgaris* produces glycoprotein which shows anticancer activity through antimetastatic immunopotentiation. Integrated biorefinary with the aim of biofuel production from microalgae along with value added compounds as coproducts is a sustainable and economically feasible approach. Table 8.1 depicts the different value added products from microalgae and their application.

8.4.2 Use of Industrial Refusals for Biomass Production: Wastewater Nutrient Medium and Flue Gases

Microalgae are simple unicellular photosynthetic organisms which utilize nutrients from growth medium and capture atmospheric CO_2 for its energy requirements and growth. More than half of energy requirement for microalgal biomass generation is utilized by carbon dioxide (CO_2) and fertilizers supplementation (Orfield et al. 2014). Economical feasibility of microalgal biomass production can be improved by use of industrial refusals like wastewater and flue gases. This strategy not only lowers the cost involved in biomass generation, but also has several environmental significances. Integrated biorefinary approach of utilizing industrial wastewater and flue gases for microalgal biomass generation provides effective resource management, environmental benefits, and makes it economically competitive. Microalgae can assimilate nutrients in wastewater and CO_2 from flue gases into cellular components like carbohydrates and lipids. Microalgal biomass generated using such industrial refusals can be directed to synthesis of various end products like biodiesel, biomethane, and animal feed.

a. Wastewater nutrient medium

Wastewater effluent after primary and secondary treatment has various organic and inorganic constituents. Release of such effluent into environment can cause severe problems like eutrophication and pollution. Inorganic constituents of wastewater effluent primarily consists of nitrogen and phosphorous. European Commission Directive 98/15/EEC have specified limits of 10 mgL^{-1} total nitrogen and 1 mgL^{-1} total phosphorous for discharge. Normal values of total nitrogen and phosphorous in wastewater effluent are 20–70 and 4–12 mgL^{-1} (Arbib et al. 2014; Cabanelas et al. 2013). Discharge of industrial and domestic wastewater are adding organic and inorganic nutrients, pathogens, heavy metals, suspended solids, and oxygen demanding material to the existing water resources. Biological treatment of such tertiary effluent is the possible solution for this problem. Availability of fresh water is facing severe risks due to rapid industrialization and socioeconomic development. In biorefinary concept cultivation of microalgae using wastewater serves dual purpose of biomass generation as well as polishing of the effluent by removing inorganic nutrients (Rawat et al. 2011; Singh et al. 2014). Microalgae cultivation using wastewater for valuable biomass generation, which can be utilized for several purposes fulfilling energy and feed requirements, is a sustainable approach. Water requirement for mi-

Table 8.2 Microalgae grown on various industrial wastewater and their biomass and lipid yields

Microalgae	Wastewater	N removal (%)	P removal (%)	Biomass ($gL^{-1}D^{-1}$)	Lipid yield (%)	Reference
Chlorella zofingiensis	Dairy wastewater	51.7	97.5	–	17.9	(Huo et al. 2012)
Microalgae consortium	Carpet mill effluent	>96	>96	0.039	12	(Chinnasamy et al. 2010)
Chlamydomonas sp. TAI-2	Industrial wastewater	100	33	–	18.4	(Wu et al. 2012)
Chlorella saccharophila	Carpet mill effluent	–	–	0.023	18.1	(Chinnasamy et al. 2010)
Botryococcus braunii	Carpet mill effluent	–	–	0.034	13.2	(Chinnasamy et al. 2010)

croalgae cultivation in open pond is as high as 11–13 million L $ha^{-1}year^{-1}$ (Chinnasamy et al. 2010). Wastewater utilization for microalgae cultivation can reduce fresh WF as well as can provide treated water for other use. Wastewater has very high concentrations of nutrients specifically N and P along with some toxic metals. Costly chemical treatment methods are required to remove these nutrients. The potential shown by microalgae to grow with minimal fresh water and accumulate nutrients and metals can be exploited to treat such domestic and industrial wastewater. Cultivation of microalgae needs water and supply of inorganic nutrients like nitrogen and phosphorous. Nitrogen and phosphorous plays a very important role in microalgal physiology, and thus needs to be supplied through growth medium. The nutrient supplementation contributes a major portion in the overall cost of microalgae cultivation. Utilization of tertiary industrial or domestic wastewater as growth medium can supply required nutrients for microalgae. Nutrients cost can be reduced if wastewater effluent is used as the nutrient medium. Various microalgal strains have been studied for their growth in wastewater nutrient medium. Most studied strain for wastewater utilization is *Chlorella* sp, due to its robustness and application in biodiesel production (Huo et al. 2012; Ramanna et al. 2014). Table 8.2 shows different microalgal strains grown on various industrial wastewater and their biomass and lipid yields. Wastewater medium has lower N and P content as compared to commercial media used for microalgae cultivation. Limitation of nutrients for stress induced lipid accumulation is a well accepted method in microalgal biofuel process. Ramanna et al. (2014) when grew Chlorella sorokiniana on wastewater medium and standard BG11 medium, lipid yield was found to be higher in wastewater grown biomass (10.7% DCW) compared to BG11 grown biomass (8.08% DCW). Utilization of wastewater growth medium for microalgal biomass generation thus provides several benefits like cheap nutrient source, enhanced lipid productivity, reducing risk of eutrophication, reduce fresh WF, and polished tertiary treated wastewater for other uses. Overall, this approach provides sustainability, commercial compatibility, and environmental benefits for microalgal biofuel production process.

b. Utilization of flue gases

CO_2 is a major contributor of green house gases (GHG) which causes global warming. Global warming poses serious threat causing climate changes, glacial melting, rise in ocean level, reduced food production, extinction of species, and many other environmental problems. Globally, it has seen as a serious issue, and thus Kyoto Protocol has been promoted by United Nations with the objective of reducing GHG emission by 5.2% on the basis of emission in 1990 (Pires et al. 2012). Several strategies are practiced for capture and sequestration of CO_2. Most widely used method is carbon capture and storage. CO_2 is captured from emission sources like power plants and cement industries. CO_2 can be captured by several methods viz., adsorption, absorption, separation

Table 8.3 Microalgae grown using flue gases and their biomass and lipid yields

Microalgae	CO_2 concentration (%)	Biomass ($gL^{-1}D^{-1}$)	Lipid yield (%)	Reference
Chlorella sp.	6–8	19.4–22.8[a]	–	(Doucha et al. 2005)
Chlorella sp. MTF7	25	0.48	25.2	(Chiu et al. 2011)
Scenedesmus obliquus SJTU-3	10	0.155	19.25	(Tang et al. 2011)
Chlorella pyrenoidosa SJTU-2	10	0.144	24.25	(Tang et al. 2011)

[a] Biomass in g m^{-2} D^{-1}

membranes, and cryogenic distillation. Captured CO_2 is transported to storage locations, and stored in geological or ocean storage or mineralized. However, with this approach of capture and storage several technological, economical, and environmental issues are related. Biological CO_2 capture is a sustainable approach and provides alternatives to conventional CO_2 capture methods.

Microalgae have the ability to fix atmospheric CO_2 through photosynthesis, with ten times greater efficiency than terrestrial plants (Pires et al. 2012). Carbon is the key component of microalgal cell which constitutes 36–56% of dry matter. 1.3–2.4 kg CO_2 is fixed by microalgae for per kg of dry biomass generation (Van Den Hende et al. 2012). Microalgae cultivated by supplying CO_2 from flue gases produce biomass which can be utilized for biofuels, value added products, and animal feed. Microalgae can be cultivated in open or closed system for biomass generation. For microalgae cultivation either CO_2 is separated from flue gases and used or directly flue gases are applied. Direct use of flue gases is beneficial in terms of energy and cost saving; however, microalgal strain should be resistant toward the high percentage of CO_2 (15%) and presence of SO_X and NO_X (Maeda et al. 1995). Maeda et al. (1995) screened several microalgal species, and found a *Chlorella sp.* strain with high growth rate at a temperature of 35 °C and 15% CO_2 concentration. Table 8.3 depicts the studies using flue gases for microalgae cultivation. CO_2 can directly diffuse through the microalgal plasma membrane. CO_2 is assimilated to 3-phosphoglycerate by enzyme rubisco (ribulose 1,5-bisphosphate carboxylase/oxygenase) in the Calvin cycle. Fixed CO_2 through various metabolic pathways assimilated into carbohydrates, proteins, and lipids in microalgae. CO_2 has low mass transfer coefficient, and thus mass transfer from gaseous phase to liquid phase could be a limiting step in application of this technology. High flow rate in closed system or proper mixing in open cultivation could be the possible solutions to overcome mass transfer limitations (Pires et al. 2012). Flue gases contain many compounds like SO_X, NO_X, CO, C_xH_y, halogen acids, and particulate matter apart from CO_2. Direct utilization of flue gases can pose problems for microalgae cultivation as some of these compounds could have toxic effect on microalgae (Van Den Hende et al. 2012). Thus, a better understanding of the effect of these individual compounds and their concentration limits on microalgal physiology is needed. Effective utilization of flue gases for microalgae cultivation can reduce environmental concerns as well as earn carbon credits. Application of innovative scientific technologies for utilization of flue gases by microalgae, can aid in improving economics of biomass production.

8.5 Conclusion

Microalgae have shown a promising future with simultaneous production of more than one biofuels viz., biodiesel, biomethane, biohydrogen, and bioethanol to cater the energy demands. High energy input and the cost associated with production of microalgal biofuels are still a bottleneck which has to be overcome for its industrial viability. The biorefinary concept could be the possible answer to this problem, where with the production of biofuels, emphasis is also given on the coproduction of value added products and utilization of refusals. For commercial feasibility of microalgal biofuel production, it is necessary to produce other valuable products along

with the biofuel production. Microalgal carbohydrate, protein, lipid, and pigments can be used in pharmaceutical, nutraceutical, food and feed industries with various applications. High energy demand for biofuels production can be reduced by producing valuable coproduct. Utilization of wastewater as a nutrient source and flue gases as a source of CO_2 for microalgal cultivation reduces the environmental concerns as well as improves carbon and WF. Biorefinary approach of microalgal biofuel production thus becomes imperative for sustainable application. Thus, practicing this approach could make biofuels production from microalgae sustainable, economically viable, and environmentally friendly.

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Oil Spill Cleanup: Role of Environmental Biotechnology

9

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Abstract

Oil spills are a major environmental concern in today's world. With the increase in anthropogenic activities, accidental and incidental spillage of oil has severely affected the environment, causing both ecological and economic damage. Mechanical, chemical, and biological approaches have been utilized as remediation strategies for oil spill cleanup. The time period just after oil spillage being the most crucial for oil spill cleanup, it is imperative that primary and secondary oil spill cleanup response and contingency plans should be in place for mediating immediate intelligent remedial action. On the basis of type of oil spilled, weather conditions, and topography of the surrounding area, careful selection of remedial methods should be done. Mechanical approaches such as booms, skimmers, and sorbents are utilized in conjunction with one another for cleanup operations and are one of the widely used primary responses. Chemical dispersants when sprayed on oil slick accelerate the rate of natural dispersion of medium- and light-weight oils and also increase the availability of oil for microbial colonization. Close monitoring of economic and ecological implications of addition of dispersants has to be done before undertaking dispersant application since they are known to be detrimental or ineffective if not applied intelligently. Biostimulation, bioaugmentation, phytoremediation, and genetically modified organisms (GMOs) have all been tried as remedial strategies for oil spills with varying success. As biological strategies are safest, we need to redesign them with the help of genomic and molecular tools to make them more successful.

Keywords

Bioremediation · Oil spill · Molecular tools

9.1 Introduction

When we talk of oil spill, liquid petroleum comes in our mind as oil and the affected area as a sea or an ocean. However, it should be noted that oils

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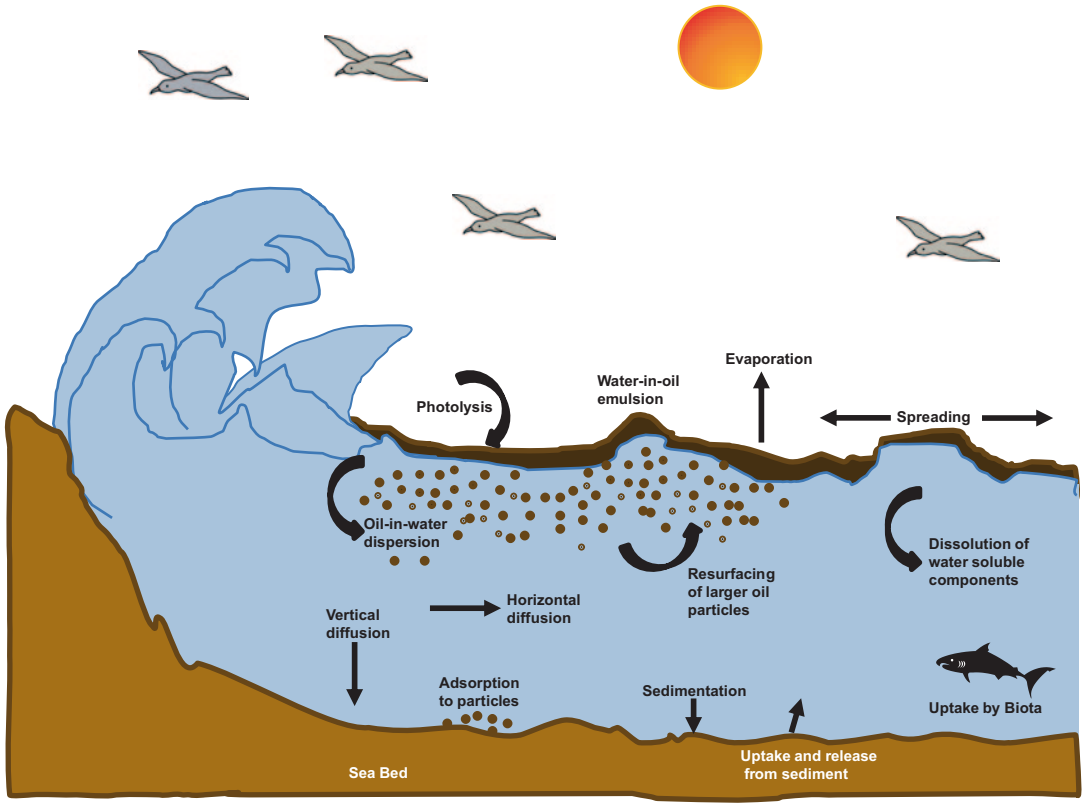

Fig. 9.1 Fate of spilled oil

can be of various types, such as vegetable oils, animal fats, and other nonpetroleum oils, which are manufactured and utilized on large scale. In addition, the affected area can be a freshwater body or land. In this chapter, we will use the term oil in the context of liquid petroleum hydrocarbon and the affected area, in major discussions, as open systems such as seas and oceans.

Crude oil consists of more than 17,000 distinct chemical components as discerned by ultrahigh resolution mass spectrometry (Marshall and Rodgers 2003). This number is an approximate value as different crude oils have different compositions, which are subject to change due to weathering and biodegradation. Crude oil composition can be broadly classified into four major fractions: saturated hydrocarbons, aromatic hydrocarbons, resins, and asphaltenes. Saturated (absence of double bonds) hydrocarbons are major constituents of crude oil; common examples of saturated hydrocarbons are alkanes (paraffin) and cycloalkanes (naphthalene). Aromatic hydrocarbons have one or more aromatic rings with or without alkyl substitution(s). Resins and asphaltenes are nonhydrocarbon polar compounds with complex chemical structures (Harayama et al. 2004). Typically, heavy oils have higher content of more polar compounds, resins, and asphaltenes and lower content of saturated and aromatic hydrocarbons, whereas light oils have higher content of saturated and aromatic hydrocarbons and lower content of resins and asphaltenes (Head et al. 2003, 2006).

Spilled petroleum on water surface is subject to many modifications and its composition changes with time. This is called weathering. Evaporation, dissolution, emulsification, and photochemical oxidation occur during weathering (Fig. 9.1). Low-molecular weight fractions (*n*-alkanes with chain length shorter than C_{14}) and monocyclic aromatic hydrocarbons (benzenes and xylenes) are subject to both evaporation as well as dis-

solution. Polar fraction increases and aromatic fraction decreases in petroleum when subjected to photochemical oxidation under sunlight (Dutta and Harayama 2000). Biodegradation of crude oil also results in similar changes in its composition, with loss of saturated and aromatic hydrocarbons and relative increase in polar fractions (Head et al. 2006).

Nature of oil (physical and chemical properties) and natural conditions (water, temperature, weather, and topography of surrounding area) prevailing at the time of oil spill influence the weathering and biodegradation of oil. Oil spills contaminate drinking water, disrupt food chain, endanger public health, destroy natural resources, and disrupt economy. Thus, it is of utmost importance to develop *in situ* strategies for cleanup of marine oil spills (Fig. 9.1).

9.2 What Are Oil Spills?

Accidental or incidental release of liquid petroleum hydrocarbon into the environment (marine or inland) due to human activity is called oil spill. During Gulf War (1991), retreating Iraqi forces opened valves of oil wells and pipelines and set them to fire in a bid to slow down onslaught of invading American forces. An estimated 240–360 million ga of crude oil flowed into Persian Gulf. This exacted little damage to the marine ecosystem as per a report by the Intergovernmental Oceanographic Commission at UNESCO. Deepwater Horizon/BP oil spill (2010) caused by wellhead blowout at the Deepwater Horizon oil rig is the most devastating and the largest marine oil spill. The explosion at the rig killed 11 men working on the platform and injured 17 others. It spilled 220 million ga of oil into the Gulf of Mexico, causing extensive damage to gulf's fishing and tourism industry. Though Exxon Valdez oil spill (1989) is the second largest oil spill in the USA, it is notable because it led to serious reexamination of policies and framework for oil spill cleanup in the country. It occurred in Prince William Sound, Alaska, when Exxon Valdez, an oil tanker, ran aground, releasing 10.8 million ga of crude oil, which impacted 1609 km of Alaskan coast. Table 9.1 lists some of the largest oil spills in the world.

9.3 Impact of Oil Spills on Environment and Society

Flora and fauna as well as the topography of the surrounding areas bear the brunt of oil spills. Animals may be smothered in oil and killed or seriously injured soon after coming in contact with oil. However, many other effects of oil spills are more subtle and long lasting.

Aquatic animals like turtle, seal, walrus, and dolphin which live close to the shore, endanger themselves when they consume oil-contaminated prey. Kelps and sea grasses are often used for food, shelter, and nesting by birds and aquatic animals. These are destroyed by oil contamination, thus affecting their reproductive cycle and nursing of the young. Direct physical contact of oil with fur of mammals causes loss of their insulating properties, leading to hypothermia-induced death. Feathers also lose their architecture as well as their insulating properties, which help birds in keeping warm, flying, and floating, when in contact with oil. Seepage of oil onto the surface of eggs often seals their pores and prevents gaseous exchange leading to the death of the embryo. Aquatic mammals and birds often starve to death when they refuse to eat oil-stained unpleasant-smelling prey (EPA 1999b).

Coral reefs, which serve as nurseries for fingerlings of fish, are often smothered in oil and risk exposure to toxic substances in oil. Tidal flats, sheltered beaches, salt marshes, and mangrove forests harbor rich biodiversity, which on exposure to oil, gets disturbed, damaged, and destroyed (EPA 1999b).

Human activities like fishing, aquaculture, recreational activities, tourism industry, and human health are adversely affected because of oil spills. Fishing and shellfish fishing are often closed to prevent catching oil-tainted fish. Severe economic losses are incurred by tourism industry and operators of recreational activities (scuba diving, angling, and boating). Water is often used for cooling purposes in nuclear desalination, and power plants. These industries risk intake of oiled water into their piping and machinery. Personnel engaged in containment of oil spills risk ill health by inhaling or touching oil products and

Table 9.1 Ten most disastrous oil spills in the world. (Cohen 2002; Briney 2011)

Sl. No.	Name and Location	Cause	Type of oil spill	Amount (million gallons)	Year	Area of damage	Comment
	Gulf War, Kuwait (Briney 2011)	Deliberate Sabotage	Inland and marine	240–360	1991	Persian Gulf	Little damage. Largest oil spill
	Deepwater Horizon/BP oil spill, Gulf of Mexico (Cleaveland 2010; Briney 2011; Cohn 2010)	Wellhead blowout at the Deepwater Horizon	Marine	205	2010	Gulf of Mexico	Extensive damage to gulf's fishing and tourism industry. Largest marine oil spill
	Ixtoc 1 oil well (ERCO 1982)	Wellhead blowout	Marine	140	1979	Bay of Campeche, Mexico	Little damage to benthic and epibenthic community. Littoral and intertidal communities affected
	Atlantic Empress, Trinidad, and Tobago (Casselman 2011; Briney 2011)	Collision of supertankers, Atlantic empress, and Aegean Captain	Marine	90	1979	–	Minor shore pollution reported
	Fergana Valley, Uzbekistan (Briney 2011; Casselman 2011)	Unknown	Inland	88	1992	Fergana Valley	Largest inland oil spill
	ABT Summer, off the coast of Angola (Casselman 2011; Briney 2011)	Explosion	Marine	80	1991	High seas, off the coast of Angola	Little damage
	Nowruz oil field, Persian Gulf (Briney 2011; Casselman 2011)	Collision and bombing	Marine	80	1983	Persian Gulf	No information
	Castellio de Bellver, off Saldanha Bay, South Africa (Briney 2011)	Fire	Marine	79	1983	Off Saldanha Bay, South Africa	Damage to local fishing stocks minimal; however, 1500 gannets who gathered on nearby island were oiled
	Amoco Cadiz, off Brittany, France (Bourne 1979; Briney 2011)	Collision	Marine	69	1978	Brittany, France	200 miles of coastline of Brittany polluted
	Odyssey oil spill, Nova Scotia, Canada (Briney 2011)	Bad weather and fire	Marine	43	1988	Off Nova Scotia coast, Canada	No information

consumers may suffer from eating contaminated fish and shellfish (ITOPF 2013).

9.4 Approaches for Cleanup of Oil Spills

Containment and recovery of oil are major concerns in the event of an oil spill. Containment aims at minimizing the area of oil spill, thus curtailing its adverse effects on lives of animals and plants, economics of surrounding area, and environment. Containment is the preliminary step that allows recovery or dispersal of oil at later stages. The following are the approaches for cleanup of oil spills.

9.4.1 Mechanical Approaches

Careful selection and proper use of equipment for combating oil spill is the key to successful oil spill cleanup. Booms, skimmers, and varieties of sorbents are utilized for such cleanup operations, which are often used in conjunction with one another. Type of oil spilled, weather conditions, and topography of the surrounding area need to be considered while choosing the method of cleanup (EPA 1999c).

Containment booms

Containment booms and skimmers are utilized to block spreading of oil, concentrating and recovering it. They are temporary floatation devices used to contain oil spills. Containment booms are most successful in gentle seas. In choppy waters, booms are prone to failure due to environmental constraints. Oil entrapped in the area under the containment boom can be scooped and collected by ships, which then return to the shore for its proper disposal and recycling (Castro et al. 2010). Wang et al. made a superhydrophobic and superoleophilic miniature oil containment boom (MOCB) based on Cu_2O film-coated stainless steel (SS) mesh. MOCB has high efficiency in excluding oil from the water surface (it repels water absolutely), is reusable, and has good water pressure and corrosion resistance. The oil contained in the MOCB can be collected and recycled (Wang et al. 2014).

Skimmers

Oil skimmers are devices that separate oil floating on water. Skimmers are of three types—weir, oleophilic, and suction skimmers. Weir skimmers allow oil floating on water to spill over the weir, which can be then entrapped in a well. They tend to get jammed and clogged by floating debris and need to be monitored. Oleophilic skimmers use mop, belts, drums, and discs made of oleophilic material to attract oil, which then can be scrapped off or squeezed out into a recovery tank for further processing. Oleophilic skimmers are effective against oil layers of varying thickness (Broje and Keller 2006). Suction skimmer is quite similar to the household vacuum cleaner and operates on the same principle: it sucks the oil floating on water, which is then pumped into storage tanks. Suction skimmers often suffer from clogging by debris and work best in scenarios where oil has been already contained against a boom.

Sorbents

Natural and synthetic sorbents are used to recover oil. Sorbents are materials that tend to absorb or adsorb liquids. Peat, corn cobs, hay, and saw dust are some examples of natural organic sorbents, which tend to absorb 3–15 times their weight in oil. These are relatively inexpensive and readily available. They tend to absorb oil as well as water causing them to sink; moreover, they tend to be loose, hence they need to be packaged for their easy disposal (EPA 1999c).

Inorganic natural sorbents include clay, glass, vermiculite, volcanic ash, and wool. They absorb 4–20 times their weight in oil and, like natural organic sorbents, are relatively inexpensive and readily available. Synthetic sorbents like polyurethane, nylon, and polyethylene can absorb 70 times their weight in oil and can be cleaned and reused several times. Factors like rate of absorption, ease of application, and oil retention are considered while choosing sorbents for oil spill cleanup. Heavy oils have slower rate of absorption than light oils. Rate of absorption also varies with the thickness of oil. Oil may be released from the pores of sorbents while recovering from

oil-saturated sorbents. Moreover, lighter oils tend to be released more easily than heavier oils. Blowers and fans are often used for application of loose sorbents. However, in windy conditions, application of clay and vermiculite is particularly difficult. Also, clay and vermiculite are injurious to health if inhaled as they spread in the surrounding area as dust (EPA 1999c; Al-Majed et al. 2012).

9.4.2 Chemical Approaches

Dispersants are mixture of surfactants and solvents, which when sprayed on oil slick accelerate the rate of natural dispersion (EPA 1999a). They are capable of rapidly removing medium- and light-weight oil from the sea surface to the water column, wherein by the action of wave energy, oil slick breaks into minute droplets and gets diluted rapidly. Microbial action is at the oil–water interface—dispersion of oil into minute droplets increases the area of microbial colonization. Microorganisms degrade the oil droplets and prevent formation of water-in-oil emulsions. Dispersants are most effective when applied immediately after oil spill, before the evaporation of lightest components of oil. Wax and asphaltene content of the oil affect the manner of weathering, emulsification, and dispersion of oil in sea (Strøm-Kristiansen et al. 1997). Therefore, knowledge of properties of spilled oil and how they change with weathering is an important factor for determining the use of dispersants (Chapman et al. 2007). The state of sea and probable weather conditions in the approaching weeks after the oil spill also affect the success of oil spill cleanup by dispersants. Rough and windy seas tend to inhibit interaction of oil and dispersant, as they are often overwashed by waves. Thus, dispersants unable to interact with oil are washed or blown off into sea. Application of dispersants in calm conditions is effective only if the wave energy is predicted to increase with reasonable time period (Nedwed et al. 2006). Use of dispersants is generally discouraged in unique ecological areas, such as Baltic Sea, where sea is shallow and is characterized by low-water exchange and low salinity (35‰). Low salinity encourages increased solubility of surfactant and, therefore, the dispersant is less available to interact with oil (Chapman et al. 2007). Efforts are currently active to increase the salt content of surfactant in order to reduce its solubility in sea water (George-Ares et al. 2001).

Close monitoring of economic implication of addition of dispersants has to be done before undertaking dispersant application. Spraying of dispersants may be decided against in areas close to aquaculture activities, where dispersant-tainted oil may interfere with spawning of fish. Dispersant spraying is often undertaken to prevent potential damage to coastal amenities, intertidal marine life, and aquatic birds, even at the cost of potentially tainting fish stocks. Therefore, the decision on whether dispersants should be applied or not has to be taken only after weighing carefully the cost effectiveness of the operation and conflicting priorities for protection of different resources from potential damage (ITOPF 2013).

Dispersants have been used with varying success in real incidents. In case of M/V Red Seagull (1998) and Sea Empress (1996), successful use of dispersants was reported, whereas in case of Natuna Sea incident in Singapore Strait (2000), it was proved ineffective. In case of M/V Red Seagull, oil spilled was light to medium Arabian crude oil that was dispersed readily on application of dispersants, whereas Nile Blend crude oil spilled in Natuna incident had high viscosity. Moreover, the weather conditions (calm weather and little wave energy) at Natuna oil spill further exacerbated the situation. Thus, it can be inferred that successful chemical dispersion results from accurate understanding of components of spilled oil, weather, and sea conditions (Chapman et al. 2007).

Dispersants suffer from limitations and, hence, their use has to be carefully planned and judiciously controlled. The effectiveness of dispersant application needs to be carefully examined and should be immediately stopped once it is no longer effective. Submerged flow-through system using ultraviolet fluorescence spectrometry (UVF) and *in situ* florometry can be used to monitor oil concentration and confirm visual observation (successful chemically dispersed oil yields a brown-colored plume) about amenability of oil to chemical dispersion. Currently dispersants are

claimed to be less toxic to marine microorganisms than in past. During Deepwater Horizon blowout, more than 700,000 ga of dispersant Corexit was applied directly at the wellhead. Corexit was reported to have increased the toxicity of oil by 52 times after 2 years of the incident (Rico-Martinez et al. 2013). The debate is still active about the perceived success of Corexit as oil spill response in Deepwater Horizon incident. Current research in this area should focus to enhance our understanding about functional interaction of dispersants with different components of oil and better technology to predict real-time information on oil removal from water surface as a result of dispersant application.

9.4.3 Biological Approaches

Biological approaches for biodegradation primarily include bioremediation (biostimulation and bioaugmentation) which enhances the rate of natural biodegradation. Phytoremediation has been suggested as one of the biological approaches. As the name suggests, biodegradation is the conversion of complex compounds by biological agents (fungi, bacteria, and yeasts) into simpler compounds for obtaining energy and nutrients. Addition of nutrients, enzymes, and naturally occurring or genetically modified microorganisms (GMOs) and application of phytoremediating plants are key biological approaches for oil spill cleanup. We will discuss bioremediation with reference to biostimulation and bioaugmentation.

Biostimulation

Biostimulation is the addition of growth-limiting nutrients and other cosubstrates to the contaminated environment for stimulating the growth of indigenous oil degraders. Biostimulation is one of the most environmentally safe methods for combating oil spills. Indigenous oil degraders generally subside on natural oil seeps and plant synthesis; however, the rate of biodegradation is slow (Fehler and Light 1970). In case of natural and anthropogenic oil spills, indigenous oil degrading bacteria are unable to degrade the oil due to limiting abiotic factors such as molecular oxygen, nitrogen, and phosphate concentrations in sea water. Therefore, P and N-based fertilizers are applied to alleviate nutrient limitation, which stimulates the growth of oil degrading bacteria. P and N fertilizers such as ammonium phosphate, nitrates, phosphates, and urea can be used. However, being highly soluble, they risk rapid dissolution and dispersion in open systems such as seas. Water soluble fertilizers are best applicable in low-energy fine-grained shorelines where water transport is limited (Nikolopoulou and Kalogerakis 2009). Thus, efforts are being made to provide a suitable alternative that can work on other conditions.

Oleophilic (oil-loving) and slow-release fertilizers have been suggested as other alternatives. Inipol EAP22 containing oleic acid, urea, and lauryl phosphate has been utilized for oil spill cleanup in the shoreline of Prince William Sound (Zhu et al. 2001). Inipol EAP22 was found to be effective in sandy beaches with coarse sediments and not with fine sediments due to its inability in penetrating fine sediments (Sveum and Ladousse 1989). Inipol EAP22 suffers from at least three problems—oleic acid component contributes as alternative carbon source, urea component dissolves in water phase and is unavailable to microbes working at the oil phase, and toxicity of 2-butoxy-ethanol component in Inipol EAP22 (Ron and Rosenberg 2014). Polymerized urea and formaldehyde formulations have been used successfully to remediate oil spill in sandy beaches. However, they were found unsuitable in open seas where they tend to sink due to their high density.

Slow-release fertilizers provide continuous source of nutrients in oil-contaminated areas, overcome washout problems characteristic of intertidal environments, and forgo the need for frequent application of fertilizers. Slow-release fertilizers are soild formulations containing inorganic fertilizers coated with paraffin or vegetable oil. Customblen, a formulation of calcium phosphate, ammonium phosphate, and ammonium nitrate, coated with vegetable oil has been used in oil spill cleanup in the shorelines of Prince William Sound along with Inipol EAP22 with moderate success. Studies on slow-release

fertilizers indicate that rapid release is detrimental to the sustenance of microbes in the long run, and very slow release rates are unsuitable for maintenance of rapid biodegradation rates. The challenge that still remains is to design a slow-release fertilizer whose release rates can be controlled to allow optimal nutrient concentrations over longer periods of time in the marine environment (Nikolopoulou and Kalogerakis 2009).

Some other exciting alternatives are: use of nitrogen-fixing, hydrocarbon-oxidizers and uric acid as a natural fertilizer. Nitrogen being a limiting factor in biodegradation following an oil spill, a strong selection for nitrogen-fixing, hydrocarbon-oxidizers is important. However, few reports exist that unequivocally demonstrate nitrogen fixation coupled with growth on hydrocarbons larger than ethane. Consortia of bacteria degrading hydrocarbon through nitrogen fixation have been reported (Foght 2010). *Azotobacter chroococcum* isolated from oil-polluted site is currently the sole example of a marine nitrogen-fixing, hydrocarbon-oxidizers microbe (Thavasi et al. 2006). Another option is addition of nutrient amendments to the oil spill using thin-filmed minerals comprised largely of Fuller's Earth Clay. Together with adsorbed N and P fertilizers, filming additives, and organoclay, clay flakes can be engineered to float on seawater, attach to the oil, and slowly release contained nutrients. Large amount of oil is converted in bacterial biofilm and there is significant reduction in alkane content (Warr et al. 2013).

Studies on uric acid, a biostimulant, during oil spill are rapidly gaining credence in scientific circles. Uric acid has low solubility in water, has adherence to hydrocarbons, is major nitrogen waste in animals, and is readily available as inexpensive commercially available fertilizer, guano (Ron and Rosenberg 2014). Many bacterial species including *Alcanivorax* strains have been documented to use uric acid as a natural source of nitrogen (Knezevich et al. 2006). Uric acid has been suggested as a potential biostimulant for bioremediation of oil spills (Ron and Rosenberg 2014).

Bioaugmentation

Seeding of microorganisms at the site of oil spill to enhance the oil biodegradation is called bioaugmentation. Of particular interest to bioaugmentation are groups of microbes that utilize hydrocarbon as sole source of carbon and energy. Such microbes are called hydrocarbonoclastic bacteria. They include strains of *Alcanivorax* (Yakimov et al. 1998; Kostka et al. 2011), *Cycloclasticus* (Dyksterhouse et al. 1995), *Oleiphilus* (Golyshin et al. 2002), *Oleispira* (Yakimov et al. 2003), *Thalassolituus* (Yakimov et al. 2004), and *Planomicrobium* (Engelhardt et al. 2001). *Alcanivorax sp.* grows only on *n*-alkanes and branched alkanes as carbon and energy source. Similarly, *Cycloclasticus* strains grow on aromatic hydrocarbons such as naphthalene, phenanthrene, and anthracene while *Oleiphilus* and *Oleispira sp.* grow on aliphatic hydrocarbons, alkanoles and alkanoates (Head et al. 2006).

The predominant growth of *Alcanivorax* after biostimulation in oil-impacted marine environment has been shown by conventional methods and also proved by 16S rRNA gene sequencing studies (Syutsubo et al. 2001; Roling et al. 2002, 2004). It has been suggested that growth is due to the higher ability of this genus to use branched-chain alkanes. *Alcanivorax borkumensis* relies exclusively on alkanes as energy source, thus it is unsurprising that it has multiple alkane-catabolism pathways including alkane hydroxylases (AlkB1 and AlkB2) and 3 cytochrome P450-dependent alkane monooxygenases (Schneiker et al. 2006). In cold marine environments, *Oleispira sp.* is the dominant alkane-degrading microbe associated with oil spills (Coulon et al. 2007) rather than *Alcanicorax sp.*, whereas in temperate environments, *Thalassolituus* spp. are the dominant species (McKew et al. 2007). Generalists (microbes capable of using alkanes and/or polyaromatic hydrocarbon as well as nonhydrocarbons) such as *Acinetobacter* (diverse array of alkane hydroxylases capable of degrading wide array of short- and long-chain alkanes is present), *Roseobacter, Marinobacter, Pseudomonos*, and *rhodococcus sp.* are important constituents of hydrocarbon-degrading community. Although *Cycloclasticus sp.* is the leading polycyclic aromatic hydrocarbon (PAH) degrader, *Vibrio, Marinobacter, Microbacterium, Pseudoalteromonas, Halomonas*, and others contribute significantly to PAH degradation (McGenity et al. 2012). In estuarine

waters enriched with naphthalene, *Cycloclasticus* and *Pseudomonas* were found to be abundant, but *Pseudomonas* appeared in the latter stages of the enrichment (Niepceron et al. 2010). It is envisaged that though there is single carbon and energy source, both species are able to coexist presumably, because not all PAHs are oxidized to CO_2 and H_2O by a single organism; intermediate oxidation products are formed, which are utilized by other microbes as carbon and energy source (McGenity et al. 2012). One of the lesser appreciated microbes in the context of oil spill bioremediation are fungi found in marine mats (Allen et al. 2009) and many of them are also reported to be salt adapted (Valentin et al. 2006), which may play a major role in degradation of coastal PAH (Frey-Klett et al. 2011). Filamentous fungal networks provide the so-called "fungal highway" of continuous liquid films in which chemoattractants provide a gradient for directional transport of hydrocarbon-degrading bacteria to the pollutant (Furuno et al. 2010). High molecular weight PAHs strongly adsorb minerals and associated organic matter and, thus their bioavailability decreases. Microbes often circumvent this problem by either colonizing on the surface of minerals or producing biosurfactants (biological surface active agents with dual hydrophobic and hydrophilic moieties), which minimize the diffusion time and enhance bioavailability and desorption of PAHs (Guerin and Boyd 1992; Perfumo et al. 2010). *A. borkumensis* produces surfactants that increase the bioavailability of PAHs for other microbes. Though it does not use the PAHs as carbon or energy source itself, the biosurfactants so produced may be helpful in reducing the stress due to accumulation of toxic PAHs (McGenity et al. 2012). Knowledge about cooperative behavior of microbes in establishing self-sufficient community, which is pivotal in biodegradation of petroleum fractions, will help choosing the microbe or consortium of microbes for bioaugmentation.

In a real-life incident of oil spill, it is essential to know which type of microbe is best suited for bioaugmentation. The above discussion throws some light on potential candidates for bioaugmentation, however, to unequivocally decide on the right candidate, it is essential to know more about microbial interactions and isolate more of the still uncultivable marine bacterial species for oil spill bioremediation. Conventional methods to cultivate marine bacteria have been unsuccessful, since many of them live as oligotrophs and do not adapt to high carbon-containing media. "Extinction culturing" has been used to isolate hydrocarbon-degrading marine bacteria. In extinction culturing, microorganisms are grown in natural sea water as medium at a density ranging from 1 to 10 cells per tube. The recovery of bacteria by this method is 2–60 % in comparison to 0.1 % obtained from conventional culturing methods (Button et al. 1993). Several culture-independent rRNA based approaches such as 16S rRNA gene (rDNA) clone libraries, fluorescence *in situ* hybridization (FISH) with rRNA-targeted oligonucleotide probes (Pernthaler et al. 2002), and denaturing gradient gel electrophoresis (DGGE) of PCR-amplified rDNA (Baker et al. 2003) have revealed a surprising diversity in marine bacteria (Harayama et al. 2004). It has been suggested that uncultivability of such bacteria in axenic culture is due to the unavailability of secondary factors (metabolites and/or signaling molecules), which are produced by other microbes. Metabolites such as biosurfactants, *N*-acyl homoserine lactones, and cyclic AMP (cAMP) have been documented to increase the cultivability of bacteria. Extracellular polysaccharides from *Rhodococcus rhodochrous* function as biosurfactant and encourage the growth of *Cycloclasticus sp.* (Iwabuchi et al. 2002). The addition of *N*-acyl homoserine lactones helps in cell-to-cell communication in Gram-negative bacteria and brings enhanced cultivability of marine bacteria (Bruns et al. 2002). Presence of cAMP (cyclic adenosine monophosphate) also increases the resuscitation (recovery of cultivability) of starved marine bacteria (Bruns et al. 2002). Anaerobic degradation of hydrocarbons can take place in environments where oxygen concentration is often limiting such as mangroves, aquifers, and sludge digesters. Despite the absence of oxygen for the activation of hydrocarbons in anaerobic species, diverse metabolic pathways exist which help petroleum hydrocarbon degradation. These

species utilize varied terminal electron acceptors such nitrate, sulfate, or Fe (III) in place of oxygen (Peixoto et al. 2011). Phototroph–heterotroph interactions are also significant in the context of degradation of petroleum fractions. Many algae produce hydrocarbons and nearly all produce volatile hydrocarbons, isoprene which may be essential in the sustenance of hydrocarbon degraders in absence of oil spill (Shaw et al. 2010; McGenity et al. 2012), thus it is unsurprising that *Alcanivorax* spp. are often associated with micro- and macroalgae (Green et al. 2004; Radwan et al. 2010). Moreover, PAHs tend to strongly adsorb to the cell surface of marine microalgae encouraging the growth of associated microbes (Binark et al. 2000). Algae produce O_2 and encourage the growth of hydrocarbon degraders, which in turn produce CO_2 and reciprocally encourage the growth of algae. Algal biosurfactants also contribute to the emulsification of hydrocarbons (Cohen 2002).

Successful bioaugmentation requires the seeding of microbes best suited to degrade the spilled oil. Choice of the microbes is entirely based on the type of oil, type of primary oil response undertaken, and characteristics of the area under the oil spill. Most of the organisms used for bioaugmentation are obtained from enriched cultures from previously contaminated sites or similar strains enriched in laboratories. Bioaugmentation has not been very effective in cleaning up oil spills. Some of the reasons for this failure are: poor survival or low activity of laboratory strains because of sudden exposure to environmental stress, absence of mutualistic interspecies interaction that improves bioavailability and biodegradation, biomass-limiting nutrients (N and P), and predation by protozoa. Moreover, in bioremediation strategies, the focus is on biodegradation strains and use of a single species for this purpose. Use of microbial consortium with complementary catabolic pathways and the ability to adapt to local environment, disperse, and increase the bioavailability of the pollutants has been proved to be more successful in the bioremediation of simulated oil spills (Gallego et al. 2007; Jacques et al. 2008).

Phytoremediation

Wetlands serve as distinct ecological areas with high biodiversity and productivity and which provide protection agencies against shoreline erosion (Mitsch and Gosselink 1986). Marshy vegetation is easily damaged by fresh light oils and oils tend to coat prop roots of mangroves which are essential for their respiration. Mangroves require decades to grow hence, once damaged they cannot be easily replaced. In light of the above discussion, it is of utmost importance to save wetlands from detrimental effects of oil spills (EPA 1999b). Phytoremediation is one of the bioremediation tools that can be utilized for this purpose. It is defined as the employment of plants and/or associated microbes to eliminate, contain, or render harmless environmental pollutants *in situ*. This strategy is environmentally friendly, cost effective, and is proved to be effective for heavy metals, radionuclides, and organic pollutants (Cunningham and Ow 1996; Cunningham et al. 1996; Dzantor et al. 2000; Njoku and Oboh 2009). However, in the context of oil spill cleanup, phytoremediation is best suited for the remediation of oil-contaminated marshes and shorelines and not for open systems such as seas. Plants through their roots oxygenate their rhizosphere and exude organic compounds, which ultimately stimulate the activity, density, and diversity of microbes in the rhizosphere. Plants have also been documented to initiate fungal degradation of PAHs through rhizosphere effect (McGenity et al. 2012). Studies on salt marsh grass, *Sparta patens* indicate its potential to phytoremediate oil spills. *S. Patens* could survive 320 mg oil/g dry sediment and, at oil doses between 40 and 160 mg/g, the oil degradation was found to be significantly higher than in control samples (Lin and Mendelssohn 2008). Rhizosphere-associated bacteria of mangroves have been studied and are found to promote plant growth as well as oil degradation (do Carmo et al. 2011). *Glycine max* (soyabean) was reported to grow in oil-contaminated soil and also enhance the degradation of crude oil. It was reported to reduce the oil toxicity as observed by the growth of weeds in soils supplemented by *G.max* and their absence where

no *G.max* was planted (Njoku and Oboh 2009). In a study, Liu et al. found that typical ornamental species including *Gaillardia aristata, Echinacea purpurea,* Fawn (*Festuca arundinacea Schreb*), Fire Phoenix (a combined *F. arundinacea*), and *Medicago sativa L.* can be adopted in the phytoremediation of oil-contaminated soil. Since these do not enter the food chain and provide ornamental cover to the revegetated land, they can serve as better alternative than using crops for phytoremediation of petroleum hydrocarbons (Liu et al. 2012). Phytoremediation might be most effective during the vegetative growth stages as greater abundance of hydrocarbon-degrading bacteria containing *alkB* and *tol* genes was observed at these stages in the phytoremediating species, *Phragmites australis* (Nie et al. 2011). A greater understanding of rhizosphere-associated bacteria and need of bioaugmentation for *in situ* bolstering of hydrocarbon-degrading bacteria are required to chart out potential candidates for the phytoremediation of oil spills in wetlands and coastal zones.

Role of genetically modified organisms

The use of GMOs, especially designed for petroleum hydrocarbon degradation, has been given serious consideration. However, due to the complex nature of oil and accompanied change in its components due to weathering and multifaceted and interconnected metabolic pathways integral to degradation of petroleum hydrocarbon, this field has remained in infancy. Moreover, GMOs do not find public acceptance and there are only few takers of such organisms. Regulatory bodies across the world have strong reservations against GMOs and the recent decline in funding of bioremediation research projects has also further impacted this field (Fox 2011). Thus, only few studies are currently available. *Cycloclasticus* strain A5 is capable of growing on naphthalenes, dibenzothiophenes, phenanthrenes, and fluorenes with or without alkyl substitution. The genes encoding the a and b subunits of an iron–sulfur protein, a ferredoxin and a ferredoxin reductase, respectively termed *phnA1*, *phnA2*, *phnA3*, and *phnA4* were isolated from it. Transformed *Escherichia coli* cells containing the *phnA1*, *A2*, *A3*, and *A4* genes were able to convert phenanthrene, naphthalene, methylnaphthalene, dibenzofuran, and dibenzothiophene to their hydroxylated forms. Furthermore, these *E. coli* cells also transformed biphenyl- and diphenylmethane, which are ordinarily the substrates of biphenyl dioxygenases (Kasai et al. 2003; Harayama et al. 2004). Exhibition of such broad substrate specificity can make *Cycloclasticus* the key player in oil-contaminated sea water (Harayama et al. 2004). Bacteriophages offer steady supply of nutrients needed for bacterial hydrocarbon degradation through phage-mediated biomass turnover. Phages, together with various mobile genetic elements, are also important tool for dissemination of valuable genetic material, including hydrocarbon-degradation genes and the generation of new catabolic pathways via lateral gene transfer (Herrick et al. 1997; Top et al. 2002). Thus, studies based on phage-mediated gene transfer can also be targeted as potential tool for transfer of genes into indigenous species. PAH detoxification can also be achieved by laccase enzyme. Taking this into account, laccase from *Myceliophthora thermophila* (MtL) was successfully expressed in *Saccharomyces cerevisiae* with the help of directed evolution in an attempt to bioremediate petroleum spills (Bulter et al. 2003). Often, the role of GMOs in bioremediation is considered as a lost cause due to steep impediments in this field. However, as Ananda Chakraborty says "oil spills are old love" (Fox 2011), this field is not dead yet; synthetic biology approaches are being made to understand the natural pathways better. Without this knowledge, it is impossible to tweak the existing underperforming metabolic pathways in the potential candidates for bioremediation (Fox 2011).

9.5 Perspective

The most crucial phase after an oil spill is the first few days. Oil if not dispersed, reclaimed, or evaporated, tends to sediment to the benthic region where it can remain for decades. Therefore, it is imperative that resources, norms, and logistics necessary for the *in situ* trial should be readily accessible to enable a quick decision to initiate

primary and secondary oil spill response. Use of conventional oil spill response is not always advisable as in the case of Deepwater Horizon BP oil spill, application of chemical surfactant, Corexit increased the toxicity of oil by 52 times (Rico-Martinez et al. 2013). Bioremediation does not remove toxic compounds from one to another environment, rather it converts them into simpler compounds that enter the biogeochemical cycle. Thus, bioremediation that is often visualized as a primary polishing step after conventional mechanical cleanup should be reconsidered after carefully evaluating its utility and potential harm on case by case basis (Atlas and Hazen 2011).

Intelligent application of genomic and molecular tools in understanding microbial community metabolic networking will be influential in unraveling hydrocarbon degradation in the context of petroleum hydrocarbon degradation. Use of enzymatic remediation has also been suggested as a suitable alternative to bioremediation, since it does away with the risk of introduction of exotic or GMOs to new environments. The use of extremozymes in cold or hypersaline environments is beneficial, since it does not suffer microbial competitiveness (Peixoto et al. 2011). Genome sequencing of *A. borkumensis* showed the presence of a membrane protein that brings iron into the cell (Schneiker et al. 2006). Information such as these can translate failure into success when we know beforehand the ingredients of the cocktail needed to stimulate these oil degraders. After all at stake are our habitat and future, in order to save them, we have to be ever vigilant in preventing pollution and committed in our pursuit of a greener and cleaner technology that is ecofriendly, useful, as well as cost effective.

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Bioelectrochemical Systems (BES) for Microbial Electroremediation: An Advanced Wastewater Treatment Technology

10

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Abstract

Bioelectrochemical systems (BES) have been employed for various applications in recent years including energy production, wastewater treatment, electrosynthesis and desalination. The present chapter emphasizes the advantages and potential applications of BES for the remediation of recalcitrant pollutants present in various types of wastewaters. Bioelectricity generated from the treatment of these wastewaters is an additional energy output from the process along with the possible environmental solution. Since, the treatment mechanism of BES is combination of both microbial and electrochemical reactions, the process can be termed as microbial electroremediation. The current chapter depicts the principles of bioelectrochemical remediation, possible mechanisms at anode and cathode. Further, a comprehensive overview on different types of wastewater as well as nutrients, pollutants and toxic substances, utilized as electron donors or acceptors for their treatment, is discussed in detail under different categories. Microbial electroremediation is still an emerging field of science aimed at harnessing energy from wastewater treatment and it has a potential to boon the waste remediation with net positive energy gain.

Keywords

Microbial fuel cell (MFC) · Microbial electrolysis cell (MEC) · Nutrient removal cathodic and anodic mechanism · Microbial desalination · Azo dye degradation · Bioelectricity

10.1 Introduction to Bioelectrochemical Systems

Globally, huge amount of capital and resources are being spent for treating trillions of litres of wastewater annually, consuming significant amounts of energy. The new strategies of environmental management are focused specifically

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on the energy-efficient or energy-gaining processes for the waste remediation. Bioprocess engineering certainly comes under environmentally benign treatment strategies. Bioelectrochemical systems (BES) are multidimensional systems that can accomplish significant change in wastewater treatment by considering them as renewable energy based repository units (ElMekawy et al. 2014). Microbial fuel cells (MFCs) and microbial electrolysis cells (MECs) are two examples of BES, which are rapidly developing towards environmental sustainability. Conventional treatment processes cannot handle some of the wastewater components, especially coloured compounds (dyes), complex organic and inorganic chemicals, toxic substances, etc. due to the metabolic limitations of the microbes. Similarly, the existing electrochemical process also has some limitations in treating this type of waste in terms of energy input and additional waste generation. At this point, BES combines both biological and electrochemical processes for waste remediation along with the energy generation in terms of electricity, hydrogen or other useful chemicals. This multifaceted application of BES has been attracting several researchers across the globe. Figure 10.1 shows the increasing interest in this field of research in terms of publications from the past decade. Combination of multiple disciplines, viz. environmental science, biotechnology, microbiology, electrochemistry, etc., involved in the development of this particular area. Microbial electroremediation is aimed at the use of biological energy generated during BES operation for the extended treatment of wastewaters and specific pollutants present in wastewater. Various types of bioreactors have been designed and operated in the literature for the targeted processes. Several microbial species are reported in such bioprocesses for their specific function towards waste/pollutant treatment. In this chapter, basic principles of microbial electroremediation processes at both the electrodes (anode and cathode) of BES are discussed in detail. Further to this, different types of wastewater used in BES are discussed followed by a comprehensive discussion on the specific pollutant, viz. nutrients, metals, dye, removal and microbial desalination processes.

10.2 Basic Mechanisms of BES

Energy generation in microbial metabolism, including both anabolism and catabolism, is combination of fermentation (substrate oxidation) and respiration (reduction) processes. This process requires an electron source (substrate) which falls in the metabolic flux of the microbe (can be utilized by the microbe) and a strong/weak electron sink (acceptor) to complete the electron transport chain. Separating these two processes (fermentation and respiration) by an ion permeable membrane (optional) in a system equipped with electrodes (artificial electron acceptors) creates an environment to harness the energy generated by the microbe in the form of current density, against the potential difference generated between these two processes (Venkata Mohan et al. 2014a). The microbes utilize the available substrate (fermentation) generating the reducing equivalents [protons (H^+) and electrons (e^-)] at anode. Protons are transported to cathode through the solution electrode interface across ion selective membrane, generating a potential difference between anode and cathode against which the electrons will flow through the circuit (current) across the external load (Pant et al. 2012). The reducing equivalents generated during BES operation have multiple applications in the energy generation as well as waste remediation areas. Broadly, BES application can be classified as a power generator, wastewater treatment unit and system for the recovery of value-added products. Reducing equivalents generated from substrate metabolism gets oxidized in presence of an electron acceptor at a physically distinct component of BES (cathode) and results in power generation. Alternatively, when the waste/wastewater functions as an electron donor or acceptor, its remediation gets manifested either through anodic oxidation or cathodic reduction under defined conditions (Pant et al. 2010). Very recently, reduction of some substrates or carbon dioxide (CO_2) as electron acceptors during BES operation is also being reported, increasing its commercial viability (Srikanth et al. 2014). The current chapter fully focuses on the remediation aspects of BES with respect to different wastewater, specific pollutants and desalination.

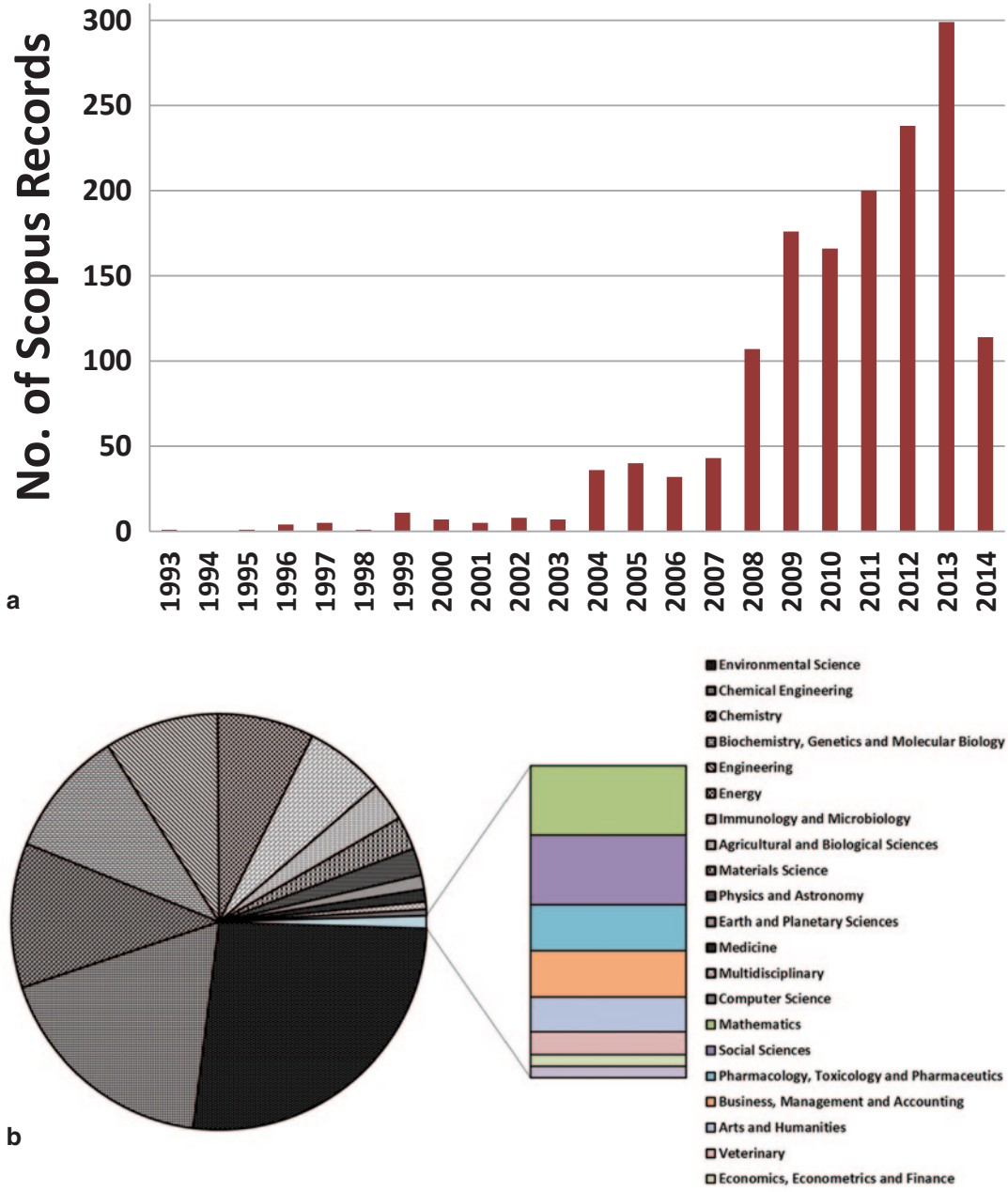


Fig. 10.1 Scopus search depicting the prominence of research on bioelectrochemical treatment of wastewater in BES. **a** Number of articles yearwise. **b** Various sciences' contribution in BES showing it as pluridisciplinary research area. (Keywords for search: bioelectrochemical systems OR microbial fuel cells AND wastewater treatment on 19 May 2014)

10.2.1 Anodic Mechanism

Anode chamber plays a pivotal role in treatment of wastewater, mainly through the microbial metabolism and partly due to the induced electrochemical oxidation (EO) mechanism. Apart from EO, direct and indirect anodic oxidation (DAO and IAO) mechanisms are two possible ways

described for the pollutants treatment at anode of BES (Venkata Mohan and Srikanth 2011). Substrate degradation in the anode chamber is mainly influenced by the oxygen in the cathode chamber acting as terminal electron acceptor (TEA). The strong electron acceptor conditions at cathode enhances the electron flow in the circuit and in turn their release from the microbial metabolism of wastewater. The presence of oxidizing agents (which gain electrons) like chlorine, bromine and ozone increases the potential differences between electrodes and thus the redox potential of the system which in turn favours EO, resulting in both pollutant as well as carbon removal. In general, pollutants are adsorbed on the anode surface and get destroyed by the anodic electron transfer reactions during DAO, while during the IAO, these pollutants will be oxidized by the oxidants (primary and secondary) formed electrochemically on the anode surface under in situ biopotential. DAO facilitates formation of primary oxidants which further react on the anode yielding secondary oxidants such as chlorine dioxide and ozone, which will have significant positive impact on treatment, especially for colour removal efficiency. Furthermore, the reactions between water and free radicals near the anode yields secondary oxidants, viz. nascent oxygen, free chlorine and hydrogen peroxide, hypochloric acid, etc. which can also help in colour/organic oxidation (Venkat Mohan and Srikanth 2011). On the other hand, these pollutants can also act as mediators for electron transfer between microbes and anode which helps in their reduction with simultaneous power enhancement.

Initially, the simple organic fraction of waste will be oxidized at anode through microbial metabolism releasing reducing equivalents [e^- and H^+], which interact with the water molecules under in situ biopotential forming hydroxyl radicals (Israilides et al. 1997; Venkat Mohan and Srikanth 2011). These hydroxyl radicals will get adsorbed onto the active sites of anode and initiates DAO, either alone or in combination with free Cl^-, (chloro hydroxyl radical) if present in wastewater. Oxygen and water molecules react with the radicals adsorbed on the electrode, forming secondary oxidants (O_3, ClO_2 and H_2O_2) which initiate the IAO process. As the concentration of primary oxidants increases, the formation of secondary oxidants also increases in the electrolysed solution (Israilides et al. 1997; Wilk et al. 1987). These oxidants also have a quite long life which can also diffuse away from the electrodes to the solution and enhance the IAO process (Israilides et al. 1997). Efficient cathodic reduction reaction also can influence the substrate degradation at anode by inducing the oxidation reaction (induced EO) at anode under in situ developed bio-potential.

General mechanism of oxidants formation:

$$O + e^- \rightarrow O^* \tag{10.1}$$

$$O^* + E[] \rightarrow E[O^*] \tag{10.2}$$

$$S + E[O^*] \rightarrow S - O^* + E[] \tag{10.3}$$

where 'O' is oxidant, 'O* is excited oxidant', 'E[]' is the electrode with active site and 'S' is the substrate.

Formation of primary oxidants:

$$H_2O + E[\,] + Cl^- \rightarrow E[ClOH^-] + H^+ + 2e^- \tag{10.4}$$

$$H_2O + E[ClOH^-] + Cl^- \rightarrow Cl_2 + E[\,] + O_2 + 3H^+ + e^- \tag{10.5}$$

$$C + E[ClOH^-] \rightarrow E[\,] + CO + H^+ + Cl^- + e^- \tag{10.6}$$

Generation of secondary oxidants:

$$H_2O + E[ClOH^-] + Cl_2 \rightarrow E[\,] + ClO_2 + 3H^+ + 2Cl^- + e^- \tag{10.7}$$

$$O_2 + E[OH^-] \rightarrow E[\,] + O_3 + H^+ + e^- \tag{10.8}$$

$$H_2O + E[OH^-] \rightarrow E[\,] + H_2O_2 + H^+ + e^- \tag{10.9}$$

10.2.2 Cathodic Mechanism

Similar to anode, cathode is also involved in effective remediation of waste streams and pollutants such as azo dyes, nitrobenzene, nitrates, sulphates etc. Hypothetically, it can be assumed that, these pollutants act as terminal electron acceptors at cathode to make the electrical circuit closed in absence of oxygen. However, their function as electron acceptor is based on the thermodynamic hierarchy. Unlike anode, cathode chamber can be maintained under different microenvironments (aerobic, anaerobic and microaerophilic) to increase the treatment efficiency based on the nature of pollutant (Venkat Mohan and Srikanth 2011; Srikanth et al. 2012). Generally, oxygen is considered as the TEA at cathodes but in biocathodes, microorganisms will be used as the catalyst for the terminal reduction reaction. The biological redox tower shows a wide range of TEA for the possible cathodic reduction reactions. Depending on the terminal electron acceptors adopted at cathode, they can be classified as aerobic and anaerobic biocathodes (He and Angenent 2006). However, the efficiency of treatment as well as energy output vary among the microenvironments studied.

In the case of aerobic biocathode operation, aerobic oxidation process undergoing in the cathode chamber results in higher substrate removal. Consumption of $H^+$and e^- during the aerobic metabolic process (along with oxygen as TEA) will be higher and this in turn helps in additional substrate removal efficiency. Manifestation of gradual substrate oxidation at anode in response to the cathodic function facilitates the maintenance of cell potential for longer periods and this also helps in increasing the treatment efficiency (Srikanth and Venkat Mohan 2012). Multiple treatment processes undergoing simultaneously in the system initiates the bioelectrochemical reactions that result in increased pollutant removal. Oxygen as terminal electron acceptor encourages the release of hydroxyl (OH^-) ion at cathode and increases the formation of oxidation species (Fig. 10.2). Formation of oxidation species and radicals at cathode under biopotential increases the possibility of other pollutant removal at cathode (Aulenta et al. 2010). The oxidizing species also react with primary cationic species, viz. $Na^+$and K^+, under biopotential leading to their removal as salt. Biocarbonates will be formed from the reaction between CO_2 (from air sparging or aerobic metabolism) and water which further reacts with the cationic species forming respective salts. These salts can also act as buffering agents (Eqs. 10.10–10.12) decreasing the chances of strong redox shifts (pH changes) during constant reduction reactions as well as the formation of oxidizing species. The possibility of salt removal at BES cathode under in situ biopotential through salt splitting mechanism was depicted in Eqs. 10.13 and 10.14.

$$\mathrm{H_2O + c - a \rightarrow c^+ + a^- + H^+ + OH^-} \tag{10.10}$$

$$\mathrm{H_2O + CO_2 \rightarrow H^+ + HCO_3^-} \tag{10.11}$$

$$\mathrm{HCO_3^- + c^+ + H^+ + OH^- + e^- + O_2 \rightarrow cHCO_3 + H_2O} \tag{10.12}$$

where ‘c’ is cationic species and ‘a’ is anionic species

$$\mathrm{c - a + E[] + H_2O \rightarrow E\left[c^+\right] + a^- + H^+ + OH^-} \tag{10.13}$$

$$\mathrm{E\left[c^+\right] + H^+ + OH^- + e^- + O_2 \rightarrow E[] + c - OH + H_2O.} \tag{10.14}$$

Maintenance of cathodic pH is very crucial to sustain the microbial activity at cathode, *in spite* of continuous reduction reactions. The in situ bicarbonate buffering mechanism formed at cathode helps to overcome this drop in cathodic pH which is essential in continuing the reduction reaction as well as maintaining the metabolic activities of microbes. Physiologically favourable redox conditions in the cathode chamber support the rapid metabolic activities of aerobic consortia, thus

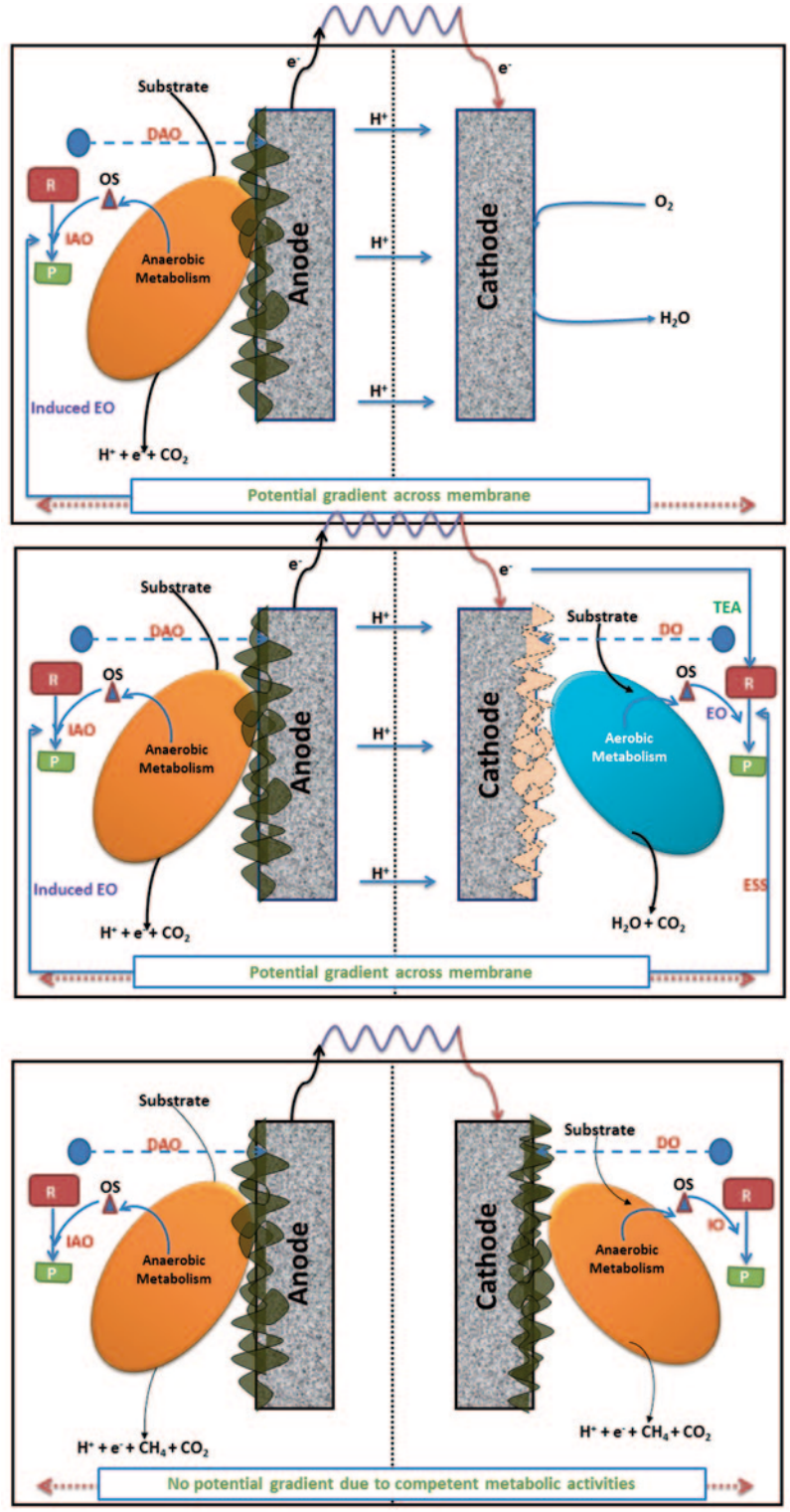


Fig. 10.2 Schematic illustration of the possible bioelectrochemical reactions happening at anode and cathode during BES operation (Venkata Mohan and Srikanth 2011)

R: Salt/Pollutant; P: Product; OS: oxidizing species; EO: Electrochemical oxidation; DO: Direct oxidation; IO: Indirect oxidation; ESS: Electrochemical salt splitting; DAO: Direct anodic oxidation; IAO: Indirect anodic oxidation; TEA: Terminal electron acceptor

resulting in higher substrate removal (Mahmoud et al. 2014; Torres 2014).

Similarly, the anaerobic biocathode chamber also supports the reduction reactions which help in the removal of pollutants and toxic components of wastewater, especially when they act as electron acceptors. Instead of oxygen, other substances like nutrients, viz. nitrogen, sulphur, and metal ions, viz. iron, manganese and chromium, will act as TEAs in the case of anaerobic biocathode. This helps in the removal of those toxic substances from the wastewater along with power generation (Clauwaert et al. 2007; Hamelers et al. 2010; Huang et al. 2011). Both the anode and cathode chambers function as anaerobic treatment units in this case except for the variation that the presence of electrodes in each chamber and connected in the circuit across an external resistance/load. Generally, the biopotential maintenance in this type of operation will be very low due to the fact that the microbes in both chambers follow the similar metabolic function and compete as electron donors, instead of one acting as acceptor. This situation will not allow the system to carry out the induced oxidation reactions. However, the strong reduction conditions prevailing in both the chambers support the substrate removal. When the wastewater contains a specific pollutant or component, viz. metal ions (Fe^{2+}, Mn^{2+}), dyes, nitrates, sulphates, etc., which can act as an electron acceptor, treatment efficiency will increase along with the power output.

On the other hand, the microaerophilic environment at cathode switches between aerobic and anaerobic microenvironments. This has an advantage over aerobic and anaerobic biocathode operations, especially in wastewater treatment sector. Some pollutants like azo dye need both the environments for complete mineralization. The anaerobic condition helps in splitting the azo bond, while the aerobic condition helps in mineralization of dye metabolites (Venkata Mohan et al. 2013). The lower DO levels maintained at cathode during this operation helps in initiating electrochemical oxidation reactions as well as maintaining strong reduction reactions. The survival of facultative microbes which can carry out both metabolic functions will increase the treatment efficiency (Srikanth et al. 2012).

10.3 Merits of BES in Microbial Electroremediation

The BES function as wastewater treatment unit has been gaining prominence more recently due to the higher efficiency of waste remediation compared to conventional anaerobic treatment process (Velvizhi and Venkata Mohan 2011; Mohanakrishna et al. 2010a). The principle of bioelectrochemical treatment (BET) relies on the fact that electrochemically active microorganisms can transfer electrons from a reduced electron donor to an electrode and finally to an oxidized electron acceptor generating power (Pant et al. 2013; Venkata Mohan et al. 2014b). Coupling of bioanode to a counterelectrode (abiotic/biotic cathode) will have positive influence on overall wastewater treatment efficiency along with energy recovery, which has to be tapped. The possibility of integrating diverse components, viz. biological, physical and chemical components, during BES operation provides an opportunity to initiate diverse reactions such as biochemical, electrochemical, bioelectrochemical, physicochemical, etc. which are cohesively termed as bioelectrochemical reactions. *In situ* generated biopotential helps in the enhancement of the degradability of different pollutants in both the anode and cathode chambers. Formation of oxidants and reactive species like OH^-, O^-, etc. is an added advantage of BES over conventional treatment systems, especially for the treatment of complex wastewater streams (Israilides et al. 1997; Mohanakrishna et al. 2010a). Sometimes the pollutants/components of wastewater themselves act as mediators in electron transfer. For instance, elemental sulphur present in the wastewater acts as a mediator at anode and converts itself to sulphate which is the easier form for degradation (Dutta et al. 2009). Similarly, azo dyes act as mediators and decolorize during reduction (Mu et al. 2009a) and estrogenic compounds get oxidized in BES system (Kiran Kumar et al. 2012). BES is also proven for considerable reduction of toxicity, colour

and TDS from wastewater, apart from carbon content (Mohanakrishna et al. 2010a; Pant et al. 2012; Venkata Mohan et al. 2014). Application of BES was also extended to treat solid waste, as well as toxic aromatic hydrocarbons under in situ biopotential (Venkata Mohan and Chandrasekhar 2011). Studies related to the mechanism of pollutant reduction and their role in electron transfer will give a spectrum of practical feasibility of this technology for the sustainable removal of toxic pollutants.

10.4 Wastewater Treatment

The nature of the substrate is regarded as one of the most important biological factors that can influence the treatment efficiency of BES, thus affecting the electron recovery. BES can utilize a wide range of substrates as electron donors/acceptors, including inorganic and organic molecules. However, the efficiency of electron recovery depends on the oxidation state of the electron donor and it's ratio to the microbe that can oxidize it. Among the simple substrates, glucose and acetate are most widely used anodic fuels but other simple substrates, viz. sucrose, starch, butyrate, dextran, peptone, ethanol, etc. were also evaluated in BES, with a prime motto of power generation. Apart from these simple substrates, BES also depicted versatility in utilizing a wide range of simple to complex organic wastes. Waste generated from different origins, viz. industries, commercial areas, residential areas, etc. were considered as potential electron donors in BES. The waste having higher biodegradability such as domestic wastewater, dairy based wastewater, food wastewater, vegetable waste, etc. will have good power generation capacity, while the industrial wastewater having low biodegradability will depict lower power output. Still wastewater is a potential substrate for MFC because of its dual advantages of converting negative valued waste into bioenergy.

10.4.1 Highly Biodegradable Wastewater

The characteristics of wastewater vary based on the raw materials used as the source for its generation. Domestic wastewater is considered to be simple and highly biodegradable in nature with low substrate load and hence its treatment in BES is highly efficient and faster but the power generation lasts only for few hours (Venkata Mohan et al. 2009a). Dairy wastewater rich in milk-based waste components such as lactate, proteins, etc. is also simple in nature and depicted higher treatment efficiency (Venkata Mohan et al. 2010a). Similarly, kitchen waste, food waste, vegetable waste, cheese waste, potato wastewater, etc. comes under highly biodegradable wastewater (Pant et al. 2010; ElMekawy et al. 2013). Wastewater from these sources mainly contains a lot of organic carbon in the form of carbohydrates and proteins, which can be easily degraded by almost all the bacteria. The energy gain from this type of wastes is also very high along with higher treatment efficiency. Moreover, microbes can also function effectively under higher organic loading rates this type of wastewaters, that avoids the necessity of feed dilution. Various types of biodegradable wastewater used in BES including their treatment efficiency are depicted in Table 10.1.

10.4.2 Complex/Low Biodegradable Wastewater

On the other end, BES can also handle highly complex and low biodegradable wastewater such as distillery-based wastewater, pharmaceutical wastewater, lignin-based wastewater etc. A detailed list of complex wastewater used as substrates in BES was provided in Table 10.1. The complex nature and low biodegradability of these wastewaters creates difficulty in conversion to the reducing equivalents, and moreover, the electrons and protons generated will be accepted by the pollutants/components of wastewater themselves (intermediary acceptors) for further oxidation, generating lower current densities. Colour removal from industrial wastewater such as dis-

Table 10.1 Detailed list of wastewaters studied in BES for their treatment

Wastewater	MFC configuration	Removal efficiency (%)	Reference
Highly biodegradable wastewater			
Domestic wastewater	Single chamber	66.7	Venkata Mohan et al. 2009a
Domestic wastewater	Double chamber	85	Jiang et al. 2012
Dairy wastewater	Single chamber	95.5	Venkata Mohan et al. 2010a
Dairy wastewater	Double chamber	90	Elakayya and Matheswaran 2013
Canteen based food waste	Single chamber	65	Goud et al. 2011
Chocolate Industry wastewater	Single chamber	95.5	Patil et al. 2009
Cereal wastewater	Double chamber	95	Oh et al. 2005
Potato processing wastewater	Three compartment tubular	62	Durruty et al. 2012
Rice mill wastewater	Double chamber	–	Behera et al. 2010
Cheese wastewater	Two chamber	59±9.3	Kelly and He 2014b
Cattle dung	Single chamber	–	Zhao et al. 2012
Dairy Manure	Three chamber	39.8#	Zhang et al. 2012
Low biodegradable wastewater			
Pharmaceutical wastewater	Single chamber	85.8	Velvizhi and Venkata Mohan 2011
Paper recycling wastewater	Single chamber	51	Huang and Logan 2008
Swine waste	Single chamber	86	Min et al. 2005
Brewery wastewater	Single chamber	87	Feng et al. 2008
Wheat straw hydrolysate	Double chamber	37#	Zhang et al. 2009
Distillery wastewater	Single chamber	72.8	Mohanakrishna et al. 2010a
Meat packing wastewater	Single chamber	87*	Heilmann and Logan 2006
Molasses wastewater	Single chamber cuboid MFC	53.2	Zhang et al. 2010
Molasses wastewater	Single chamber	59	Sevda et al. 2013
Vegetable wastewater	Single chamber	62.9	Venkata Mohan et al. 2010b
Composite chemical	Single chamber	66	Venkata Mohan et al. 2009b
Cassava mill wastewater	Single chamber	20#	Kaewkannetra et al. 2011
Slaughter house wastewater	Dual chamber	93	Katuri et al. 2012
Penicillin wastewater	Single chamber	90	Wen et al. 2011
Palm oil mill effluent	Two chamber	96.5	Cheng et al. 2010
Integration with fermentation process			
Mixed volatile fatty acids	Double chamber	39	Freguia et al. 2010
Fermented vegetable waste	Single chamber	80	Mohanakrishna et al. 2010b
Anaerobic food waste leachate	Single chamber	91	Li et al. 2013
Primary effluent	Single chamber	84	Yang et al. 2013
Fermented sludge	Single chamber	94	Yang et al. 2013
Dark fermentation effluent	Single chamber	72	ElMekawy et al. 2014

#Coulumbic efficiency; *Removal based on BOD

tillery and pharmaceutical wastewater is one of the critical aspects of wastewater but BES can easily remove colour at anode. Similarly, toxic halogens and other hydrocarbons are recalcitrant to aerobic remediation but they also can serve as electron acceptors in BES under anaerobic respiration. Solid wastes such as kitchen waste, food waste, vegetable waste, etc. were also can be utilized by BES without higher dilutions for the efficient treatment and power generation. Similarly, lignocellulosic biomass (Ren et al. 2007; Wang et al. 2009), dye wastewater (Sun et al. 2009),

landfill leachates (Kjeldsen et al. 2002; Zhang et al. 2008; Gálvez et al. 2009; Greenman et al. 2009), cellulose and chitin (Yazdi et al. 2007), and reed mannagrass (Strik et al. 2008), etc., also studied in MFC as electron donors. MFC can also be operated with the substrate in solid phase (Venkata Mohan and Chandrasekhar 2011).

10.4.3 Integrated Process for Additional Treatment

BES were also reported to be used for the degradation of effluent from fermentation and preliminary treatment processes, which contain the acid and solvent metabolites of first process along with the residual organic carbon. Few studies have been reported in the literature based on utilizing organic acids (pure/mixed) and effluents from different processes as primary substrates for the power generation in MFC. Table 10.1 depicts the comparative MFC performances in various studies reported. All these studies were carried out in a membrane based single/dual chambered fuel cell configurations. The conversion efficiencies of the system were similar to the regular fuel cells, indicating the higher efficiency of this system. All the studies have reported the coulombic efficiency (CE) between 12–75%, but the studies with real fermentation effluents range only between 12 and 45%, which is comparable to the regular wastewater. The biocatalyst enriched in presence of acid metabolites such as acetate and butyrate is reported to depict higher treatment efficiencies and power output which could effectively oxidize the higher concentrations of metabolites present in the effluents (ElMekaway et al. 2014; Mohanakrishna et al. 2010b). Especially, the treatment gained in this type of system is additional to the first process, which increases the valorization capacity of the waste.

10.5 Specific Pollutant Remediation

The possibility of utilizing waste as both electron donor and acceptor in BES, raised a choice of treating toxic and recalcitrant pollutants from wastewater. This treatment is in addition to the treatment that can happen with any other biological treatment process. The unique ability of chemotrophic (autotrophic/heterotrophic) microbes to utilize various pollutants at anode (electron donors) or at cathode (electron acceptors) facilitates effective remediation of these substances along with power generation. Removal of pollutants such as sulphide (Rabaey et al. 2006), nitrates (Clauwaert et al. 2007; Virdis et al. 2008), perchlorate (Thrash et al. 2007) and chlorinated organic compounds (Aulenta et al. 2007) were also reported in BES. In absence of oxygen, these compounds can also function as electron acceptors at cathode to accomplish the terminal reduction reaction (respiration) which facilitates their remediation. Some of the compounds, viz. sulphur, metals, estrogens, etc. can also act as electron carriers at anode which also results in their treatment (Chandrasekhar and Venkata Mohan 2012; Kiran Kumar et al. 2012). Nitrates are the best known electron acceptors after O_2 accounting for denitrification, while some microbes and archea use sulphate and elemental sulphur as their electron acceptor and reduce them. On the other hand, some microbes oxidize (assimilatory reduction) or reduce (dissimilatory reduction) metal ions as electron acceptors or donors. BES can also use the coloured dye compounds as alternate electron acceptors which results in their removal. Apart from these, nitrobenzenes, polyalcohols and phenols have also been studied for their treatment either through oxidation or reduction in BES. The comprehensive table depicting some of the specific pollutants treatment in BES was reported in Table 10.2. Detailed discussion pertaining to the removal of these specific pollutants was made in the further sections of this chapter.

10.5.1 Nitrogen/Sulphate Removal

Nitrogen is one of the common and key contaminants of wastewater. Its overload can cause eutrophication of a water body that also threatens aquatic life and biogeochemistry associated with water body (Camargo and Alonso 2006). Reduc-

Table 10.2 Detailed list of pollutants treated in BES at cathode or anode

Pollutants treated at anode				
Specific pollutant	*TEA at cathode*	*MFC configuration*	*Removal efficiency (%)*	*Reference*
Phenol	Oxygen	Double chamber	90	Luo et al. 2009
Polyalcohols	Oxygen	Single chamber	90	Catal et al. 2008
Indole	Ferricyanide	Double chamber	88	Luo et al. 2010
Estriol	Oxygen	Single chamber	54	Kiran Kumar et al. 2012
Ethenylestradiol	Oxygen	Single chamber	38	Kiran Kumar et al. 2012
2–fluoroaniline	Oxygen	Single chamber	43	Zhang et al. 2014
Pollutants treated at cathode				
Specific pollutant	*Electron donor at anode*	*MFC configuration*	*Removal efficiency (%)*	*Reference*
Nitrate	Acetate	Double chamber	84	Lefebvre et al. 2008
Sulfide	Acetate	Double chamber	87	Dutta et al. 2009
Perchlorate	Acetate	Double chamber	97	Butler et al. 2010
Azo dye	Glucose	Double chamber	77	Mu et al. 2009a
Nitrobenzene	Acetate	Double chamber	98	Mu et al. 2009b
Selenite	Acetate	Double chamber	99	Catal et al. 2009
Nitrophenols	Acetate	Double chamber	70	Zhu and Ni 2009
Pyridine	Glucose	Double chamber	95	Zhang et al. 2009

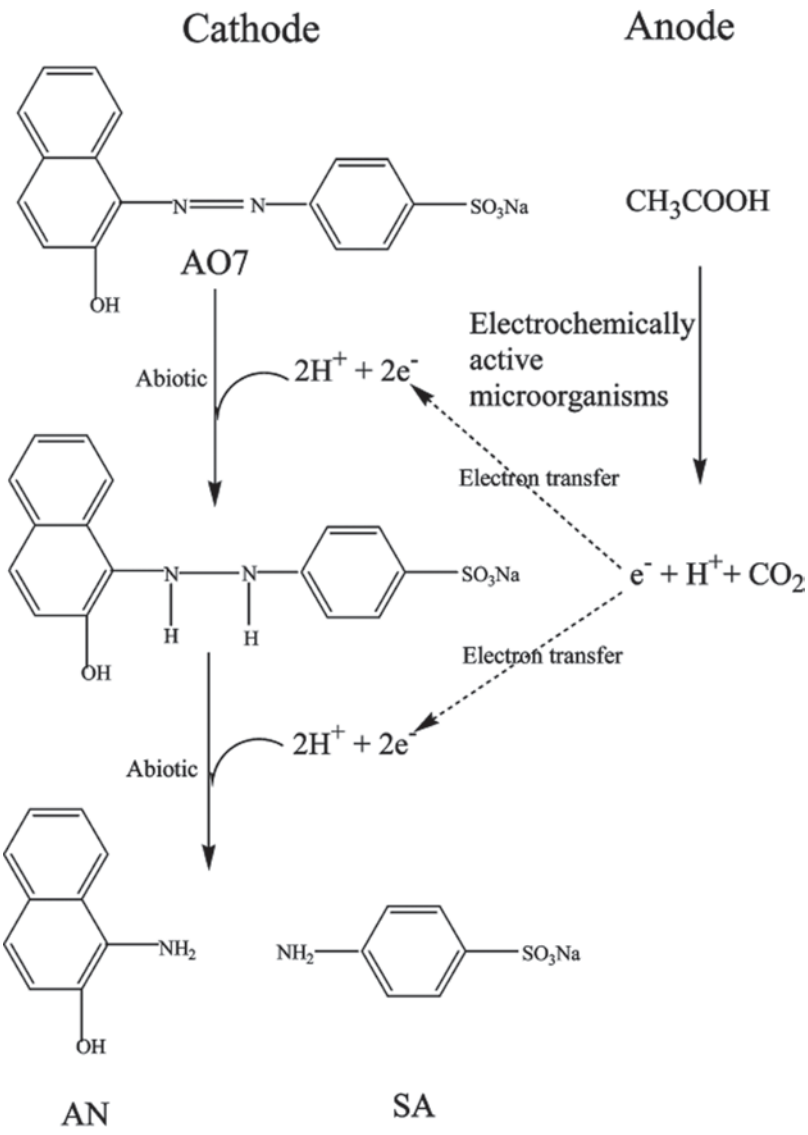
ing nitrogen concentration in the treated effluent is critical factor to achieve the concerned environmental regulations. Compared to physical and chemical methods, biological processes such as nitrification and denitrification are widely applied for nitrogen removal in wastewater due to their low cost and effectiveness (Peng and Zhu 2006). Integrating the electrochemical process with biological process (in BES) is found to be more cost-effective and efficient for nitrogen removal, especially from high nitrogen strength wastewater (Kelly and He 2014a). An investigation by Zhang and He (2012) resulted in more than 96% ammonium removal in 150 days using a dual cathode-tubular MFC consisting of two biocathodes to accomplish nitrification in its outer cathode and denitrification in the inner cathode while the total nitrogen removal was between 66.7 and 89.6%, largely affected by the remaining nitrate in the effluent of the inner cathode. This operation also resulted in 96% of COD removal. In another study, a submerged desalination denitrification cell (SMDDC) for in situ removal of nitrate from groundwater, production of electric energy and to treat wastewater was operated in subsurface environments. The SMDDC produced 3.4 A/m^2 of current density, while removing 91% of nitrate from groundwater within 12 h of hydraulic retention time (HRT) (Zhang and Angelidaki 2013). Clauwaert and Verstraete (2009) suggest that enhanced denitrifying biocatalytic activity requires appropriate pH-neutralizing actions since the bioelectrochemical active microorganisms tend to deteriorate their own environment. Continuous monitoring of cathode pH helps to achieve effective nitrogen removal.

The pharmaceutical and paper production wastewater contains higher concentration of sulphate, which is harmful to the environment and human health if not handled properly. Biological sulphate reduction process is energy intensive as it requires electron donors. Recently, MFCs, and MECs were observed as suitable process by using sulphate-reducing bacteria (SRB) for the treatment of sulphate-rich compounds (Su et al. 2012). As the SRB are sensitive to pH changes, it was also observed that pH 4.5 as the optimum for SRB in MFC. In the case of MEC operation, at cathode, sulphate reduction consumes H^+ ions which results in increase in pH (Coma et al.

2013). By employing the *Desulfovibrio desulfuricans* which is a sulphate-reducing bacteria demonstrated electricity generation along with 99% of sulphate removal (Zhao et al. 2008). Sharma et al. (2013) investigated various materials such as activated carbon fabric and stainless steel for cathodic SRB biofilm formation, and it was reported that stainless steel as the more suitable material for sulphate reduction.

10.5.2 Metal Oxidation/Reduction

Metal oxide-reducing bacteria have been discovered over the last 30 years. The microbes, capable for metal oxide reduction, were called as dissimilatory metal-reducing bacteria (DMRB). These bacteria have more interest due to their applications in geobiological phenomena, bioremediation and biotechnology. Organisms such as *Clostridium* (Park et al. 2001), *Geobacter* (Bond and Lovley 2003; Holmes et al. 2006), *Aeromonas* (Pham et al. 2003), *Rhodoferax* (Chaudhuri and Lovley 2003), *Desulfobulbus* (Holmes et al. 2004), and *Shewanella* (Chang et al. 2006) included in DMRB group. All of these DMRB have also been shown to produce current in MFC systems (Bond and Lovley 2003; Logan et al. 2006) as well as provens as good biocatalysts to produce higher current densities. *Shewanella oneidensis* MR-1 is a Gram-negative facultative anaerobe capable of utilizing a broad range of electron acceptors for bioelectricity generation. *S. oneidensis* MR-1 can reduce Mn(IV) and Fe(III) oxides and can produce current in MFCs. Deletion mutants of this bacteria were generated and tested for current production and metal oxide reduction was evidenced that cytochromes play a key role in bioelectricity generation (Bretschger et al. 2007). Metal oxidation is also possible in biocathode configured BESs. Microorganisms present on biocathode assist the oxidation of transition metal compounds, such as Mn(II) or Fe(II), for electron delivery to oxygen. In addition, bacteria in the cathode benefited the reaction by supplying oxygen. Rhoads et al. (2005) have operated a MFC in which glucose was oxidized by *Klebsiella pneumoniae* in the anodic compartment and biomineralized manganese oxides were deposited through electrochemical reduction reaction in the cathode compartment by *Leptothrix discophora*. The cathodic reduction reaction occurs directly by accepting electrons on graphite electrode surface. These depositions of manganese oxide do not need any mediators. It was also demonstrated that biomineralized manganese oxides are superior to oxygen by two times. To further explore the viability of such a biocathode, Shantaram et al. (2005) also used manganese anode sediment MFC, which is different from conventional MFCs. Here, the oxidation of manganese helps to drive the electrons from magnesium oxidation. On complete oxidation, the anode needs to be replaced. Due to the high redox potential of manganese oxide, this BES produced a maximum voltage of 2.1 V. The voltage was further amplified to 3.3 V, which was sufficient to power a wireless sensor. The study demonstrated, for the first time, the application of BES to power small electronic sensors and manganese compounds as promising biocathodes for sediment BES. Iron, which is also an abundant element also showed its function in biocathode reduction. Although iron compounds have been used as electron mediators in abiotic cathodes, previous studies have revealed that Fe(II) is oxidized to Fe(III) through microbial activity by *Thiobacillus ferrooxidans* (Nemati et al. 1998). Researchers have adopted this process to oxidize organics in an electrolytic cell in which electrical energy is converted into chemical energy, requiring an external voltage supply (Lopez-Lopez et al. 1999). In the cathode chamber of this reactor, *T. ferrooxidans* was grown to regenerate the ferric irons by obtaining energy from the reaction and methanol was oxidized in anode. A study by Lefebvre et al. (2013) used metal scraps as cathodes and it was found that metal scraps can be recycled in BES for energy generation. Even though this study was not focused on any remediation, but it is providing future possibilities microbial electroremediation of metals oxides.



Scheme 10.1 Proposed bioelectrochemical decolorization mechanism for AO7 was elucidated by Mu et al. (2009a)

10.5.3 Azo Dye Degradation

Residual dyes present in textile wastewater have attracted a lot of interest due to their intense colour which is also closely associated with toxicity and aesthetics of the discharged effluents (Pant et al. 2008; Venkata Mohan et al. 2013). Textile dyes exhibit high resistance to microbial degradation. Particularly azo dyes are readily converted to hazardous aromatic amines under anoxic conditions (Yemashova and Kalyuzhnyi 2006). These dyes are highly stable under light, during washing and also resistant to microbial degradation. The aromatic compounds with one or more –N=N– groups present in azo dyes makes them recalcitrant. Azo dyes and their break down products are toxic and mutagenic (Scheme 10.1; Mu et al. 2009; Solanki et al. 2013). About 10–15% of the dyes used in textile industry are discharged in the effluents. (Rajaguru et al. 2000). An electron donor is required for the anaerobic biological decolorization of azo dyes to create reductive conditions. Generally it can be an organic cosubstrate. The decolorization rate of conventional

anaerobic biological methods is very slow. Moreover, the cosubstrate addition makes the process noneconomical. Addition of organic cosubstrate also leads to the methane formation (van der Zee and Villaverde 2005; Mu et al. 2009).

The application of BES for azo dye degradation in cathode compartment is showing an advantage of BES processes. It was already known that in an electrochemical cell, the chromophoric linkage of azo dyes can be reduced by accepting the cathodic electrons. The resultant colourless aromatic amines are more biodegradable (Frijters et al. 2006). A similar mechanism prevails in BES, which acts for the degradation of azo dyes. (Mu et al. 2009; Ding et al. 2010). But the azo dye reduction occurs at high cathodic over potential that imparts system efficiency (Mu et al. 2009). Several dyes such as methyl orange, acid orange 7, active brilliant red X-3B, amaranth, congo red, etc. were studied for the degradation in BES (Table 10.3). The concentration of dyes was varied between 10–900 mg/l concentrations in single and double chamber BES. The reduction of dyes in a conventional biological reactor follows different decolorization mechanisms involving enzymes, low molecular weight redox mediators, chemical reduction by biogenic reductants like sulphide or a combination of these (Pandey et al. 2007). The mechanism of dye degradation in cathode is similar to the anaerobic anodic degradation, except that there is an additional mode of electron and proton transfer to the dye, through the external circuit and the membrane respectively. In BES, the colour removal was primarily observed due to biodegradation rather than biosorption by living cells (Sun et al. 2009). Mu et al. (2009) proposed the decolorization mechanism of AO7 (Scheme 10.1). At the anode, the substrate is oxidized by bacteria to produce protons and electrons, which are transferred to the cathode via proton exchange membranes and external circuit respectively. The azo bonds of dye are broken at cathode by using proton and electron generated in anode, resulting in the formation of toxic intermediates. Ding et al. (2010) reported on methyl orange reduction via photogenerated electrons in a BES containing an irradiated rutile-coated cathode.

The performance of a BES for decolorization depends on the concentration and the type of dye used. Mu et al. (2009) investigated the effect of concentration of azo dye acid orange 7 (AO7). Circuit configuration also showed a considerable effect on dye degradation. It was shown that during closed-circuit operation, decolorization efficiency decreased from 78 to 35% with an increase in influent dye concentration from 0.19 to 0.70 mM, while the dye decolorization rate increased from 2.48 to 4.08 mol m^{-3} NCC d^{-1} with an increase in the influent dye concentration from 0.19 to 0.70 mM, maintained at constant HRT and pH. The BES power output increased from 0.31 to 0.60 W/m^3 with increase in AO7 concentration from 0.19 to 0.70 mM. Sun et al. (2009) reported that the percent decolorization decreased with increase in ABR-X3 (Active Brilliant Red X-3B). The decolorization rate decreased slightly from 90 to 86% as ABRX3 concentration increased from 100 to 900 mg/L within 48 h. It was predicted that decolorization efficiency decreases with increase in dye concentration. Besides concentration of azo dye, other factors like operating pH, structure of dye, HRT, type of wastewater used in the anode and cathode etc., also influence the process of dye degradation in BES. These factors were also found to influence the power generation capacity of the BES.

10.6 Microbial Desalination

Application of BES in desalination of saline water and industrial wastewater is found to be a promising technology that utilizes the microbiological energy from the wastewater treatment to drive the ions through ion exchange membranes (IEMs), resulting in desalination (ElMekawy et al. 2014). This new method that can reduce or completely eliminate the electricity requirement for desalination is called as microbial desalination cell (MDC). The main feature of the MDC is that exoelectrogenic microorganisms produce electrical potential from the degradation of organic matter, which can then be used to desalinate water by driving ion transport through IEMs (Cao et al. 2009; Kim and Logan 2013). When

Table 10.3 List of microbial desalination studies with various configurations and operation parameters and the results

BES design	Anolyte	Catholyte	Saline water concentration	Desalination efficiency (%)	References
Three chamber	Sodium acetate	Ferricyanide	NaCl—35 g/l	93	Cao et al. 2009
Three chamber (CEM-MDC)	Sodium acetate in domestic wastewater	Tap water	NaCl—10 g/l	46.3	Ping et al. 2013
Three chamber (PEM-MDC)	Sodium acetate in domestic wastewater	Tap water	NaCl—10 g/l	78.7	Ping et al. 2013
Three chamber	Sodium acetate	Phosphate buffer solution	NaCl—20 g/l	63	Mehanna et al. 2010a
Three chamber	Sodium acetate	Phosphate buffer solution	NaCl—20 g/l	37	Mehanna et al. 2010b
Three chamber	Sodium acetate	Sulphuric acid	NaCl—30 g/l	100	Jacobson et al. 2011
Three chamber	Sodium acetate	Sulphuric acid	NaCl—30 g/l	11	Jacobson et al. 2011
Three chamber	Sodium acetate	Sulphuric acid	NaCl—35 g/l	94	Jacobson et al. 2011
Three chamber	Sodium acetate	Sulphuric acid	Sea salt—35 g/l	74	Jacobson et al. 2011
Three chamber	Dye wastewater	Ferricyanide in Phosphate buffer	NaCl—10–50 g/l	62	Kalleary et al. 2014
Three chamber	Sodium acetate	Phosphate buffer solution	NaCl—10 g/l	98	Luo et al. 2011
Three chamber	Municipal wastewater	Ferricyanide	NaCl and $NaHCO_3$—14.2 g/l	66	Luo et al. 2012
Three chamber	Xylose	Phosphate buffer solution	NaCl—20 g/l	55	Qu et al. 2012
Three chamber	Sodium acetate	Ferricyanide	Hard water- 0.22–2.08 g/l as $CaCO_3$	96	Brastad and He 2013
Two chamber MOFC	Sodium acetate	Sodium chloride	NaCl-35 g/l	35	Werner et al. 2013
Five Chamber	Sodium acetate	NaCl	NaCl—10 g/l	100	Chen et al. 2013
Stalk MDC	Sodium acetate	Phosphate buffer solution	NaCl—20 g/l	72	Chen et al. 2011
Stalk MDC	Sodium acetate	None	NaCl—35 g/l	44	Kim and Logan 2011
Stalk MDC	Sodium acetate	None	NaCl—35 g/l	98	Kim and Logan 2011
Bipolar MEDC[a]	Sodium acetate	NaCl	NaCl—10 g/l	86	Chen et al. 2012
Hydraulic-MDC	Xylose	Anode effluent	NaCl—20 g/l	97	Qu et al. 2013
Osmotic MDC	Sodium acetate	Ferricyanide	Sea salt—35 g/l	63	Zhang and He 2012
Osmotic MDC	Sodium acetate	Ferricyanide	NaCl—20 g/l	60	Zhang and He 2012
OsMFC Integrated with MDC	Sodium acetate	Acidified water	NaCl—10–50 g/l	96	Zhang and He 2013

OsMFC forward osmotic MFC, *MOFC* microbial osmotic fuel cell

[a] Microbial electrolysis desalination cell (MEDC) with bipolar membrane. The design includes an additional bipolar membrane (BPM)

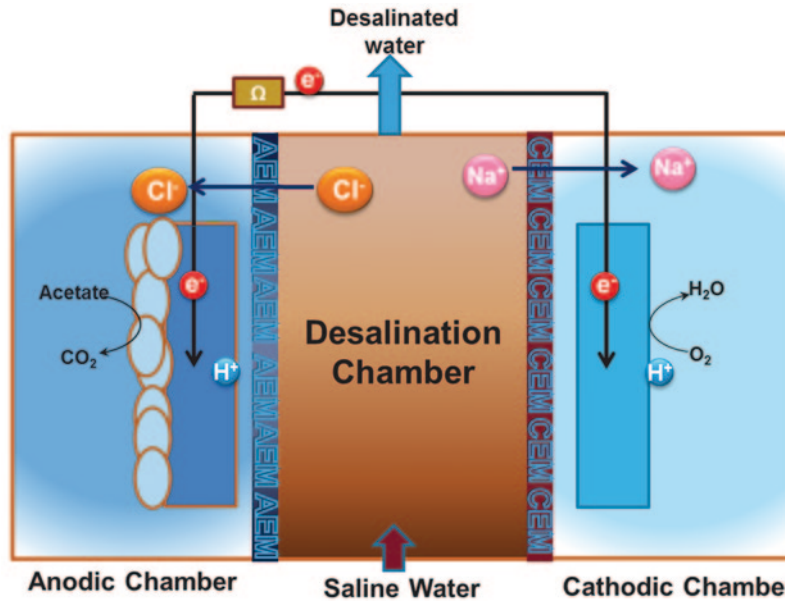


Fig. 10.3 Schematic diagram of microbial desalination cell (*AEM* anion exchange membrane, *CEM* cation exchange membrane)

wastewater is used as the source of the organic matter that required for development of potential gradient, the MDC can achieve three goals such as desalination, energy production and wastewater treatment (Kim and Logan 2013). Basic design of MDCs consists of three chambers separated by two membranes (Fig. 10.3, Table 10.3). As the desalination chamber is fixed in middle, both anode and cathode chambers were attached to the both sides of the desalination chamber. Anode and desalination chambers separated by anion exchange membrane (AEM) whereas, cathode and desalination chambers separated by cation exchange membrane (CEM). In another way, it can be viewed as inserting an AEM next to the anode and a CEM next to the cathode of a MFC, with the salt solution to be desalinated filled in the middle desalination chamber. The electricity-generating mechanism of MDC is similar to that of MFC. Current is generated by the bacteria on the anode from oxidization organics, and electrons and protons are released to the anode and anolyte, respectively (Logan et al. 2006; Chen et al. 2011). As cations are prevented from leaving the anode chamber by the AEM, anions (such as Cl^-) move from the middle desalination chamber to the anode. In the cathode, protons are consumed in the reduction reaction of oxygen, while cations (such as Na^+) in the middle chamber transfer across the CEM to the cathode. This proceeds to water desalination in the middle chamber, without consuming additional external energy. On top of it, electricity can be produced from the treatment of wastewater by exoelectrogenic bacteria in anode (Cao et al. 2009; Chen et al. 2011). The electrode reactions create an electric potential gradient up to about 1.1 V (open circuit condition with acetate as organic source at pH = 7 and partial pressure of oxygen in air is 0.2 atm) (Kim and Logan 2013). This potential drives the process of desalination as explained above.

On compilation of various studies for the minimum and maximum salinity removal by MDCs, it was found between 11% and 100%, respectively, using 30 g/L salt water (Jacobson et al. 2011). Salinity removals can be above 90% when the salt water concentration is increased to 35 g/L NaCl solutions which have similar conductivities like marine water (Cao et al. 2009; Kim and Logan 2011). However, very high salinity removals require large volume of nonsalty water in both anolyte and catholyte with 55–133 times the volumes of desalinated water (Kim and Logan 2013). The use of stacked MDCs can reduce the need for large amounts of nonsalty elec-

trolyte. Up to 98 % salinity removals from 35 g/L NaCl were achieved using stacked MDCs consist of five pairs of cells. These results imply that, for practical applications, MDCs are more likely to be used for partial salt removal from seawater. The requirement of fresh water also depends on the initial salinity of salt water. MDCs can also be used for brackish water desalination. Many studies were performed using acetate as the organic substrate in anode and phosphate buffer as catholyte. Few other studies also considered real-field wastewater as anolyte. Microbial oxidation of organics was the sole mechanism involved in electric potential in anode, whereas oxygen reduction reaction (ORR), ferricyanide reduction reaction and HER were considered for cathodic reduction mechanism. The maximum CE of MDC mechanism is found to be 80 % (Kim and Logan 2011)

A system consisting of two membrane-based bioelectrochemical reactors, an osmotic microbial fuel cell (OsMFC) containing a forward-osmosis (FO) membrane and MDC that had ion exchange membranes was designed to treat wastewater and to desalinate saline water. Both the reactors were coupled hydraulically. This design significantly improved desalination efficiency through both dilution in the OsMFC and salt removal in the MDC along with extended organic removal efficiency (Zhang and He 2013). Other systems were also developed with stalk design using more than one membrane pair between electrodes (Chen et al. 2011) and similar to the stack design used for electrodialysis (ED) desalinating systems. The IEM stack consists of alternating AEMs and CEMs, creating repeating pairs of desalting and concentrating (concentrate) cells (Chen et al. 2013). The MDC stacks should be designed potential energy generated by exoelectrogens with oxygen reduction and the resistance of individual cell pairs. Chen et al. (2011) found that the rate of desalination with two cell pairs was faster than that with three cell pairs by increasing the inter membrane distance compared to electrodialysis systems (0.2–3 mm) (Strathmann 2004). Many MDCs designed were having intermembrane distance between 1 and 2.4 cm, resulting in very high internal resistances (Mehanna et al. 2010a, b; Chen et al. 2011; Luo et al. 2011, 2012; Qu et al. 2012). Performance can be improved by reducing the internal resistance with minimized intermembrane distance. The internal resistance of an MDC also increases with the number of IEM pairs in the stack. In an ED system, the applied voltage is controllable depending on the stack size. In an MDC, however, the voltage used for desalination is limited to that produced by the electrode reactions, and therefore the voltage per cell pair decreases with an increase in the number of cell pairs (Kim and Logan 2013).

Another design, submerged microbial desalination denitrification cell (SMDDC) to in situ remove nitrate from groundwater and to produce electric energy along with treatment of wastewater (Zhang and Angelidaki 2013). The SMDDC can be easily applied to subsurface environments. When current was produced by bacteria on the anode, NO_3^- and Na^+ were transferred into the anode and cathode through anion and cation exchange membranes, respectively. The anode effluent was directed to the cathode where NO_3^- was reduced to N_2 through autotrophic denitrification. This design was removed 90.5 % of nitrate from groundwater in 12 h and generated 3.4 A/m^2 of current density. External nitrification was beneficial to the current generation and nitrate removal rate, but was not affecting total nitrogen removal (Zhang and Angelidaki 2013). Photosynthetic MDC was designed and operated using algae as catalyst in cathode (biocathode) which enhanced the COD removal and utilized treated wastewater as the growth medium to obtain valuable biomass for high value bioproducts (Kokabian and Gude 2013). The increase in salinity concentrations in anode chamber provide more favourable conditions for certain types of microbes than others resulting in enrichment of selective bacteria with simultaneous elimination of the bacteria that can withstand saline conditions (Mehanna et al. 2010). Integration of multiple bioprocess with diverse products can be beneficial in enhancing the sustainability of microbial desalination cells. Besides advantages of MDCs in desalination along with wastewater treatment at low energy consumption, few limitations were also associated. They can be listed as salt removal can be very high (>95 %) but it

requires large amount of wastewater and fresh water, low current densities, pH, membrane integrity and fouling, and safety issues. Addressing these issues with relevant investigations helps to commercialize MDC as the technology (Kim and Logan 2013; Ping et al. 2013).

10.7 Future Directions

Among the multifaceted applications of BES, treatment of recalcitrant pollutants present in wastewaters is quite interesting and already few studies have been reported with synthetic as well as real field substrates. The unique ability of these systems to treat complex pollutants, which are difficult to treat in conventional processes, is based on the integrated function of microbial metabolism with electrochemistry in a single reactor. Important fact is that the application of BES for the removal of toxic pollutants and xenobiotics is currently being extensively studied to enhance the treatment efficiency. Experiments with real-field wastewater differs a lot compared to the synthetic pollutants, especially in terms of energy recovery. Application of BES for the treatment of real-field wastewater should be more focused, considering the energy recovery as one of the objective, to make the system/process economically viable. Treatment of petroleum based chemicals such as aromatic hydrocarbons and pharmaceutical based wastewater are some of the burning problem of the industrial sector. Application of BES to treat complex structures to simple carbon chains (breaking aromatic rings) would be very interesting. Similarly, application of BES for the treatment of solid wastes such as kitchen-based, vegetable, slaughter house, municipal etc., would be very innovative and reduces the pretreatment costs. Treatment of chlorinated aliphatic hydrocarbons such as trichloroethene (TCE) and perchloroethylene, widely used solvents and degreasing agents, is also being studied by few researchers in BES. Detailed studies towards complete elimination of these highly toxic substances (carcinogenic also) from being disposed into soil and groundwater by treating them in BES would be highly interesting. On the other hand, BES can also be integrated to the effluents of conventional treatment process (rich in acid metabolites) to generate value added chemicals and solvents under small applied potential. Multiple advantages of BES are mainly limited by the problems in upscaling, especially with the design issues. Working in the direction of constructing BES to treat large volumes and higher loading rates is very important to make this technology competitive to the existing conventional processes.

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