BIOREMEDIATION TECHNOLOGY

RECENT ADVANCES

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Top – Effluent channel let out by industrial belt (Chembur, Mumbai, India).
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I dedicate this book to my mother

POWARA

for the inspiration, motivation and blessings

Preface

Global society in 21st century is facing the challenge of improving the quality of environment. Environmental pollutants have become major global concern due to rapid growth of industrialization, urbanization, modern agricultural development and energy generation. These resulted in indiscriminate exploitation of natural resources for fulfilling the human desires and needs, which have contributed in disturbing the ecological balance on which the quality of our environment depends. Human beings in true sense are the product of their environment. Man-environment relationship indicates that pollution has a social origin. The modern technological advancements in chemical processes have given rise to new products and new pollutants in much abundant level which are above the self-cleaning capacities of environment. One of the major issues in recent times is the threat to the human life caused due to the progressive deterioration of the environment. The contamination of ground water, surface water, soil and air with hazardous and toxic chemicals is one of the major problems the industrialized world faces today. As far as the solution to this problem is concerned, there is an urgent need to develop technologies that consume fewer resources, less time and would be environmental friendly. Therefore, biological approaches received great deal of attention in the recent years. One among the effective biological approaches to deal with the environmental contamination is bioremediation. Bioremediation is an attractive and potential alternative for treatment of contaminated environment. Bioremediation has been proved effective for treating soil and water contamination at numerous sites throughout the world, and is accepted as a viable remediation technology by the United States Environmental Protection Agency, Environment Canada, and other regulatory agencies worldwide. Around the world bioremediation technologies are categorized as the "innovative technologies".

This book discusses bioremediation technology based remediation used on genetically modified microorganisms to restore contaminated sites and protect the environment. It studies the opportunities for more efficient biological processes in molecular biology and ecology. Notable accomplishments of these

studies include the cleaning up of polluted water and contaminated land. The book identifies the bioremediation techniques other than traditional methods of waste treatment such as incineration, absorbent/adsorbent techniques, catalytic disruption, and destruction of pollutants etc. The book discusses in detail microorganisms that enzymatically attack the pollutants and convert them into harmless products. It analyses biodegradative pathways and biotransformation manipulations through plasmid strains of microorganism, employing recombinant DNA technology. The book includes invited papers by eminent contributors who provide cost effective bioremediation strategies to immobilize contaminants for cleanup of environment.

Bioremediation technology summarizes Introduction to Bioremediation; Biodegradation techniques for pesticides treatment; Remediation of low level nuclear waste; Environmental nanotechnology for remediation of contaminants; Lignins and polyphenols in bioremediation; Biosorption in environmental remediation and Genetic engineering. It also describes application for remediation of environmental contaminants; Microorganisms and genes in the environment; Ecology of microbial consortium for biodegradation; Global status of environmental pollution; Improving plants for acquisitism of heavy metals; Bioengineering for bioremediation; Bioremediation of hydrocarbons in the environment; Bioaccumulation and biotransformation of heavy metals; Genomic approaches for bioremediation and Recombinant DNA technology for bioremediation. The book is directed towards postgraduate students in biotechnology/life sciences/environmental sciences/biosciences and researchers in universities, research institutes and industries.

I express my gratitude to honourable Vice Chancellor, Prof. Vijay Khole, University of Mumbai and Pro-Vice Chancellor, Prof. A.D. Sawant for providing the necessary facilities and encouragement to compile this volume.

I would like to acknowledge my family members with love and affection in particular my wife Dr. (Mrs.) Kalpana, children Jaya, Jyoti and Vinay and brothers Sacchidanand, Dilip, and Pawan.

The technical support and constant encouragement received from my PhD students from the Environmental Biotechnology Laboratory, University of Mumbai are acknowledged with thanks.

I strongly believe that the successful completion of this manuscript is possible because of the blessings of "Almighty God".

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Contents

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Global Status of Environmental Pollution and Its Remediation Strategies

M.H. Fulekar

1.1 Introduction

Environmental pollution has become a major global concern due to rapid growth of industrialization, urbanization and modern agricultural development. Energy generation which have exploited natural resources for fulfilling human desires and needs, resulted in disturbing the ecological balance on which the quality of environment depends. Technological innovations and advancements in products and processes in industries have given rise to new products and new pollutants in abundant level which are above the self cleaning capacity of the environment. It is the industrial revolution that gave birth to environmental pollution. Pollution is introduction of contaminants into an environment that causes instability, disorder, harm and discomfort to the ecosystem i.e. physical systems or living organisms. Pollution has become a popular issue after World War II when the aftermath of atomic warfare and testing made evident the perils of radioactive pollutant. Then a conventional catastrophic event, the smog of 1952 in London killed at least 8000 people. This massive event promoted some of the first major modern environmental legislation like the clean air act of 1956. Pollution began to draw major public attention in the United States between the mid-1950's and early 1970's when Congress passed the noise control act, the clean air act, the clean water act and the national environmental policy. Environmental pollution sources include: chemical plants, coal-fired power plants, oil refineries (Beychook, 1967), petrochemical plants, nuclear waste disposal activity, incinerators, large line stock farms, PVC factories, metal production factories, plastics factories, many industries and spraying of

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pesticides. About 400 million metric tonnes of hazardous wastes are generated each year out of which the United States alone produces about 250 million metric tonnes. Americans constitute less than 5% of the world's population, but produce roughly 25% of the world's CO_2 and generate approximately 30% of world's waste.

1.2 Waste Disposal

Wastes notably include municipal solid waste, construction and demolition waste, institutional waste, commercial waste, industrial waste, medical waste, radioactive waste, electronic waste and biodegradable waste. Waste is directly linked to human development, both technologically and socially. The composition of different wastes has varied over time and location directly linked to waste materials. Wastes are materials that are not prime products for which the generator has no further use in terms of production transformation or consumption, and therefore need to be disposed off. Wastes may be generated during the extraction of raw materials, the processing of raw materials into intermediates and final products, the consumption of final products and other human activities. The waste disposal onto land causes contaminants of the soil environment.

1.3 Water Pollution

Water pollution is a major global problem due to disposal of waste in soil-water environment. It has been suggested that it is the leading worldwide cause of deaths and diseases and that it accounts for the death of more than 14,000 people daily. An estimated 700 million Indians have no access to a proper toilet, and 1000 Indian children die due to diarrheal sickness every day. Some 90% of China's cities suffer from degree of water pollution (Chinadaily.com.cn), and nearly 500 million people lack access to safe drinking water. In addition to the acute problems of water pollution in developing countries, industrialized countries continue to struggle with pollution problems as well. In the most recent national report on water quality in the United States, 45% of assessed stream miles, 47% of assessed lake acres, and 32% of assessed bay and estuarine square miles were classified as polluted.

Water pollution is the contamination of water bodies such as lakes, rivers, oceans, and groundwater. All water pollution affects organisms and plants that live in these bodies of water and in almost all cases the effect is damaging not only to individual species and populations but also to the natural biological communities. It occurs when pollutants are discharged directly or indirectly into water bodies without adequate treatment.

1.4 Biotechnological Approaches

The present treatment technology involving physico-chemical and biological methods are not efficient and/or effective to treat the contaminants to acceptable level. Today, biotechnology is being considered as an emerging science for environmental protection. The technology involves the use of microorganisms for biological treatment of air, water and soil pollutants. Biotechnological treatment is carried out at lower temperature and pressure which requires less energy than the conventional physico-chemical treatment technology. The industries generating hazardous wastes have found beneficial measures from the emerging trend of biotechnological treatment. Biotechnological innovations for treatment of hazardous waste under controlled environmental conditions have been found cost-effective means of reducing the pollution potential of waste water, leading to enhanced public acceptance and compliance with environmental legislation (Fulekar, 2009). Environmental pollution such as contaminated soil or surface/ground water can be solved by bioremediation and/or phytoremediation by use of biological living organisms and green plants.

1.5 Bioremediation Technology

Bioremediation technology uses microorganisms to reduce, eliminate or transform contaminants present in soils, sediments or water. Bioremediation depends on the presence of specific microorganisms in the correct amounts and combination and in the appropriate environmental conditions. Microorganisms already living in contaminated environments are often well adapted to survive in the presence of the existing contaminants and to the temperature, pH and oxidation/reduction potential of the site. These indigenous microbes tend to utilize the nutrients and electron acceptors that are available, provided liquid water is present. Water also acts as a vehicle to transport both microorganisms and dissolved substances including contaminants and their breakdown products. Bioremediation process involves biotransformation and biodegradation by transforming contaminants to non-hazardous or less hazardous chemicals. Often, the microorganisms metabolize the chemicals to produce carbon dioxide or methane, water and biomass. Biotransformation is any alteration of the molecule or structure of a compound by microorganisms. Biodegradation is the breaking down of organic or bioaccumulation and biotransformation of inorganic compounds into environmental friendly compounds.

1.6 Phytoremediation

The term phytoremediation is a combination of the words *phyto* meaning plant and *remediation* which means remedy. Phytoremediation is the use of plant's natural ability to contain, degrade or remove toxic chemicals and pollutants from soil or water. Phytoremediation describes various mechanisms by which

living plants alter the chemical compositions of soil matrix in which they are growing. It can be used to cleanup metals, pesticides, solvents, explosives, crude oil and other contaminants. The plants are often used in combination with other traditional techniques. The interactions between the plants and microorganisms that live in the soil can also contribute to phytoremediation, often called rhizosphere bioremediation. Phytoremediation takes advantage of a plant's natural ability to absorb, accumulate, or metabolize certain organic chemicals and metals. When the plants have absorbed and accumulated contaminants, they can be harvested and discarded. Incineration is the most common method used to dispose plants that have absorbed/accumulated large amounts of contaminants. This process produces ashes, which can then be discarded at appropriate waste disposal sites.

1.7 Genetic Engineering Approach—Bioremediation/Phytoremediation

Scientists are currently looking into certain genetically engineered microorganisms to increase their ability to metabolize specific chemicals such as hydrocarbons and pesticides. The possibilities of using genetic engineering for improvement of bioremediation process had an early boost in the late 1980's. Recombinant DNA techniques have been studied intensively to improve the degradation of hazardous wastes under laboratory conditions. The genetically engineered microorganisms have higher degradative capacity and have been demonstrated successfully for the degradation of various pollutants under defined conditions. Genetically modification technology has resulted often in a wide variety of current and potential applications for use in the process of bioremediation. Bioremediation explores gene diversity and metabolic versatility of microorganisms. The genetic architecture of these organisms makes them valuable in biodegradation, biotransformation, biosorption and bioaccumulation. The necessary blue print of genes encoding for biodegradative enzymes is present in chromosomal and extrachromosomal DNA of such microbes. Recombinant DNA techniques facilitate to evolve the ability of an organism to metabolize a xenobiotic by detection of such degradative genes and transforming them into an appropriate host via suitable vector under the tight control of appropriate promoters. It depends on susceptibility to alteration and exchange of genetic information. The recombinant DNA technology explores PCR, anti-sense RNA technique, site directed mutagenesis, electroporation and particle bombardment techniques. The biotechnology armed with recombinant DNA technology is now fine tuning the bioremediation technology by improving pollutant-degrading microbes through strain improvement and genetic modification of specific regulatory and metabolic genes that are crucial in developing effective, safe and economical techniques for bioremediation.

Recent advances involve the examining of extract mechanisms surrounding metal transport in plants and when some plants can absorb and tolerate high amounts of toxic metals while others cannot. Many different genes are suspected to be involved in regulating metal absorption in plants. The genes control the solubility of metals in the soil surrounding the roots, as well as the transport proteins that move metals into root cells and up into the plant's shoot. Researchers have identified genes and cloned genes to develop genetically modified plant to tolerate metal contamination. In some cases, plants are being genetically modified with bacterial genes. Improved knowledge of the genes, molecular mechanisms and roles microorganisms play in governing phytoremediation allows scientists to apply plants to decontaminate the environment. Plants have the innate capabilities of remedying hazardous contaminants from the environment (bioremediation) but the rate of bioremediation is directly proportional to plant growth rate and the total amount of bioremediation is correlated with a plant's total biomass, making the process very slow. This necessitates the identification of a fast growing (largest potential biomass and greatest nutrient responses) and more strongly metal accumulating genotypes. Genetic engineering approach has successfully facilitated to alter the biological functions of plants through modification of primary and secondary metabolism by adding new phenotypic and genotypic characters to plants with the aim of understanding and improving therein phytoremediation.

1.8 Environmental Nanotechnology

Environmental nanotechnology would be the new innovation to remediate and treat the contaminants to the acceptable levels. Environmental scientists and technologists are already working with nanoscale structure to manipulate matter of atomic or molecular scale that has cut across disposing off chemistry, physics, biology and even engineering. An important challenge in nanotechnology is to engineer particles with desired optical and electronic properties by controlling their size and shape. Nanotechnology involves the design, characterization, production and application of structures/particles by controlling their size and shape at nanoscale. Natural weathering of metals, iron oxides and silicate and microorganisms such as bacteria and algae produce nano celluloids, which include dispersions of nano sized particles in media with special properties that can be important in the fate, transport, transformation and bioavailability of environmentally harmful substances. Recently, the emerging field of nanotechnology has also contributed significantly in remediating the soil and water pollutants into environment friendly compounds. Nanotechnology offers great promise for delivering new and improved environmental technologies.

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2

Bioremediation of Xenobiotics-contaminated Sites: Concept and Selected Case Studies

T. Chakrabarti and T.K. Ghosh

2.1 Introduction

Amongst the knowledge-based technologies, biotechnology is a frontier technology, which has the potential to provide substantial benefits to society in a wide range of sectors such as agriculture, medical and health, forestry, animal husbandry, environment protection, and improving the quality of products and services. Industrialization in developing countries is causing considerable environmental damage, as the conventional responses of end-of-pipe pollution controls are inefficient and expensive. The social and economic costs of environmental damage caused by the prevailing industrial growth in India have been estimated to be much higher than the required expenditure of 0.5-1.0 percent of GNP for pollution control. The clean up of xenobiotics and anthropogenic contaminants, which are introduced in the environment following rapid industrialization, is one of the environment related problems currently being encountered globally. The least cost clean-up technology involving bioremediation originates in environmental biotechnology, which warrants interdisciplinary approach involving such disciplines as environmental and chemical engineering, earth sciences, chemistry, toxicology, ecology, microbiology and biochemistry. In addition, the demographic compulsions and declining per capita natural resources necessitate the developing countries to optimize land and water use and restore environmental quality but, at the same

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time, to produce more food, fibre, fuel, fodder and fertilizer to meet the growing demands. Thus inevitable rapid industrialization and higher production required for human survival call for adoption of technologies that are environmentally sustainable. Bioremediation is an environmentally benign technology that can be safely employed for ecorestoration.

2.2 Biotechnology—A *Sine Qua Non* for Environmental Security

For the majority of Earth's six billion years history, microbes ruled on the globe, from ocean's floor to arctic permafrost, under extreme environmental conditions. This abundant biodiversity may be the key to helping the planet by putting these rich microbial communities to work for serving the needs of society through environmental biotechnology.

Environmental biotechnology has been around for almost a century, first adapted widely in the 1910s and 1920s, when wastewater was cleaned up by a bacterial-laden sludge that speeds up the breakdown of the organic material in sewage and industrial wastewaters. The beginnings of microbial ecology started back in the 1940s and 1950s, when microbial cultures were initially sorted by morphology encompassing mainly size and shape. The function of a microorganism was assigned by selective culturing on agar plates or a nutrient-rich broth and selecting on the basis of metabolic function, which turned out largely to be a hit-or-miss approach. The first use of modern molecular biology tools began in the early 1980s, with the advent of polymerase chain reaction (PCR) amplification of microbial DNA and a new view of the evolution of organisms based on their ribosomal RNA.

2.3 Environmental Site Assessment (ESA)

The three main investigation phases involved in ESA are summarized in Table 2.1.

Table 2.1: The three main investigation phases, and major tasks

Main investigation phases	*Major tasks*
Preliminary site Investigation/Phase 1 Environmental site assessment (ESA 1)	Gathering and reviewing of available data (historical data, existing site data and assessment, regulatory agency file data) Site inspection (site walkover) and review of waste handling Interviews with site owners, personnel and tenants Data evaluation and reporting Does not normally include sampling and chemical analytical testing.

(*Contd.*)

Detailed site investigation/ Phase 2 Environmental site assessment (ESA 2)	Site-specific sub-surface investigation (may include drilling, test pits, monitoring wells, air, surface water, ground water, soil sample collection and chemical analyses) Data evaluation, delineation of contaminated area and reporting Note: Phase 2 ESAs may require more than one stage, depending on the nature and distribution of environmental impacts.
Site clean up/Phase 3 Remedial design and implementation (ESA 3)	Discussion of remedial objectives (with owner and/or regulatory agencies) Establishment of clean-up criteria Reviewing of appropriate alternative remedial technologies Selecting and designing of remedial action plan Obtaining required approvals Conducting performance monitoring and verification sampling Report

There exists a relationship between soil contamination and air and water quality. Soil contaminants interact with air through volatilization and with water through dissolution and leaching to ground water or runoff to surface water. Mobile soil contamination that is adding contaminant mass to air or water is automatically considered a source. Therefore it must be remediated, or the contaminant release from the soil must be controlled.

2.4 Scope for Site Remediation in India

The country has, so far, identified 172 abandoned dump sites located in various states which require remediation. So far, bioremediation in India appears techno-economically feasible because of the prevailing tropical climate almost throughout the year in most of the states and union territories. Phytoremediation in India is being extensively used for restoration of environmental quality. However, there exists ample scope to modify the process through biostimulation and bioaugmentation as well as through better understanding of the behaviour of microbial community. Also, the potential for generation of carbon credit through phytoremediation intervention as well as through solid waste composting (instead of land filling) needs to be identified and applied wherever possible.

2.5 Biodegradation and Bioremediation

2.5.1 Concept

The clean-up of xenobiotics and anthropogenic contaminants that are introduced in the environment following rapid industrialization is one of the most important

problems currently being encountered globally. The least cost clean-up technology involves biodegradation and bioremediation.

Biodegradability of a compound requires that the compound in question must possess a chemical structure, which will enable to be accepted by the existing catabolic enzymes of the microorganisms present in the environment. The energy and the carbon released by the reaction are used to support microbial growth. Further, the compound must be capable of inducing or derepressing the synthesis of the enzymes, which will degrade it. When biodegradation is complete, the elements of which the original molecule was composed are released into the environment in inorganic forms.

Bioremediation may be defined as the use of biotechnological routes for transformation, degradation and detoxification of waste constituents that result in environmentally acceptable waste assimilation while assuring the protection of public health. The remediation should be based on the risk which the residual contaminant(s) is/are likely to pose. Risk-based remediation goal is required to be set while delineating the strategy for remediation of a contaminated site. Ecotoxicity, genotoxicity, carcinogenicity and endocrine disruption potentials of contaminant(s) are considered while determining the risk.

Out of available options, in situ bioremediation has several advantages, which include:

- Minimized site disturbance
- Less expensive compared to ex situ bioremediation
- Application of bioventing which optimizes bioremediation of unsaturated zone with air and reduces volatile organic emissions.

It is noted that future belongs to sustainable technologies, which would optimize the full life cycle of products also including environmental, social and economic issues. However, there would remain great need to use in situ bioremediation technology to restore already contaminated sub-surface water and soil for some time to come.

2.5.2 Approach

The essential pre-requisites for a successful in situ bioremediation are delineation of contaminated subsurface plume size using geophysical techniques, development of suitable microbial systems that can stabilize the xenobiotics and anthropogenic chemicals to a no observed adverse effect level (NOAEL) or to a lowest observed adverse effect level (LOAEL) as determined by the risk-based remediation goal to be set-up before any bioremediation is planned, and bioremediation engineering. The microbial systems must have thresholds which enable the system to grow, proliferate and degrade at low substrate concentrations usually encountered in contaminated niches, especially in subsurface contaminated water. The process must then be controlled by providing suitable energy source(s), electron acceptor(s) and nutrients. A requirement for co-metabolite(s) must be determined if the threshold of

the microbial system needs to be reduced thereby enabling the system to metabolize the xenobiotic and anthropogenic contaminant(s) in question. Lastly, bioremediation involves engineering which warrants development of injection system(s) to provide electron acceptors, donors and nutrients to the resident microorganisms, in situ microbial enrichment technology and establishment of an intimate contact between substrates, electron acceptors, other additives (nutrients and cometabolites) and microbial systems in situ.

It is not out of place to emphasize that most approaches look at remedial measures as either microbial treatment or a combination of physico-chemical interventions into existing microbial processes. Since the health of terrestrial environment is linked to groundwater contamination, the conceptualization of a combination of phytoremediation and in situ bioremediation-based technological approach will be logical. This is more so as in places such as India which has vast unexplored natural resources in terms of biodiversity of microorganisms (deeper subsurface zones may contain greater diversity of microbial life) and green plants that can be utilized to mitigate subsurface pollution.

The basic difference between the design principles of biological wastewater treatment processes and those for bioremediation lies in the bioreaction system. Bioremediation reaction is confined in a geological boundary whose size and geometry is different from that of bioreactors used in wastewater treatment system. Further, the threshold for microbial system(s) suitable for bioremediation is usually much lower than that of wastewater treatment systems. The microbial systems in bioremediation, therefore, should essentially comprise oligotrophs compared to heterotrophs prevailing in waste treatment systems. Thus, from the point of view of bioreaction system as well as microbial system, bioremediation is different from biological wastewater treatment. Hence, research is required to undertake a total system approach suitable for bioremediation.

With recent advances in biology, materials, computing and engineering, environmental biotechnologists now are able to use microbial communities for a wealth of services to society. These include detoxifying contaminated water, wastewater, sediment, or soil; capturing renewable energy from biomass; accelerating biocomposting process; sensing contaminants or pathogens; and protecting the public from dangerous exposure to pathogens. These technologies have advanced into high-throughput genomic and proteomic protocols that can detect specific genes and their metabolic functions with great precision and detail. The culture-independent genomic analysis of microbial communities can now assist in reconstructing entire genomes of what were once "unculturable" microbes.

2.5.3 Bioremediation is Aimed to Increase Threshold Assimilative Capacity

Though biodegradation is a natural process, any ecosystem can be defined for its threshold assimilative capacity for any xenobiotic chemical. However,

through appropriate biostimulation, the xenobiotic chemicals can become a substrate for assimilation by microorganisms in an ecosystem. Biochemical processes may be controlled by:

- Stimulation of existing microflora via enrichment as well as by moisture, aeration and nutrient control (biostimulation)
- Exogenous organism (natural or recombinant) addition to increase the versatility of the system (bioaugmentation)
- Biodegradation essentially requires microorganism(s), electron acceptor and electron donor which are required in right proportion *a priori* for successful bioremediation.

2.5.4 Paradigm shifts—Remediation

These include:

- Conventional microbial approach to metagenomics
- Monitoring natural attenuation (MNA) to programmed remediation
- Conventional bioremediation to chemo-bio approach

2.5.5 Metagenomics: Application of Genomics to Uncultured Microorganisms

The realization that most microorganisms cannot be grown readily in pure culture, forced microbiologists to question their belief that the microbial world had been conquered. It has been realized later that the uncultured microbial world far outsized the cultured world and that this unseen world could be studied.

Metagenomics (also referred to as environmental and community genomics) has opened new avenues of research by enabling unprecedented analyses of genome heterogeneity and evolution in environmental contexts and providing access to far more microbial diversity than has been viewed in a petri dish. Metagenomics provide a second tier of technical innovation that facilitates study of the physiology and ecology of environmental microorganisms. Novel genes and gene products, discovered through metagenomics, include the first bacteriorhodopsin of bacterial origin; novel small molecules with antimicrobial activity; and new members of families of known proteins, such as an $Na^+(Li^+)/H^+$ antiporter, RecA, DNA polymerase, and antibiotic resistance determinants.

Reassembly of multiple genomes has provided insight into energy and nutrient cycling within the community, genome structure, gene function, population genetics and microheterogeneity, and lateral gene transfer among members of an uncultured community. The application of metagenomic sequence information will facilitate the design of better culturing strategies to link genomic analysis with pure culture studies.

The diversity of soil bacteria, demonstrated with DNA-DNA reassociation techniques, revealed that the complexity of the bacterial DNA in the soil was at

least 100-fold greater than could be accounted for by culturing. So far, 52 phyla have been delineated, and most are dominated by uncultured organisms.

2.5.6 Bioremediation Process

The process of bioremediation enhances the rate of the natural microbial degradation of contaminants by supplementing these microorganisms with nutrients, carbon sources or electron donors. This can be done by using indigenous microorganisms or by adding an enriched culture of microorganisms that have specific characteristics that allow them to degrade the desired contaminant at a quicker rate. Ideally, bioremediation results in the complete mineralization of contaminants to H_2O and CO_2 without the build up of intermediates.

Bioremediation processes can be broadly categorized into two groups: ex situ and in situ. Ex situ bioremediation technologies include bioreactors, biofilters, land farming and some composting methods. In situ bioremediation technologies include bioventing, biosparging, biostimulation, liquid delivery systems and some composting methods. In situ treatments tend to be more attractive to vendors and responsible parties because they require less equipment, generally have a lower cost and generate fewer disturbances to the environment. However, the difficulties associated with implementing in situ processes have limited their application in the field. Bioremediation using white-rot fungi to innoculate contaminated media is a promising technology that is currently being researched. This technology can be used in an ex situ or in situ manner. Generally, this fungus is used to innoculate a composting process, but it does have other bioremediation applications.

Composting involves the mixing of the contaminated soil in a pile with a solid organic substrate, which serves as a carbon source for the indigenous aerobic soil microorganisms. Composting is a means for the remediation of pesticide contaminated sites and several large companies, such as W.R. Grace and Astra Zeneca, have developed and patented successful composting technologies. For ex situ treatment, the soil is excavated, screened and formed into windrows or some form of pile. In situ treatment is also possible for composting but is not used frequently. The soil is then supplemented with the organic substrates, nitrogen and phosphorous. Moisture, pH and redox potential are monitored while the soil is mixed on a regular basis to maintain homogeny and aeration. The piles may also be kept anaerobic by covering them with plastic sheets and encouraging the aerobic microorganisms to utilize all of the oxygen remaining underneath. Once the oxygen in the pile has been depleted, anaerobic microorganisms will become active, degrading the organic pollutants that were non-degraded by the aerobic microbial population. Figure 2.1 shows an aerobic windrow composting system.

The terms land farming, land spreading, land application and land treatment are often used interchangeably to refer to the same process. It is a full-scale bioremediation technology where contaminated solid media, such as soil, sludge

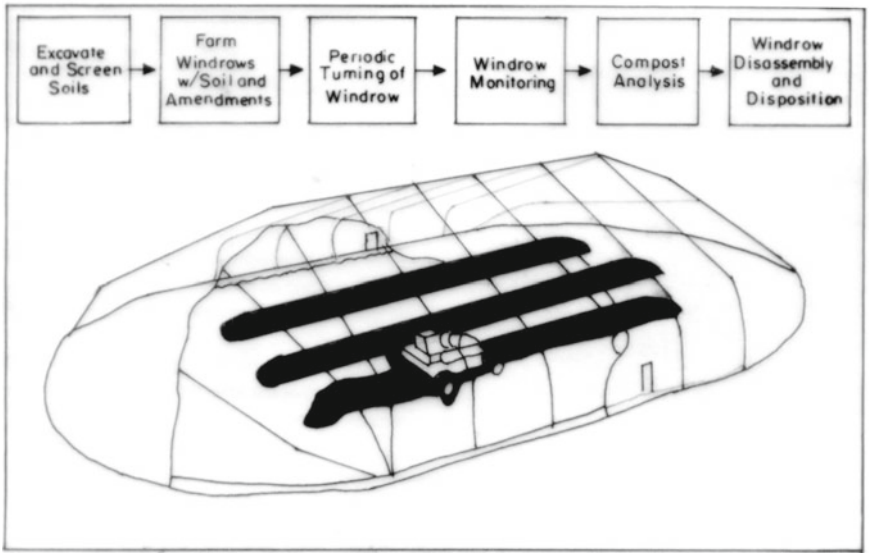

Fig. 2.1: Anaerobic windrow composting system.
Source: Frazar, 2000

or sediment, are applied to uncontaminated soil. Mixing of the contaminated media with the soil allows the indigenous microorganisms to interact with the contaminant and degrade it. The rate of application is calculated so as to avoid concentrations that would be unsafe in soil, groundwater or crops.

Generally, the rate is similar to the label rate, which is the suggested rate of application of pesticide per unit of land or soil that is on the pesticide label. The size and location of the spreading operation is then chosen based upon the application rate. Finally, a cover crop may be added to the land farming operation. A cover crop allows a farmer to continue to use these productive fields while remediation occurs, and it may enhance rhizosphere degradation, which will be discussed in the phytoremediation section. Often it is necessary to add nutrients in order to enhance biodegradation by these indigenous organisms. In addition, it is important to monitor soil moisture and oxygen levels. Although the land farming process is slow, it is a very low cost technology, which makes it attractive to small waste generators, such as farmers. Land spreading has been used successfully throughout the United States, particularly in the Midwest to remediate a variety of different pollutants. It is the most widely used ex situ bioremediation treatment process (EPA, 1997). Before a farmer can begin land spreading, he must obtain a permit and fully outline his intentions, including the quantity of contaminant and the soil characteristics of the land where it will be applied. When land spreading, it is required that all guidelines on the label, including rate of application and season of application be followed. The state of Wisconsin requires the oversight of the land spreading process by a certified

applicator. Because pesticides reach the soil through normal application, land spreading at application rates generally doesn't require a lined bed. However, land spreading of pesticides at significantly higher concentrations or land spreading of other hazardous wastes occurs on a lined bed to collect leachate. A typical system for the land treatment of hazardous wastes is shown in Fig. 2.2.

Land spreading of some hazardous compounds can result in their volatilization, which necessitates a cap for the system to control emissions. White-rot fungi, particularly those of the family *Phanerochaete*, are becoming recognized for their ability to efficiently biodegrade toxic contaminants. Most studies focus on the ability of *Phanerochaete chrysosporium* to degrade persistent compounds, but *Phanerochaete sordida*, *Pleuotus ostreatus*, *Phellinus weirii*, and *Polyporus versicolor* have also been successful in laboratory studies (Bumpus and Aust, 1987; Safferman et al., 1995). These fungi are effective because of an extracellular enzyme that catalyzes a reaction that can degrade lignin, an aromatic plant compound. In order to catalyze these powerful reactions, the enzyme requires hydrogen peroxide which is produced by the fungus. These fungi are capable of degrading chlordane, lindane and DDT, which make them useful for the remediation of pesticide-contaminated sites (Alexander, 1999). White-rot fungi could be used to inoculate a composting operation. However, large quantities of the fungus are required to remediate a site due to the very slow nature of compound degradation (Bumpus and Aust, 1987). Other studies have demonstrated the ability of white-rot fungi to degrade DDT in aqueous cultures.

Bioventing and biosparging are very similar in situ processes. Both methods involve the introduction of O_2 into permeable soil to increase the activity of aerobic microorganisms. Bioventing introduces the O_2 to the vadose, or unsaturated zone, while biosparging introduces O_2 below the water table into

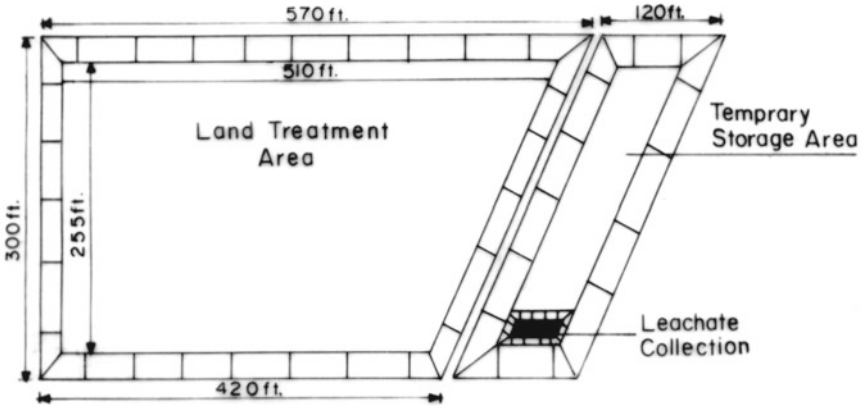

Fig. 2.2: Typical lined-bed land treatment setup for the remediation of hazardous wastes.
Source: Frazar, 2000

the saturated zone. Neither of these processes is suitable for compounds which may volatilize too quickly. Biosparging can force volatile contaminants out of the water table and up into the unsaturated zone, wherein the vapours can be recovered. Because of this, it is necessary to monitor off gases. Biosparging also introduces O_2 to the saturated zone, which will increase the rate of biodegradation. These procedures have not been used frequently with pesticide-contaminated sites.

Monitored natural attenuation is the remediation of contaminated media by indigenous microorganisms without active treatment. This remediation process requires a longer time frame to reach remediation goals than active bioremediation methods. Due to the longer time frame, a more intensive monitoring programme needs to be implemented to assure that attenuation is occurring.

2.5.7 Phytoremediation

A significant amount of work has been conducted to examine the ability of plants to remediate heavy metal contaminated soils. Plants are often capable of the uptake and storage of significant concentrations of some heavy metals and other compounds in their roots, shoots and leaves, referred to as phytoextraction. The plants are then harvested and disposed off in an approved manner, such as in a hazardous waste landfill. This technique results in upto a 95% reduction in waste volume over the equivalent concentration of contaminated soil.

Phytotransformation occurs when plants transform organic contaminants into less toxic, less mobile or more stable forms. This process includes phytodegradation, which is the metabolism of the organic contaminant by the plant enzymes and phytovolatilization, which is the volatilization of organic contaminants as they pass through the plant leaves. The release of these pollutants into the air results in the exchange of one form of pollution for another. Phytostabilization immobilizes the contaminants and reduces their migration through the soil by absorbing and binding leachable constituents to the plant structure. This process effectively reduces the bioavailability of the harmful contaminants. Almost any vegetation present at contaminated sites will contribute to phytostabilization (Arthur and Coats, 1998).

At the soil-root interface, known as the rhizosphere, there is a very large and very active microbial population. Often the plant and microbial populations provide needed organic and inorganic compounds for one another. The rhizosphere environment is high in microbial abundance and rich in microbial metabolic activity, which has the potential to enhance the rate of biodegradation of contaminants by the microorganisms. Generally, the plant is not directly involved in the biodegradation process. It serves as a catalyst for increasing microbial growth and activity, which subsequently increases the biodegradation potential. However, the rhizosphere can be limited in its remediation potential because it does not extend far from the root. This process is often referred to as phytostimulation or plant-assisted bioremediation.

2.6 Case Studies

2.6.1 Evaluation of HCH, DDT and Endosulfan Levels in Soil and Delineation of Management Strategy

POPs contaminated site: In USA, many agrochemical facilities, including pesticide manufacturing and storage sites, have become superfund sites. In this case study, the study locations were inside a pesticide manufacturing plant in South India, having facilities to produce technical and formulated grade pesticides such as DDT, HCH and endosulfan. The industry manufactured/produced 1344 MT/KL of technical grade DDT, 2688 MT/KL of formulated DDT, 1600 MT/KL of technical grade endosulfan and 1910 MT/KL of formulated endosulfan. The manufacturing of HCH was stopped in 2004 as per Government's directives. DDT is produced for use in vector control purpose only.

(A) *Sampling*

Soil samples were randomly collected and references of each sampling sites are given in Fig. 2.3. Top soil samples were collected from the surface at east, north–east and south–west directions. Samples were also collected from these sites at a depth of 30 cm (sub-soil).

(B) *Analytical Procedure*

Sample Processing: Soil samples, collected from the industrial locations, were screened for large objects such as sticks, leaves and gravel. These were hand-picked and removed. Soil samples were then dried at below 30°C, sieved to pass through a 2.8 mm mesh size and milled using a ball mill.

Soil characteristics were determined in terms of moisture, pH, organic matter, organic carbon and total nitrogen as per the methods of Jackson (1973). Ten grams of the sample was thoroughly mixed with 10 g of anhydrous sodium sulfate in a 100-ml beaker for extraction.

Extraction: The sample was transferred to an extraction thimble and subsequently inserted in the Soxhlet extraction apparatus. The solvent used was a mixture of hexane: acetone (1:1 v/v 100 ml). The extraction process was continued for an eight-hour period. The extract was then concentrated in a rotary evaporator until the final volume was reduced to 1 ml. The sample was transferred to a graduated glass vial with glass stopper.

Analysis of organochlorine residues in the soil by GC-MS: The GC-MS system was optimized for separation of a standard mixture of 11 pesticides. The system comprised a Varian Model Saturn 2200 GC-MS-MS system with CP-3800 gas chromatograph equipped with 1079 injector and electronic flow controller (EFC). The injector port was programmed with initial temperature of 100ºC, ramp 180ºC/min, and final temperature at 300ºC. Column flow was maintained at 1.1 ml/min; while split ratio was kept off. Column oven was

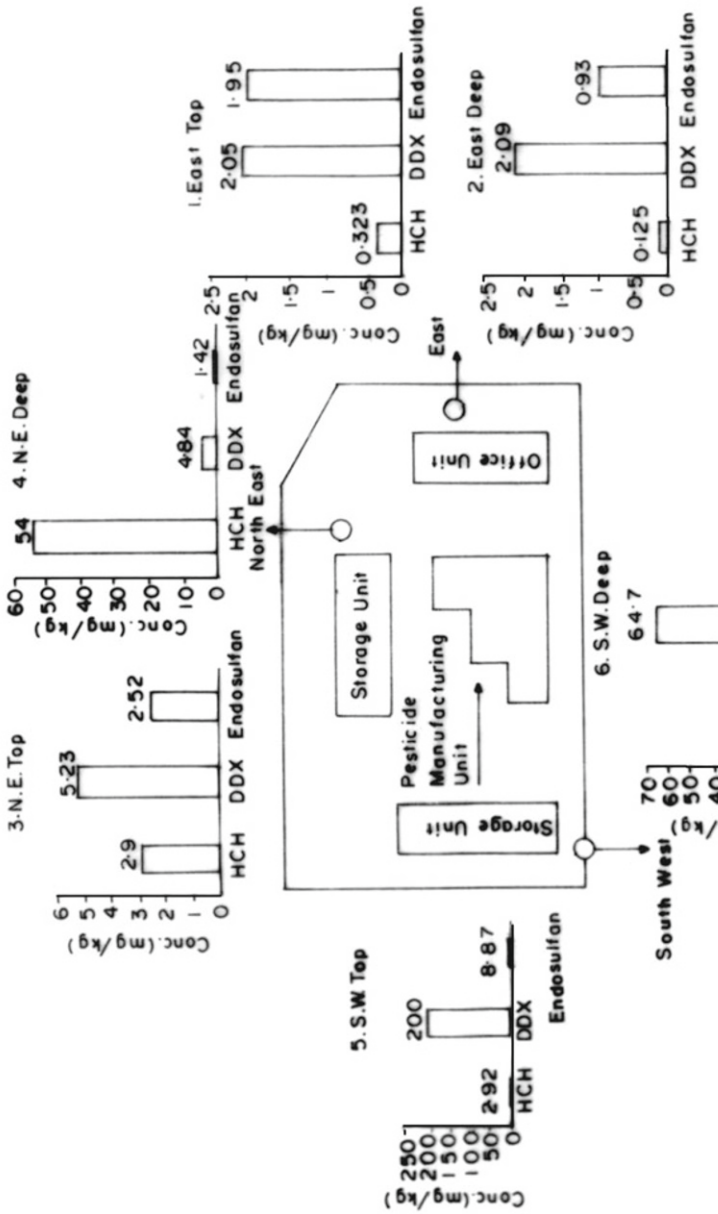

Fig. 2.3: Sampling locations and concentrations of Σ DDX, Σ HCH and Σ Endosulfan.

programmed with initial temperature of 70ºC for 1.50 min, ramp to 200ºC at 10.0ºC/min, hold for 0.0 min, and then ramp to 280ºC at 5ºC/min, and hold for 14.50 min. MS operating conditions were: Trap temperature 200ºC, manifold 80ºC, and transfer line 300ºC. EI was tuned by FC-43 (Table 2.2).

Table 2.2: MS/MS instrumental conditions for the analysis of pesticides

Ionization Parameters	
Ion-Trap temperature	200ºC
Mannifold temperature	80ºC
Transfer line temperature	300ºC
Multiplier offset	300 volts
Emission current	30 µA
Scan rate	0.35 s/scan
Target TIC	1200 counts
PreScan ionization time	1500 µs
Count threshold	2 count
Ion Preparation Parameters	
Mass isolation window	3.0 m/z
CID waveform	Non-resonant
Excitation time	20 msec
Isolation time	5 msec
Low edge offset	6 steps
High edge offset	2 steps
Ejection amplitude	20 volts
High edge amplitude	30 volts

A standard Pesticide Mix 2 containing 11 pesticides from Supelco (Cat No.4-8196) was injected in the GC to obtain a well resolved chromatogram of 11 pesticides. Figure 2.4 summarizes the separated pesticides in the mixture.

The conditions were developed experimentally to get the best sensitivity and spectral identification for each one of the pesticides analyzed. A calibration curve for at least three concentrations of each pesticide was developed. These conditions were further applied for analyzing real soil samples collected from various locations inside the industry manufacturing pesticides. A quantitative relation over a range of observed responses was established correlating each of several known concentrations to corresponding signal, thus yielding a response curve. Using this calibration curve, the value of the unknown analyte was determined. The base-peak ion was used for quantification, and three qualifier ions were used for confirmation. After qualitative criteria were met,

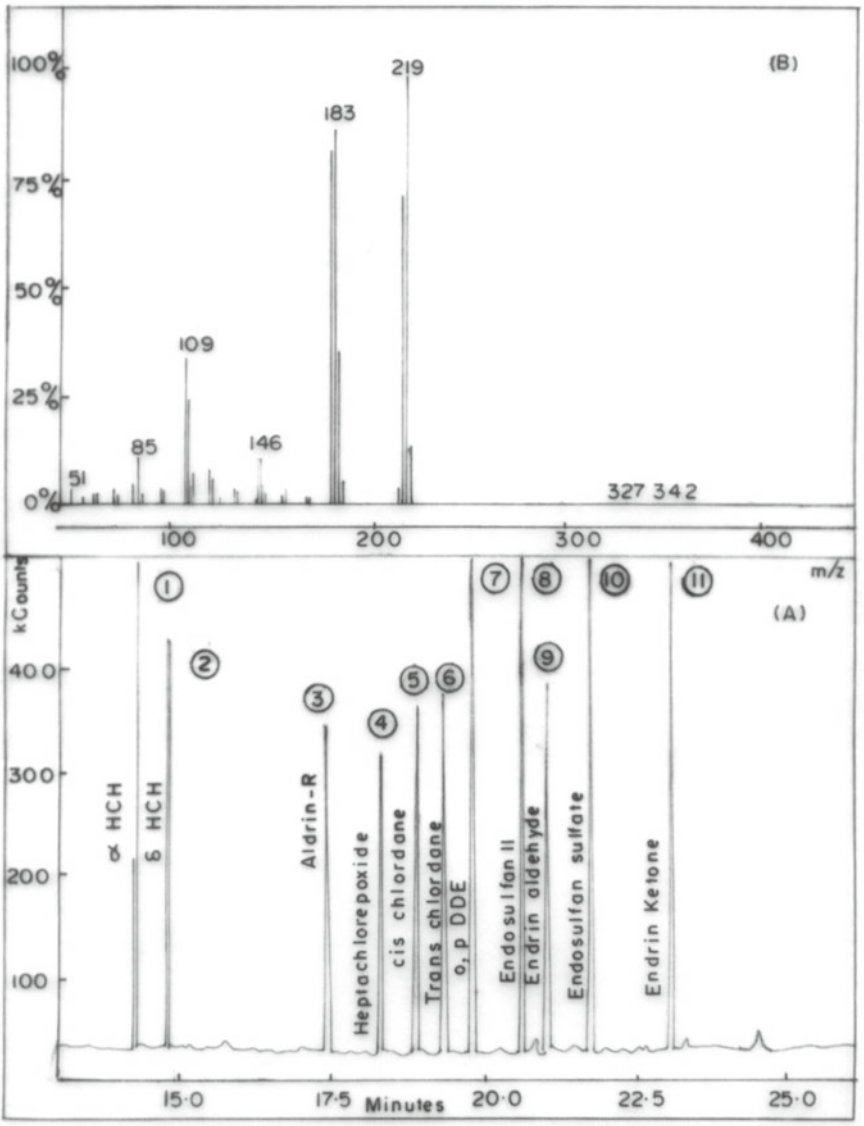

Fig. 2.4: (A) TIC chromatogram of calibration mixture of pesticides. 1: α-HCH, 2: δ-HCH, 3: Aldrin-R, 4: Heptachlor epoxide, 5: cis chlordane, 6: Trans chlordane, 7: o,p DDE, 8: Endosulfan II, 9: Endrin aldehyde, 10: Endosulfan sulfate, 11: Endrin Ketone. (B) Fragmentation pattern for α-HCH.

compound concentrations were calculated from three point calibration curves using external standards. Identification of individual pesticides was achieved through the combination of a retention time match with known standard and match of experimental EI mass spectrum, after background subtraction to a reference spectrum of standard library spectrum (NIST Library, USA).

QA/QC: Once the method parameters were optimized, calibration curves were obtained for each pesticide in the standard mix in the concentration range of 0 to 10 ng/µl. A good linearity was obtained for all the pesticides with correlation coefficient ranging from 0.9935 to 0.9986 (Table 2.3). The precision was established by determining the Relative Standard Deviation (RSD). The RSD ranged between 8.23 and 22.66 percent.

Recovery studies were calculated on spiked soil at 5–10 µg/kg concentration levels after aging the soil for four days. Good recovery values were obtained for all the pesticides in the range of 80–109% (Table 2.3). The analytical results pertaining to pesticide quantification are reported in Table 2.4. Organochlorine compounds other than DDT, HCH, endosulfan and their derivatives identified in the soil by GC-MS are listed in Table 2.5.

Table 2.3: QA/QC of the GC/MS method used for pesticide analysis in soil samples

S. No.	*Pesticides*	*RSD (%)*	*Determination coefficients (r^2)*	*Recovery (%) at 5–10 µg/kg*
1.	α-HCH	11.77	0.9974	87
2.	δ-HCH	15.19	0.9959	85
3.	Aldrin	8.23	0.9986	82
4.	Heptachlor epoxide	10.99	0.9977	91
5.	Cis-Chlordane	13.05	0.9969	97
6.	Trans-Chlordane	14.29	0.9964	95
7.	o,p′ DDE	12.57	0.9940	109
8.	Endosulfan II	17.62	0.9959	87
9.	Endrin aldehyde	16.11	0.9965	80
10.	Endosulfan sulfate	13.08	0.9976	90
11.	Endrin ketone	22.66	0.9935	98

MS/MS Conditions: MS/MS conditions were optimized by carrying out Automated Method Development (AMD) and subsequently MS/MS. The conditions are summarized in Table 2.6. Various conditions required for MS/MS were optimized, leading to ion formation, parent ion isolation, product ion formation and product ion mass scanning.

(C) *Observations*

Levels and transformation of target chemicals: The soil samples were characterized and the pH of the soils was found to vary in the range of 5.0 to 6.0, and the moisture being in the range of 25–35 percent. The organic matter, organic carbon and total nitrogen varied from 1.4 to 1.7, 0.8 to 1.1 and 0.073 to 0.081 percent respectively. Although microbial and abiotic decomposition processes operate in the soil, the OCs have been observed in the soils,

Table 2.4: Pesticides concentration in μg/kg of dry soil obtained in a monitoring programme of different sampling points inside the industry

S.No.	*Location*	*Pesticide*	*Concentration* (μg/kg)*
1.0	East top soil	α-HCH	159 ± 2
		δ-HCH	164 ± 3
		p,p′DDT	363 ± 4
		op′DDT	428 ± 6
		p,p′DDE	127 × 10 ± 11
		Endosulfan sulfate	199 × 10 ± 20
	East deep soil	δ-HCH	125 ± 6
		p,p′-DDT	182 ± 10
		o,p′-DDT	909 ± 6
		p,p′-DDE	100 × 10 ± 4
		Endosulfan sulfate	936 ± 7
2.0	North-East top soil	α-HCH	120 × 10 ± 35
		δ-HCH	172 × 10 ± 20
		p,p′-DDT	153 × 10 ± 24
		o,p′ DDE	$660 \times 10^2 \pm 33$
		p,p′-DDE	$133 \times 10^3 \pm 93$
		Endosulfan II	316 × 10 ± 10
		Endosulfan sulfate	571 × 10 ± 30
	North-East deep soil	α-HCH	$158 \times 10^2 \pm 89$
		δ-HCH	$381 \times 10^2 \pm 70$
		p,p′-DDT	109 × 10 ± 6
		p,p′-DDE	354 × 10 ± 31
		Endosulfan sulfate	142 × 10 ± 18
3.0	South-West top soil	α-HCH	290 × 10 ± 21
		p,p′-DDT	236 × 10 ± 7
		o,p′ DDT	864 ± 6
		o,p′-DDE	200 × 10 ± 6
		Endosulfan II	784 ± 5
		Endosulfan sulfate	173 × 10 ± 20
	South-West deep soil	α-HCH	195 × 10 ± 13
		p,p′-DDT	$500 \times 10^2 \pm 59$
		o,p′-DDE	$147 \times 10^2 \pm 109$
		Endosulfan sulfate	$209 \times 10^2 \pm 98$

*Concentrations are expressed as Mean ± Standard deviation of three samples at the same location.

Table 2.5: Other organochlorinated compounds identified in the soil of the industry

S. No.	*Pesticide identified*	*Formula*	*Molecular weight*
1.	Pentachlorobenzene	C_6HCl_5	248
2.	Cyclopentene Octachloro	C_5Cl_8	340
3.	Benzene hexachloride	C_6Cl_6	282
4.	Benzene,1,4, dichloro-3phenoxy	$C_{12}H_8Cl_2O$	238
5.	Bicyclo (2,2,1)eptene, heptachloro	$C_7H_3Cl_7$	332
6.	Benzene 1,2, dichloro-4-tetrachloromethyl	$C_7H_3Cl_5$	262
7.	Bromociclen	$C_8H_5BrCl_6$	390
8.	Mitotane	$C_{14}H_{10}Cl_4$	318
9.	1,3 cyclopentadiene 1,2,3,4,5,5 Hexachloro	C_5Cl_6	270

Table 2.6: GC/MS conditions for operation in the MS/MS mode including the appropriate time segments and respective parent ion and quantification ions

Retention time, Min	*Pesticide*	*Segment*	*Channel*	*Parent ion M/z*	*Quantification ion*	*CID Amplification, v*	*Excitation storage level, v*
14.22	α-HCH	2	4	219	183	80	96
17.39	Aldrin	4	6	263	191/193	100	116
18.26	Heptachlor epoxide	6	6	353	261/263	100	156
18.85	Chlordane	8	6	375	301	100	165
19.31	o,p DDE	10	6	318	246/248	100	140
20.14	Endosulfan-II	12	10	339	195/197	70	149
21.10	Endrin aldehyde	14	4	345	279	80	152
21.68	Endosulfan sulfate	16	4	387	287/289	80	171
23.09	Endrin ketone	18	4	317	281	80	140

indicating their persistent nature. The parent substances such as p,p′-DDT, hexachlorocyclohexane and endosulfan were found in various top soil and subsoil samples collected from different locations within the industrial premises (Table 2.4). Some organochlorine compounds (OCs) were also identified in the soil samples and these are summarized in Table 2.5.

The distribution of various organochlorine pesticides in the soil samples collected from three different locations reveals a wide range of fluctuations, as delineated in Table 2.4. The levels of various pesticides were in the following range: HCH 0.125–54 mg/kg; DDX 2.06–200 mg/kg; and endosulfan 0.93–20.9

mg/kg. Hexachlorocyclohexane (HCH) normally exists in different forms called isomers. One of the forms, gamma-HCH (or γ-HCH, also known as lindane), is a well known insecticide. It is observed that the α-HCH and δ-HCH were predominant in the samples, collected at various locations. The industry produces technical grade HCH and their presence as isomers may be attributed to the emissions into the air as vapour and depositions as small particles in the soil. Being a pesticide manufacturing unit, these materials are also released during packaging and transportation of finished products. DDTs were detected in most of the soil samples. However, various forms of DDT, such as p,p′-DDT, o,p′-DDT, were less significant than the metabolites of DDT such as p,p′-DDE, o,p′-DDE, whose concentrations varied from place to place. The minimum value of DDT was recorded at the east location and the maximum at the south-west location.

The relative concentrations of the parent DDT compared to its biological metabolites DDE can be used as indicative indices for assessing the possible pollution sources of the soil. Since the ratio of _DDT/_DDE at north-east top soil is 0.0076 and north-east sub-soil is 0.3097, it appears that these sites have not received any fresh input of DDT as small ratio (i.e., ratio<1) indicates aged (microbially degraded) DDT. On the other hand, in the south-west locations, the ratio of DDT/DDE is 1.607 in the top soil and 3.396 in the sub-soil. The ratios greater than one very much corroborates with the earlier findings (Harner et al., 1999). This indicates that these locations are very near to the DDT manufacturing plant; hence a continuous influx of DDT is possible. Higher concentration of pp_DDT (2.36 mg/kg) has been observed in the south-west topsoil as compared to other sites. The north-east top soil recorded the highest concentration of degraded products such as op_DDE 66.0 mg/kg and pp_DDE 133.0 mg/kg, suggesting that the location has the presence of highest old pesticide inputs. This observation agrees well with the ratio values of less than one, indicating that the pesticides in the north-east soil are subjected to the transformation processes, as could be seen in some old dump sites.

Endosulfan was detected in most of the soil samples at medium concentration, mainly represented by the β-isomer (endosulfan-II). Endosulfan sulphate was also detected in the soil samples. Its concentration was always higher than that of endosulfan-II, which is the major soil microbial degradation product. The toxicity risk, however, still remains (Fig. 2.5), since it is known to be as toxic as the parent compound (Luchini et al., 2000; WHO, 1984).

One noticeable observation was the presence of large DDT and its metabolites and endosulfan levels at the south-west sub-site and HCH at the north-east sub-soil site. On enquiry, it was revealed that the industry buried its off quality and date-expired pesticides in the soil at these sites.

Considering the behaviour of these pesticides in soil, one may conclude from Fig. 2.5 (illustrating the probable pathways) that the aerobic degradation of parent compound DDT leads to production of metabolic intermediate DDE; in case of HCH one observes pentachlorocyclohexene, and in case of endosulfan, the metabolic intermediate is endosulfan sulfate. These intermediates have been

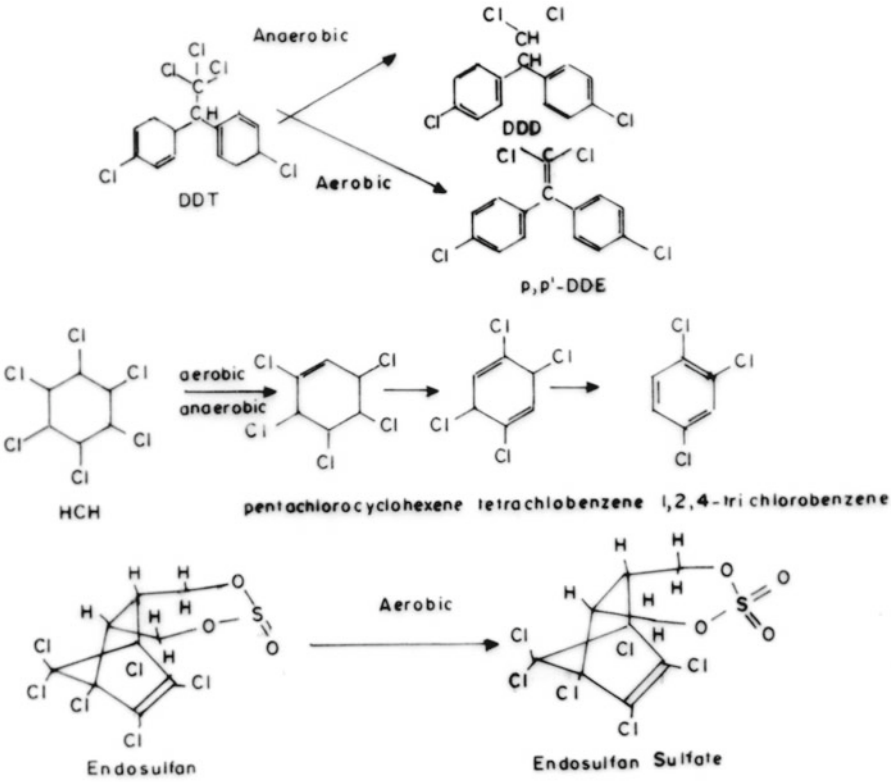

Fig. 2.5: Probable metabolic pathways of DDT, HCH and endosulfan.

identified in the soil samples from the various sites, showing that the soil has the required microorganisms to carry out partial biotransformation of DDT, HCH and endosulfan.

MS/MS Analysis: The optimized condition for obtaining the MS/MS for pesticides is summarized in Table 2.6. The programme was divided into 18 segments with a total time of 25 minutes in the Auto Electron Impact (EI) mode and MS/MS modes. Nonresonant waveform was adopted for dissociation with an excitation storage level as illustrated in Table 2.6. The maximum excitation amplitude was determined by scanning the range in the 0–100 volts for non-resonant excitation. The excitation amplitude varied depending on the mass of the ions and the excitation mode. The resulting MS and MS/MS of the pesticides are summarized in Figs 2.4 and 2.6 respectively.

(D) *Assessment of POPs concentrations relative to the standards in the Hazardous Waste and Contaminated Sites Regulations*

India is yet to evolve risk-based remediation goal although the Hazardous Waste (Management, Handling and Transboundary Movement) Rules 2008 mandate

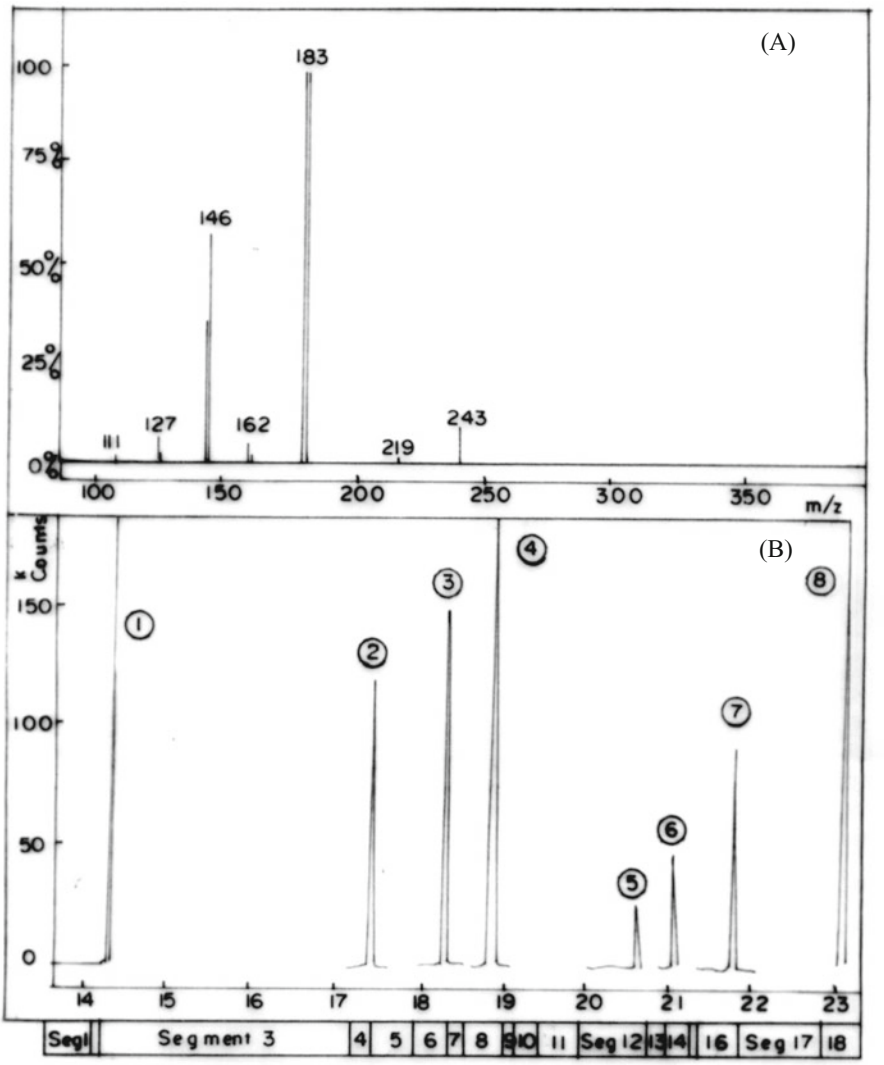

Fig. 2.6: (A) MS/MS fragmentation pattern of the m/z 219 ion of α-HCH. (B) 1: MS/MS TIC chromatogram of pesticides. α-HCH, 2: Aldrin, 3: Heptachlor epoxide, 4: Chlordane, 5: Endosulfan II. 6: Endrinaldehyde, 7: Endosulfan sulfate, 8: Endrin ketone.

that any site which is contaminated must be remediated. In order to understand whether a site is contaminated or otherwise, one must consider some limit values of the POPs in question. The limits proposed in Austrian Guidelines may be considered for this purpose.

PCB (includes also PCT, PBB, etc.): In various countries a limit value of 50 ppm is already established to define PCB wastes. In a footnote to entry A3180 of Annex 8 of the Basel Convention this is already acknowledged although

it is also stated that several countries have fixed a more stringent limit value (e.g. 20 ppm). An additional argument for the 50 ppm limit value is that any limit value fixed should be enforceable. There exist different quick tests for PCB in oil and also for PCB in soil. Typically these tests can be used down to 20 to 5 ppm PCB content. This fact which would ease the enforcement of any regulation should also be kept in mind.

PCCD/PCDF: Unlike most other POPs these substances are produced only unintentionally by various processes, mostly but not only by incineration. Different sources produce different pattern of PCDD/PCDF. Some synthesis (e.g. the production of pentachlorophenol) produce mainly the most toxic "Seveso Dioxin" (2, 3, 7, 8 tetrachlorodibenzo [1, 4] dioxin) while incineration processes produce also higher chlorinated and less toxic dioxins and furans.

There exists a well established system of "toxicity equivalent" (TE) to define the toxicity of a mixture of different dioxins and furans. Any limit value should be based therefore on this TE. In several countries (including the EU Member States) at least for stack emissions of PCDD/PCDF a limit value of 0.1 ng TE/m³ for incineration plants was introduced.

The Basel Convention lists PCDD (in Annex 1 PCDF under Y43 and PCDD under Y44). In the OECD Decision (92)39 Final wastes containing PCDD/PCDF were categorized as "red list wastes". In the rational to this OECD Decision a limit value of 10.000 ng TE/kg was proposed as a definition of "PCDD/PCDF" waste. Different guidelines for the use of PCDD/PCDF contaminated areas give a limit value of 10.000 ng TE/kg in soil for industrial sites as an acceptable concentration (while for domestic areas a lower value of 1.000 ng TE/kg is fixed).

"Pesticide" POPs: The acute toxicity of pesticide POPs vary widely (over a range of 1 to 2 magnitudes). This may lead to the conclusion that different limit values are necessary. Fixing one limit value for all pesticide POPs would allow to use adequate sum parameters (like EOX extractable organic chlorine compounds or AOX absorbable organic chlorine compounds) for screening investigations and the enforcement of the limit value.

A review of the "clean up" values in Europe (as far as chloro-organic pesticides are addressed) gives limit values in the range of 1-5-10 ppm. If the limit value is fixed with 5 ppm, the amount of analytical work could be reduced. The standards followed in some of the countries are provided below:

Germany 1994: DDT acceptable soil level: industrial sites: 4 mg/kg TS, domestic area 0.8 mg/kg TS (background level: Baden-Württemberg, DE, 1993: 0.03 mg/kg)

Netherlands (1988): HCB 10 mg/kg TS for further investigation, severe ecological danger (1993) 30 mg/kg TS

Netherlands (1993): Aldrin, Dieldrin, Endrin: severe ecological danger 4 mg/kg TS

(E) *Status of the Site*

From the limit values of DDT described in the Austrian Guidelines, the site is contaminated and must be remediated. India is still producing and using DDT. In the past, large amounts of DDT were used to be applied in agriculture but now it is mainly used as an intermediate in the production of dicofol, as an additive for marine antifouling paint, and for prevention and control of malaria. At present, India does not have complete regulations and standards on POPs related to food or foodstuff and electrical and mechanical equipment, which are not favourable for the protection of human health and of animals and plants. On the one hand, without adequate laws and regulations, it is difficult to carry out supervision of domestic products and to prevent foreign products containing POPs coming into India. On the other hand, the issue of POPs residues in products has become one of the obstacles to export of Indian products. As European and American countries stop uses of POPs and reduce their releases, background values of POPs in the environment are gradually decreasing in the developed countries and they will take more rigorous restrictive protection measures for the trading of related commodities, especially foods. As a result, India will face even more severe challenges in foreign trade. These issues should prompt India to decontaminate POPs contaminated site using BAT/ BEP.

(F) *Remediation Strategy*

Since the preliminary site investigation (PSI) reveals that the site is contaminated and DDT and its derivatives are over the level of 50 mg/kg, it is necessary to conduct a detailed site investigation (DSI) to determine the extent to which the remediation measures are required to be taken to decontaminate the site. DSI requires:

- Field investigation: Site screening methods (non-intrusive geophysical investigations), comprehensive subsurface investigations (excavation, borehole drilling, construction of monitoring wells)
- Analysis of field samples (delineates nature and extent of contamination)
- Remedial investigations (delineation of remediation options based on risk assessment)
- Implementation of remediation options (In situ, Ex situ)
- Post remediation monitoring: Since natural attenuation of POPs is an extremely slow process because of the recalcitrant nature of POPs, a programmed remediation approach is required. The soil is required to be excavated from the hotspots and is to be transported to a secured landfill with a double liner system and equipped with leachate detection and collection systems in accordance with the designed parameters of USEPA and the guidelines published by the Central Pollution Control Board (CPCB) of India for hazardous waste management. The contaminated soil containing POPs at levels higher than 50 mg/kg but within the limit of 100 mg/kg may be subjected to active oxidation/reduction process, bioremediation and phytoremediation. An attempt to degrade DDT using zero valent metals

was made in the National Environmental Engineering Research Laboratory, Nagpur and the results are as under.

DDT Experiment: 10 g of processed soil was taken in 250 ml beakers and about 25 ml of distilled water was added to make soil slurry. Six sets of experiments were initiated.

- Set I was kept as a control; under similar conditions as test without the addition of metals and acids, in order to account for any spontaneous dechlorination in soil.
- Set II was kept as another control with addition of only acetic acid to soil slurry.
- Set III, 2.0 g (80 mM) of zero-valent Mg^0 metal powder was added to the soil slurry.
- In set IV, 2.0 g of Mg^0 powder along with acetic acid was added to the soil slurry. The pH was adjusted to 3.0 with acetic acid, every time it turned alkaline.
- In set V, 4.5 g (80 mM) of zero-valent Fe^0 metal powder was added to the soil slurry.
- In set VI, 4.5 g of Fe^0 was added with acetic acid and pH was adjusted to 3.0, every time it turned alkaline.

All the beakers were kept on an orbital shaker at 130 rpm under ambient conditions. Soil was maintained in slurry with addition of distilled water, whenever it turned dry. Precautions were taken to exclude oxygen or reduce redox potential of the reaction mixtures. Entire reaction mixtures were processed after 30^{th} day, air dried.

Results: The experimental observations are delineated hereunder.

- Both the isomers, i.e. pp'DDT and op'DDT showed similar kinds of degradation in each of the treatments.
- Degradation with Mg^0 metal was around 41 and 39 percent for pp'DDT and op'DDT respectively.
- Around 50 percent degradation was observed in both pp'DDT and op'DDT with $Mg^0 + CH_3COOH$.
- Around 10 percent degradation was observed in both pp'DDT and op'DDT with Fe^0.
- The degradation was around 20 and 24 percent for pp'DDT and op'DDT respectively with $Fe^0 + CH_3COOH$ treatment, followed by extraction for residual pesticide analysis.

From the laboratory data it appears that this approach to remediate DDT using acetic acid and zero valent metals may not be techno-economically feasible.

(G) *Remediation of POPs Contaminated Sites*

Bioremediation and phytoremediation can provide a low cost alternative to other remediation techniques. It also results in the complete destruction of the

organic compounds, as opposed to other techniques, which may result only in the stabilization or disposal of the contaminant. However, in the document titled "Bioremediation and Phytoremediation of Pesticide Contaminated Site," prepared by Frazar (2000) for USEPA, the author describes the limitations of bioremediation and phytoremediation to decontaminate pesticide-contaminated site. The document states about Daramend technology which appears suitable for the remediation of POPs-contaminated sites. Patents have been issued to W.R. Grace & Co. for the DaramendTM process and additional remediation projects are underway.

This process has been used in a technology demonstration at a chemical manufacturing plant contaminated with chlorinated pesticides in Ontario, Canada (Raymond, 2000). According to the technology developer, DDT, DDD, DDE, 2,4-D and 2,4,5-T were reduced from 250 tons of contaminated soil by 99.5 percent (Grace & Co., 1999). At a site in Charleston, South Carolina, the DaramendTM process was used successfully in an in situ pilot-scale demonstration to remediate toxaphene and DDT. According to the technology developer, toxaphene and DDT were reduced by 98 and 90 percent respectively from contaminated spoil.

Persistent organic pollutants (DDX, HCH, and endosulfan) were quantified in top soil and sub-soil of a pesticide manufacturing industry (Pandya et al., 2006). It was also possible to identify the presence of some other organochlorinated compounds (OCs) in the soil.

A suitable multi-residue analysis of persistent organic pollutants (POPs) in soil samples was developed based on soxhlet extraction and gas chromatography–mass spectrometry for quantifying parent DDT compounds and degradation products, namely OCs and other miscellaneous pesticides. The quantification protocol was developed using Programmed Temperature Vaporization (PTV) and GC/MS/MS as identification tools. Extraction, PTV and MS/MS conditions were optimized for 11 pesticides with unambiguous spectral confirmation. The protocol has been applied to a large number of environmental samples and has proved to be reliable. The degradation ratios between the parent substances and their metabolites (DDX and HCH isomers) were calculated to determine whether there were any fresh inputs of parent pesticide at the site. Pesticide concentrations in the low to high concentration range (159 μg/kg to 133 mg/kg) have been measured. The investigations clearly indicate pesticide contamination in the soil.

2.6.2 Control and Remediation of Acrylonitrile Contaminated Area at J.R. Enterprises, Kandla, Gujarat

(A) *Background*

The earthquake in Kutch region of Gujarat on January 26, 2001 resulted in the spillage of acrylonitrile (AN) due to development of cracks at the bottom of a storage tank. Potential threat lied in contamination of soil and groundwater in

the surrounding area. Studies were undertaken for controlling the spillage and remediation of contaminated area. The approach included:

- Assessment of release of AN (2584 KL of AN)
- Recovery of the spilled material (70%)
- Spill simulation (to study the spread of AN)
- Assessment of contamination (1 ha, 30 cm deep)
- Remediation of contaminated area

(B) *Selection of Remedial Option*

Ex situ remediation: Excavation and transport of contaminated soil from 1 ha of land upto a depth of 30 cm. This is uneconomical and, therefore, was not selected.

In situ remediation (Bioremediation): This option was selected due to high biodegradability of AN and the local climatic conditions at site were supportive of biodegradation. The steps included:

- Stimulation of existing microflora via enrichment with nutrient as well as by moisture control.
- Exogenous organisms (acclimated) added to increase the versatility of the system. Acrylonitrile degrading bacterial culture C1 (gram negative small rods) and C2 (gram positive cocci) were isolated in the laboratory. Both the cultures were able to utilize AN upto a concentration of 2000 mg/L as the sole source of carbon and nitrogen.
- The concentration of AN was brought down to below detectable limit in five days when contaminated soil was mixed with AN degrading culture (K=1.465 mg/kg/d).
- Acrylonitrile, in contaminated soil in the field, degraded slowly under natural conditions. However, when it was mixed with AN degrading culture alongwith farmyard manure, the soil was completely remediated.

2.6.3 Elimination of Crude Oil Spill

(A) *Problem*

Oil spills are a major menace to the environment as they severely damage the surrounding ecosystems. Spills can take place on the land due to leakage from terrestrial pipelines and pilferage activities. This may cause a three-fold menace: fire hazards, groundwater pollution due to percolation, and air pollution due to evaporation.

Apart from the accidental spills of crude oil, oily sludge—hydrocarbon waste generated in huge quantities by oil refineries—also creates environment pollution. Oil refineries need a well-planned oily sludge management strategy to manage oil sludge. A straightforward approach may be to dump the oily sludge into specially constructed pits. Since the possibility of seepage cannot be ruled out, the ideal sludge pit should incorporate a leachate collection

system and a polymer lining to prevent the percolation of contaminants into the groundwater. Such pits are not only very expensive, but are also needed in large numbers for a single refinery. Since there is a limit to the area available within a refinery, alternative solution for the eradication of oily sludge is microbial bioremediation.

(B) *Approach*

In situ bioremediation is a process that employs microorganisms capable of degrading toxic contaminants for the reclamation of polluted sites. It has the potential to treat the contaminants on-site (in situ) thus ensuring that the contaminant is not merely moved from one place to another. Studies conducted by The Energy and Resources Institute (TERI) revealed that an efficient bacterial consortium degrades crude oil and oily sludge very fast, especially when oxygen and fertilizers supplement the growth of the microorganisms (www.teriin.org/index.php?option=com_casestudy).

This bacterial consortium was developed by mixing five bacterial strains, which could degrade aliphatic, aromatic, asphaltene, and NSO (nitrogen, sulphur, and oxygen compounds) fractions of crude oil and oily sludge. Crude oil and oily sludge degrading efficiency of the developed bacterial consortium was tested under laboratory conditions and field conditions. A feasibility study on the bioremediation of soil contaminated with crude oil/oily sludge was carried out at the Mathura oil refinery (India) with six different treatments in a 25 square metre land area, contaminated with crude oil/oily sludge, prior to full scale bioremediation. The indigenous crude oil/oily sludge degrading bacterial population was only 104 cfu/g soil in the feasibility study. Of the six treatments, the application of bacterial consortium and nutrients gave maximum response, which resulted in 48.5 percent biodegradation of TPH (total petroleum hydrocarbons) in four months as compared to only 17 percent biodegradation of TPH in soil treated with nutrients alone. Based on the feasibility study, the treatment consisting of the application of bacterial consortium and nutrients was selected for full-scale bioremediation.

Fig. 2.7: Application of Oilzapper at oil spill site in Gujarat.

A microbial consortium was developed from five bacterial isolates. These isolates were obtained from hydrocarbon-contaminated sites using enrichment methods. The microbial consortium developed was immobilized with a suitable carrier material, namely powdered corncob, which is an environment-friendly, biodegradable product. The survivability of the consortium in the immobilized condition was determined and was found to be three months at ambient temperatures. The immobilized culture was put into sterile polythene bags and sealed aseptically and transported to the place of requirement. This immobilized bacterial consortium was named Oilzapper. The site was tilled thoroughly to mix the oily sludge uniformly with the soil and oilzapper applied onto it. The land was tilled again and watered to maintain proper aeration and moisture levels. The land was tilled at regular intervals to facilitate faster degradation. The problem of heterogenous distribution of the oily sludge was solved by extensive tilling prior to the application of the oilzapper.

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3

Biosorption in Environmental Remediation

Maria Gavrilescu

3.1 Introduction

The harmful effects of organic and inorganic pollutants on ecosystems and on human health are well known and much expenditure is devoted to industrial treatment methods to prevent or limit discharges (Gadd, 2009).

Alternative pollutants removal and/or recovery methods are being considered which are based on sequestering properties of certain natural materials of biological origin. Sorption technology is currently being used extensively for the removal of organic and inorganic micropollutants, in soluble or insoluble forms from aqueous solutions (Lin and Juang, 2009). Biological treatment, based on living or non-living microorganisms or plants, offers some advantages, such as low operating cost and high efficiency (Volesky, 2001). Biological materials have emerged as an economic and eco-friendly option.

Biosorption, as a particular biological treatment, is a physico-chemical process, which involves the removal of various pollutants, such as metal or metalloid species, compounds and particulates from solution by biological material and is a property of both living and dead organisms (and their components) (Gadd, 1993). As a specific term, biosorption is adopted to indicate a number of metabolism-independent processes (physical and chemical adsorption, ion exchange, complexation, chelation, and micro-precipitation) taking place essentially in the cell wall (Lin and Juang, 2009).

Pollutants accumulative bioprocesses generally are divided into two broad categories (Veglio and Beolcmi, 1997; Volesky, 2001):

- biosorption (passive process); and
- bioaccumulation (active process).

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Certain types of microbial biomass can retain relatively high quantities of metal ions or other pollutants by "passive" sorption and/or complexation. This is commonly known as biosorption, which is *a non-directed physicochemical interaction that may occur between pollutant species and microbial cells*. Biosorption as a biological method of environmental control using non-living biomass can be considered an alternative to conventional contaminated water treatment facilities. It also offers several advantages over conventional treatment methods including cost effectiveness, efficiency, minimization of chemical/biological sludge, requirement of additional nutrients, and regeneration of biosorbent with possibility of metal recovery (Gavrilescu, 2004; Hima et al., 2007; Sari et al., 2007b; Zaied et al., 2008; Sari and Tuzen, 2008; Uluozlu et al., 2008; Hlihor and Gavrilescu, 2009b).

3.2 Equilibrium Studies in Biosorption

Proper analysis and design of adsorption/biosorption separation processes requires relevant adsorption/biosorption equilibria as one of the vital information. In equilibrium, a certain relationship prevails between solute concentration in solution and adsorbed state (i.e., the amount of solute adsorbed per unit mass of adsorbent) (Febrianto et al., 2009).

At the first stage of biosorption, a rapid equilibrium is established between adsorbed species on the cell and unadsorbed species in solution. This equilibrium can be represented by sorption isotherms. The single component sorption isotherm is the mathematical function describing quantitatively, at a constant temperature, the relationship between residual (equilibrium) metal concentration left in solution after binding (C_{eq}) and amount of metal bound to the biomass (q_{eq}) usually determined by difference (Aksu et al., 1997).

Isotherm Models

The equilibrium concentrations are a function of temperature. Therefore, the adsorption equilibrium relationship at a given temperature is referred as sorption isotherm. Several sorption isotherms originally used for gas phase adsorption are available and readily adopted to correlate adsorption equilibria in heavy metals biosorption.

Langmuir, Freundlich and Redlich-Peterson models were used to determine the sorption equilibrium between the solid biosorbent and various pollutants.

The isotherm equations for all models proposed are listed in Table 3.1. The *Langmuir model* assumes that a monomolecular layer is formed when biosorption takes place without any interaction between the adsorbed molecules (Aksu, 2002; Malkoc and Nuhoglu, 2005). Freundlich isotherm is an empirical equation based on a heterogeneous surface (Arica et al., 2001; Koumanova et al., 2002). The Redlich-Peterson isotherm (Dursun, 2006) incorporates the

features of the Langmuir and the Freundlich isotherms and has three parameters K_{rp}, a_r and β.

One of the isotherms proposed to describe equilibrium and competitive adsorption for such a system is a modified Langmuir model based on the same hypotheses as for the single-component Langmuir model and also assumes identical saturation capacities for all components. The extension of the basic Langmuir model to competitive adsorption of multi-component mixtures is written as in Table 3.1 (Aksu et al., 1997). Bellot and Condoret (1993) have defined an interaction term *i* in the Langmuir model, which is a characteristic of each species and depends on the concentrations of the other components. Tempkin and Pyzhev (cited by Yurtsever and Sengil, 2009) considered the effects of some indirect adsorbate/adsorbate interactions on adsorption isotherms and suggested that because of these interactions the heat of adsorption of all the molecules in the layer would decrease linearly with coverage so that the Tempkin isotherm can be developed.

Table 3.1 summarizes some of the most frequently applied simple sorption isotherm models. The great majority of these isotherms can be applied in linearized forms (Table 3.2).

Usually two-parameter models such as Langmuir and Freundlich isotherm can fit the data reasonably well, but these models are just "mathematical functions", they hardly reflect the sorption mechanism (Caliman and Gavrilescu, 2008). Furthermore it has been reported that the process does not depend on the viability of the biomass, which is an advantage, because waste biomass can be used for this purpose (Arief et al., 2008). However, for practical applications, multimetal biosorption models have to be judiciously used (Table 3.1).

Langmuir, Freundlich and Dubinin-Radushkevich (D-R) models were applied by Sari and Tuzen (2009) to describe the biosorption isotherm of the metal ions by *A. rubescens* biomass. Langmuir model fitted the equilibrium data better than the Freundlich isotherm. The maximum biosorption capacity of *A. rubescens* for Pb(II) and Cd(II) was found to be 38.4 and 27.3 mg/g, respectively.

The experimental data obtained by Khambhaty et al. (2009) on biosorption of hexavalent chromium by dead fungal biomass of marine *Aspergillus niger* were analyzed using five two-parameter isotherms (Langmuir, Freundlich, Dubinin-Radushkevich, Temkin and Halsey). It was observed that Langmuir model exhibited the best fit to experimental data.

Sorption equilibrium studies made by Aravindhan et al. (2007) for the biosorption of basic blue dye on to the green macro algae *Caulerpa scalpelliformis,* demonstrated that the biosorption followed Freundlich isotherm model, which implies a heterogeneous sorption phenomenon. Optimized parameters were used to treat the commercial effluent containing the dye. Complete colour removal was observed in two stages of treatment with the seaweed.

Table 3.1: Frequently used single-component adsorption models

Isotherm	*Equation*	*Advantages*	*Disadvantages*
Langmuir	$q = \frac{bq_m C_e}{1 + bC_e}$	Interpretable parameters	Not structured monolayer sorption
Langmuir (Multi-component)	$q_i = \frac{b_i q_{mi} C_i}{1 + \sum_{i=1}^{N} b_i C_i}$		
Freundlich	$q = KC_e^{1/n}$	Simple expression	Not structured no leveling off
Tempkin	$q_e = \frac{RT}{b}(\ln AC_e)$	Considers the effects of some indirect adsorbate/ adsorbate interactions	
Combination (Langmuir-Freundlich)	$q = \frac{bq_m C_e^{1/n}}{1 + bC_e^{1/n}}$	Combination of above	Unnecessarily complicated
Radke and Prausnitz	$\frac{1}{q} = \frac{1}{aC_e} + \frac{1}{bC_e^{\beta}}$	Simple expression	Empirical, uses three parameters
Reddlich-Petterson	$q = \frac{aC_e}{1 + bC_e^n}$	Approaches Freundlich at high concentration	No special advantages
Brunnauer (BET)	$q = \frac{BCQ^0}{(C_S - C)\left[1 + (B-1)C/C_s\right]}$	Multilayer adsorption inflection point	No "total capacity" equivalent
Dubinnin-Radushkevich	$\frac{W}{W_0} = \exp\left[-k\left(\frac{\varepsilon}{\beta}\right)^2\right]$ (*volume adsorbed*)	Temperature independent	No limited behaviour in Henry's Law
LAST: Ideal Adsorbed Solution Theory	$\frac{1}{q_t} = \sum \frac{Y_i}{q_i^0}$		
SCM: Surface Complexation Model	$q \approx f(C_e)$		

Dye adsorption isotherms fitted Langmuir model well and the maximum adsorption capacities at 20 °C were calculated to be 160 mg g^{-1} for Reactive Blue 19, 122 mg g^{-1} for Reactive Red 241 and 137 mg g^{-1} for Reactive Yellow

Table 3.2: Equilibrium isotherm models proposed in linear form

Models	*Equation*	*Linear equation*	*References*
Langmuir	$q_e = \frac{q_{\max} K_c C_e}{1 + K_c C_e}$	$\frac{C_e}{q_e} = \frac{1}{q_{\max} K_c} + \frac{C_e}{q_{\max}}$	Malkoc and Nuhoglu (2005), Aksu (2002)
Freundlich	$q_e = K_f C_e^n$	$\ln q_e = \frac{1}{n} \ln c_e + \ln K_f$	Arica et al. (2001), Koumanova et al. (2002)
Redlich-Peterson	$q_e = \frac{K_{rp} C_e}{1 + a_c C_e^{\beta}}$	$\ln\left(K_{rp} \frac{C_e}{q} - 1\right) = \beta \ln (C_e) + \ln a_r \; \beta \; (0 < \beta < 1)$ For $\beta = 1$, the equation converts to the Langmuir form	Dursun (2006)

145, respectively when biosorption was tested using mycelium pellets of *Penicillium oxalicum* as biosorbent (Zhang et al., 2003).

Deng et al. (2008) used *Penicillium chrysogenum* as raw materials to prepare the aminated adsorbent for pentachlorophenol (PCP) and 2,4-dichlorophenoxyacetic acid (2,4-D) sorption from aqueous solution. The maximum sorption capacity indicated were 1.23 mmol/g for PCP and 1.22 mmol/g for 2,4-D.

3.3 Kinetic Modelling

The kinetic of sorption describes the rate of pollutant uptake on the sorbent, which controls the equilibrium time. The kinetic parameters are helpful for the prediction of sorption rate, which gives important information for designing and modelling the processes (Hameed et al., 2008).

Different types of kinetic models including *(pseudo)-first order, (pseudo)-second-order* and *intraparticle diffusion* were used to investigate the mechanism of biosorption and potential rate controlling steps such as mass transport and chemical reaction processes (Yurtsever and Sengil, 2009).

The first-order rate equation of the Lagergren is given as (Ho and McKay, 1999c; Arica et al., 2001; Cheung et al., 2001) (Eq. 1):

$$\log\left(q_e - q_t\right) = \log\left(q_e\right) - \frac{k_1}{2.303} t \tag{1}$$

where q_e and q_t are the amounts of adsorbed cadmium(II) ions on the biosorbent at equilibrium and at time t (respectively mg/g) and k_1 is the first-order biosorption rate constant (min^{-1}). The kinetic parameters can be determined graphically (Fig. 3.1).

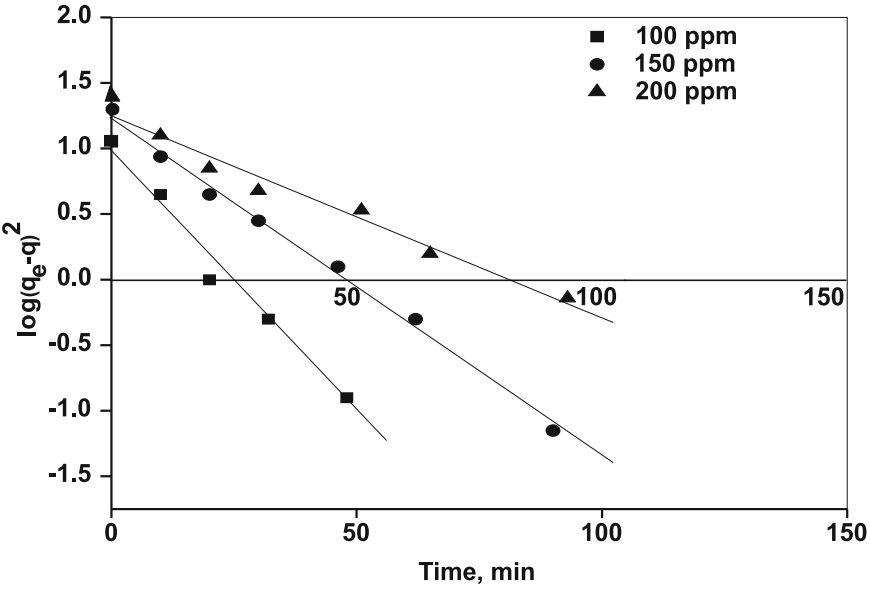


Fig. 3.1: Pseudo-first order kinetics of powered *Ocimum americanum* L. seed pods for different initial concentrations at optimum pH 1.5 (Levankumar et al., 2009).

The pseudo-second-order equation is also based on the sorption capacity of the solid phase and is given as (Hamadi et al., 2001; Arica et al., 2001; Levankumar et al., 2009) (Eq. 2):

$$\frac{t}{q_t} = \frac{1}{k_2 q_c^2} + \frac{1}{q_e^2} t \tag{2}$$

where k_2 is the second-order biosorption rate constant (g/mg min), q_e is the biosorption capacity calculated by the pseudo-second-order kinetic model (mg/g).

If the following relation is considered (Eq. 3):

$$h = k_2 q^2 e \tag{3}$$

where h is the initial sorption rate in mg/g min, the values of k_2 and h are determined from the intercept of second order Eq. (2) from graph shown in Fig. 3.2 (Levankumar et al., 2009).

The equation defining the Elovich model is based on a kinetic principle assuming that the adsorption sites increase exponentially with adsorption, which implies a multilayer adsorption (Yurtsever and Sengil, 2009). The linear form of Elovich equation is given by (Elovich and Larinov, 1962) (Eq. 4):

$$q_t = \frac{1}{\beta} \ln(\alpha\beta) + \frac{1}{\beta} \ln t \tag{4}$$

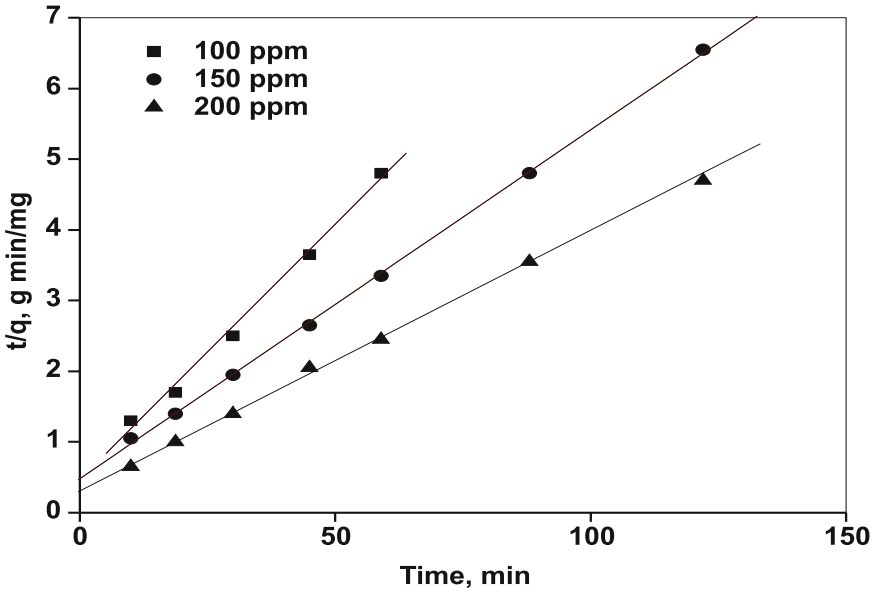


Fig. 3.2: Pseudo-second order kinetics of powered *Ocimum americanum* L. seed pods for different initial concentrations at optimum pH 1.5 (Levankumar et al., 2009).

where α and β, known as the Elovich coefficients, represent the initial sorption rate (mg g^{-1} min^{-1}) and is related to the extent of surface coverage and activation energy for chemisorption (gmg^{-1}), respectively. The Elovich coefficients could be computed from the plots of q_t versus ln t.

The fitting of the above four models to experimental data was examined by each linear plot of q_t versus $t^{1/2}$, ln $(q_e - q_t)$ versus t, (t/q_t) versus t, and q_t versus ln t, respectively.

The intraparticle diffusion model can be described as (Weber and Morris, 1962; Yurtsever and Sengil, 2009) (Eq. 5):

$$q_t = k_{int} t^{1/2} \tag{5}$$

where k_{int} is the intraparticle diffusion rate constant and q_t is the amount of dye adsorbed (mg g^{-1}) at time (t). Values of k_{int} (mg g^{-1} $h^{1/2}$) can be calculated from the slope of the linear plots of q_t versus $t^{1/2}$. In a liquid-solid system, the fractional uptake of the solute on particle varies according to a function of diffusivity within the particle and the particle radius (Yurtsever and Sengil, 2009).

When the biosorbent is treated as a porous material in aqueous solution, the diffusion process can affect the biosorption process (Weber, 1985). Therefore the intraparticle diffusion equation was introduced to indicate the behaviour of intraparticle diffusion as the rate limiting step in the biosorption (Sharma and Foster, 1994). The equation is given as (Eq. 6):

$$R = K_s t^b \tag{6}$$

where R is the percent metal adsorbed, t is the contact time (min), b is the gradient of linear plots and K_s is the intraparticle diffusion constant.

The kinetic of the adsorption data are frequently analyzed using four different kinetic models (Hameed et al., 2008; Apostol and Gavrilescu, 2009; Mathialagan and Viraraghavan, 2009; Nadavala et al., 2009; Nkedi-Kizza et al., 2006; Yu et al., 2009) (Table 3.3).

Sari and Tuzen (2009) tested experimental data obtained for the biosorption of Pb(II) and Cd(II) from aqueous solution by macrofungus (*Amanita rubescens*) biomass, in terms of biosorption kinetics using pseudo-first-order and pseudo-second-order kinetic models. The results showed that the biosorption processes of both Pb(II) and Cd(II) followed well pseudo-second-order kinetics. Table 3.4 presents the sorption parameters of some organic pollutants on natural sorbents.

Table 3.3: Kinetic models applied for description of pollutant uptake on sorbents (Apostol and Gavrilescu, 2009)

Kinetic model	*Equation*		*Characteristics*
The pseudo-first order	$\ln (q_e - q_t) = \ln q_e - k_1 t$	(7)	q_e and q_t (mg/g) are the amounts of adsorbate adsorbed at equilibrium and at any time, t (h), respectively, and k_1 is the adsorption rate constant
The pseudo-second-order	$dq/dt = k_2(q_e - q)^2$	(8)	k_2 (g/mg h) is the constant of the rate of second-order adsorption. The second-order rate constants were used to calculate the initial sorption rate given by: $h = k^2 q_e^2$
Elovich equation	$q_t = (1/b) \ln (ab) + (1/b) \ln t$	(9)	where a and b are the constants for this model obtained from the slope and intercept of the linear plot of q_t versus ln t
Intraparticle diffusion model	$q_t = k_{id} t^{1/2} + C$	(10)	q_t (mg L^{-1}) is the amount adsorbed at time t (min), and K_{id} (mg g^{-1} min^{-1}) is the rate constant of intraparticle diffusion. C is the value of the intercept, which gives an idea about the boundary layer thickness, i.e., the larger the intercept, the greater is the boundary layer effect

Table 3.4: Sorption parameter for removal of POPs by natural sorbents

Organic pollutant	*Sorbent*	*Isotherm parameter*	*Kinetic parameter*	*References*
Pentachlorophenol	Pine bark	$K_F = 8.6 \pm 1.6$	$q_e = 7.65$	Bras et al. (2005)
	Peat	$K_F = 4.47$	–	Tanjore and Viraraghavan (1997)
	Aspergillus niger	–	$k_1 = 168.86$	Mathialagan and Viraraghavan (2009)
2,4-dichlorophenol	Phanerochaete chrysosporium	$K_F = 0.232$	$k_1 = 0.085$	Wu and Yu (2006)
Endosulfan sulfate	Bamboo canes	$K_F = 12.86 \pm 1.56$	$k_1 = 0.29 \pm 0.05$	Bakouri et al. (2009)
	Olive stones	$K_F = 13.54 \pm 1.13$	$k_1 = 0.25 \pm 0.06$	
	Peanut	$K_F = 11.06 \pm 1.44$	$k_1 = 0.21 \pm 0.04$	

K_F (mg g^{-1}(mg L^{-1})) is Freundlich constant isotherm
q (mmol g^{-1}) is the monolayer sorption capacity (Langmuir isotherm)
q_e (mg/g) is the amount of adsorbate adsorbed at equilibrium
K_{id} (mg g^{-1} min^{-1}) is the rate constant of intraparticle transport, k_1 is the adsorption rate constant

3.4 Thermodynamic Parameters

The standard free energy ($\Delta G°$), enthalpy change ($\Delta H°$) and entropy change ($\Delta S°$) thermodynamic parameters have been estimated to evaluate the feasibility of the adsorption process.

The positive value of change in enthalpy ($\Delta H°$) indicates that the adsorption is an endothermic process, while positive value of change in entropy ($\Delta S°$) reflects the increased randomness at the solid/solution interface.

The Gibbs free energy change of the process is related to the K_c by Eq. 11:

$$\Delta G^o = -RT \ln K_c \tag{11}$$

K_c can be expressed as in relation (12):

$$K_c = \frac{C_a}{C_e} \tag{12}$$

where K_c is the distribution coefficient for the adsorption, C_a the amount of adsorbate (mg) adsorbed on the adsorbent per litre of the solution at equilibrium and C_e is the equilibrium concentration (mg L^{-1}) of solution.

In order to determine the thermodynamic parameters, experiments have to be carried out at different temperature (303-313 K) for biosorption. The free energy change ($\Delta G°$) of the sorption reaction is given by Eq. (11).

The thermodynamic parameters such as changes in standard free energy enthalpy change ($\Delta H°$) and entropy change ($\Delta S°$) are determined by using (Eq. 13):

$$\ln K_c = \frac{\Delta S° - \Delta H°}{R} \frac{1}{T} \tag{13}$$

where T is the temperature (K); R is the gas constant (8.314 J mol K), K_c is the equilibrium constant obtained from Langmuir isotherm. The values of enthalpy ($\Delta H°$) and entropy change ($\Delta S°$) can be obtained from the slope of the plot of ($\Delta G°$) versus T.

Thermodynamic parameters can be determined in batch experiments. For example Yurtsever and Sengil (2009) described experiments carried out at different temperatures in the range of 296–366 K for Pb adsorption on quebracho tannin resin (QTR) (Fig. 3.3). The values of $\Delta H°$ and $\Delta S°$ are calculated from the slope and intercept of the plots of ln K_c versus $1/T$.

Results are shown in Table 3.5. The Gibbs free energy change ($\Delta G°$) negative values increased with temperature, indicating the feasibility and spontaneity of the adsorption process of Pb^{2+} ions on QTR. The positive $\Delta H°$ indicated the endothermic nature of the adsorption process and the positive $\Delta S°$ an increase in the randomness in the system interface solid/solution during the adsorption process.

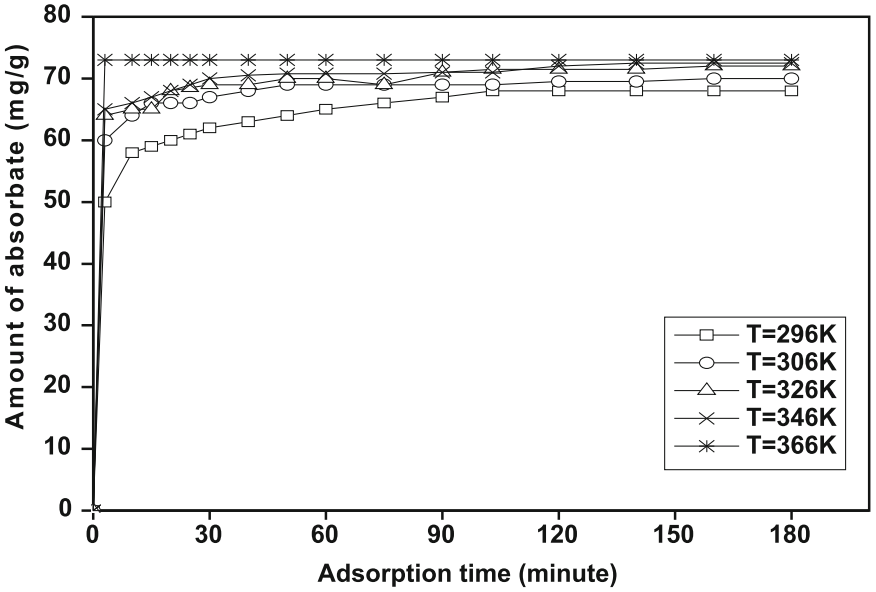


Fig. 3.3: Temperature influence on the Pb^{2+} uptake by quebracho tannin resin (C_0 = 75 mg L^{-1}, particle size = 38-53 μm, pH 5, biosorbent doze: 1 g L^{-1} (Yurtsever and Sengil, 2009).

Table 3.5: Thermodynamic parameters for Pb^{2+} ions adsorption on QTR (C_0 = 75 mg L^{-1}, pH 5, 38-53 μm particles and 350 rpm agitation rate) (Yurtsever and Sengil, 2009)

	Temperature, T(°K)				
	296	*306*	*326*	*346*	*366*
K_c	9.29	13.88	30.40	57.23	118.90
$\Delta G°$ (kJ mol^{-1})	– 5.43	– 6.63	– 9.17	– 11.54	– 14.42
$\Delta H°$ (kJ mol^{-1})	31.84				
$\Delta S°$ (kJ mol^{-1})	127.02				

Sari and Tuzen (2009) evaluated the mean free energy values for the biosorption of Pb(II) and Cd(II) from aqueous solution by macrofungus (*Amanita rubescens*) biomass. The results indicated that the biosorption of Pb(II) and Cd(II) onto *A. rubescens* biomass was taking place by chemical ion-exchange. The calculated thermodynamic parameters, $\Delta G°$, $\Delta H°$ and $\Delta S°$ (Table 3.5) showed that the biosorption of Pb(II) and Cd(II) ions onto *A. rubescens* biomass was feasible, spontaneous and exothermic under examined conditions.

3.5 Pollutants Removed by Biosorption

3.5.1 Heavy Metals

Environmental pollution by toxic metals occurs globally through military, industrial (especially fuel and power), agricultural processes and waste disposal that generates 5.15 million tons of As, Cd, Cr, Cu, Hg, Ni, Pb, Se, V, and Zn in total per annum all around the world (Brower et al., 1997; Zamil et al., 2009). Many industries including metal plating, mining, battery, precious metals manufacture, tanneries, pigment, dyestuff and chemical industries release heavy metals in waste streams which can lead to the contamination of freshwater and marine environment (Zhang and Ke, 2004; Mack et al., 2007).

Heavy metal ions are nowadays among the most important pollutants in surface and ground water (Gavrilescu, 2004; Baysal et al., 2009; Brinza et al., 2009; Hlihor and Gavrilescu, 2009a). Metals are essential minerals for all aerobic and most anaerobic organisms, but it has been proven that in large amounts some of them, such as copper, lead, cadmium, or mercury, seriously affect human health. The human body cannot process and dispose the metals. As a result they are deposited in various internal organs and may cause adverse reactions and serious damage to the body (Gavrilescu, 2004; WHO, 2007). Some of the health effects of heavy metals are illustrated in Table 3.6.

Table 3.6: Some effects of heavy metals on human health

Heavy metal	*Effects in human body*	*References*
Cr(VI)	Carcinogenic, adverse potential to modify the DNA transcription process, headache, nausea, epigastric pain, hemorrhage, severe diarrhea	Kurniawan et al. (2006a); Quaiser et al. (2007); Arief et al. (2008); Namasivayam et al. (2008); Hlihor and Gavrilescu (2009a, b)
Cr(III)	Cancer, allergic skin reactions	Vilar et al. (2007); Arief et al. (2008)
Zn(II)	Depression, lethargy, neurological effects (seizures and ataxia)	Kurniawan et al. (2006); Hlihor and Gavrilescu (2009a)
Cu(II)	Insomnia, liver damage, Wilson's disease	Kurniawan et al. (2006b); Arief et al. (2008)
Cd(II)	Renal disorder, kidney damage, hepatic damage, cancer, hypertension	Kurniawan et al. (2006a); Kurniawan et al. (2006b); Arief et al. (2008); Igwe and Abia (2007)
Pb(II)	Seizure and metal retardation, encephalopathy, hemoglobin production reducing	Quaiser et al. (2007); Arief et al. (2008); Igwe and Abia (2007)
Ni(II)	Dermatitis, chronic asthma, coughing, bronchial hemorrhage, gastrointestinal distress, nausea, weakness and dizziness	Kurniawan et al. (2006a); Kurniawan et al. (2006b); Vilar et al. (2007); Arief et al. (2008); Dahiya et al. (2008)

The most appropriate solution for controlling the biogeochemistry of metal contaminants is sorption technique, to produce high quality treated effluents from polluted wastewater according to Devaprasath et al. (2007).

Lead

At present, lead pollution is considered a worldwide problem because this metal is commonly detected in several industrial wastewaters (Davydova, 2005). Lead is a more toxic element for human and animal lives. The presence of even low levels of lead in water is a concern primarily because it tends to bioaccumulate in the food chain (Adriano, 1986; Prasad et al., 2008). Pb(II) heads the list of environmental threats because, even at extremely low concentrations, it has been shown to cause brain damage in children. Many physicochemical methods have been proposed for their removal from industrial effluents, such as chemical precipitation (Solmaz et al., 2007), adsorption (Matlock et al., 2001), biosorption (Matheickal and Yu, 1999), electrodialytic process (Ferreira et al., 2005) and so on. A major drawback with precipitation is sludge production. Ion exchange is considered a better alternative technique for such a purpose (Kazemipour et al., 2008).

Biosorption of Pb(II) was adopted by researchers as an efficient and environmentally sound process.

Schiewer and Balaria (2009) investigated the uptake of Pb(II) by processed orange peels, a pectin-rich by-product of the fruit juice industry. Potentiometric titrations showed a significantly higher negative surface charge of protonated peels compared to original peels, with acidic groups around pH 4, 6, and 10. FTIR spectra of peels were similar to those of pectin. The carboxylic group peak shifted from 1636 to 1645 cm^{-1} after Pb(II) binding, indicating the involvement of carboxyl groups in Pb(II) binding. Equilibrium was achieved in 30 min to 2 h, depending on the particle size. The first-order model fitted experimental data less than the second- or third-order models. The obtained rate constants were much higher for smaller particles, while the capacity was similar for all sizes. Low pH, increased ionic strength, or competing co-ions reduced Pb(II) binding at low sorbent dosages, but at high sorbent dosages removal remained above 90%. The Pb(II) uptake at 300 ppm was 2 mmol/g (40% dry weight). Due to high uptake, favourable kinetics and good stability, citrus peel biosorbents hold high promise for industrial applications. Overall, this study suggests that biosorption of Pb(II) ions by orange peels can be an inexpensive and effective way of metal ion treatment and should be investigated further for its practical application.

The removal of poisonous Pb(II) from wastewater by different low-cost abundant adsorbents (rice husks, maize cobs and sawdust) was investigated by Abdel-Ghani et al. (2007) at different adsorbent/metal ion ratios, pH, contact time, metal concentration, and adsorbent concentration. The adsorption efficiencies were found to be pH dependent, increasing by increasing the solution pH in the range from 2.5 to 6.5. The equilibrium time was attained after 120 min and the maximum removal percentage was achieved at an adsorbent loading weight of 1.5 gm. The equilibrium adsorption capacity of adsorbents used for lead were measured and extrapolated using linear Freundlich, Langmuir and Temkin isotherms and the experimental data were found to fit the Temkin isotherm model (Abdel-Ghani et al., 2007).

Meunier et al. (2002) examined the efficiency with which some natural adsorbents (cocoa shells, cedar bark, pine bark, spruce bark, vermiculite and volcanic rocks) can remove heavy metals, especially lead, from very acidic leachate produced during soil decontamination by a chemical leaching process using hydrochloric acid. Cocoa shells were the most efficient biosorbent with a maximal capacity q_{max} = 2.60 mg Pb/g (initial pH=1.59 and $[Pb]_i$=45.4 mg/L). Cedar bark can also be used for metal removal in very acidic solutions but are less efficient than cocoa shells. Kinetic measurements of lead removal by cocoa shells have revealed that sorption equilibrium was obtained after approximately 4 h of contact (Meunier et al., 2002).

With the goal of identifying innovative, low-cost adsorbents, Rashed (2006) evaluated some sorption technologies, by determining the suitable conditions for the use of peach and apricot stones, produced from food industries as solid

waste, as adsorbents for the removal of lead from aqueous solution. Chemical stability of sorbents, effect of pH, adsorbents dose, sorption time and equilibrium concentration were studied. The results reveal that adsorption of lead ions onto peach stone was stronger than onto apricot stone up to 3.36% at 3 h adsorption time. Suitable equilibrium time for the adsorption was 3–5 h (% Pb adsorption is 93% for apricot and 97.64% for peach). The effective adsorption range for pH in the range was 7–8. Application of Langmuir and Freundlich isotherm models show high adsorption maximum and binding energies for using these adsorbents for the removal of lead ions from contaminated water and wastewater. The results of this study could be useful in scale-up and designing cost-effective treatment plants for the removal of Pb(II) ions from wastewater.

Chromium

The chromium pollution is increasing due to the generation of water from mining, leather tanning, cement, dying, electroplating and corrosive paint industries (Ho et al., 2000; Sankalia et al., 2004; Acosta et al., 2004; Ahalya et al., 2005; Hamadi et al., 2001). The chromium related industries are facing the problem of safest disposal of large quantity of chromium containing wastewater. Chromium exists in two states as Cr(III) and Cr(VI). Trivalent chromium is relatively less toxic and less mobile (Anderson, 1997; Deng et al., 2009), while hexavalent chromium is toxic, carcinogenic, and mutagenic to animals as well as humans (Costa, 2003). The hexavalent chromium is 500 times more toxic than the trivalent form (Kowalski, 1994). This has become a serious issue because Cr(VI) has been classified as a Group I human carcinogen by International Agency for Research on Cancer (IARC) and as a Group A inhalation carcinogen by US Environmental Protection Agency (EPA) (Levankumar et al., 2009). Chronic exposure to Cr(VI) causes cancer in digestive tract and lungs, and may cause epigastric pain, nausea, vomiting, severe diarrhea, and hemorrhage (Mohanty et al., 2005). Therefore, the removal of Cr(VI) from environment by biosorption has been a research topic of great interest.

Fiol et al. (2003) used waste products from industrial operations, such as yohimbe bark, grape stalks, cork and olive stones for the removal of Cr(VI) from aqueous solutions. Equilibrium batch experiments at room temperature were performed. Metal uptake showed a pH-dependent profile and optimum uptake at initial pH between 2.0 and 3.0. Slight influence of NaCl on metal uptake was observed. The sorption data fitted well to the Langmuir model within the concentration range studied. Grape stalks proved to be the most efficient sorbent followed by yohimbe bark, cork and olive stones (Fiol et al., 2003).

Babu and Gupta (2008) studied Cr(VI) removal from aqueous solutions using neem leaves activated by heat treatment as well as acidic treatment concentrated hydrochloric acid (36.5%). Batch adsorption studies demonstrate that the adsorbent prepared from neem leaves has a significant capacity for adsorption of Cr(VI) from aqueous solution. The parameters investigated in this study include pH, contact time, initial Cr(VI) concentration and adsorbent

dosage. The adsorption of Cr(VI) is found to be maximum (99%) at low values of pH in the range of 1-3. A small amount of the neem leaves adsorbent (10 g/L) could remove as much as 99% of Cr(VI) from a solution of initial concentration 50 mg/L. The adsorption process of Cr(VI) is tested with Langmuir isotherm model. Application of the Langmuir isotherm to the system yielded maximum adsorption capacity of 62.97 mg/g.

Devaprasath et al. (2007) evaluated the sorption capacity of *Prosopis spicegera,* a readily available tree leaves for removal of Cr(VI) from aqueous media. Adsorption studies were performed by batch experiments as a function of process parameters such as sorption time, pH, and concentrations of sorbate and sorbent. Freundlich model fitted best with the experimental equilibrium. The adsorption of Cr(VI) was found dependent on initial concentration of metal ion, pH, adsorbent dosage and agitation time. The maximum removal of Cr(VI) was observed at pH 2 (95%).

Levankumar et al. (2009) investigated the removal of hexavalent chromium from aqueous solutions by *O. Americanum* L. Seed pods. The powdered *O. Americanum* L. Seed pods were reported as an effective adsorbent for the treatment of hexavalent chromium for the very first time. It was capable of removing 100% of chromium from the aqueous solutions of concentrations 100 mg/L, 150 mg/L and 200 mg/L. The predicted maximum chromium adsorption capacity as 83.33 mg/g showed that adsorbent prepared from the *O. Americanum* L. Seed pods have reasonable chromium removal efficiency.

The ability of low-rank Turkish brown coals to remove Cr(VI) from aqueous solutions was studied by Arslan and Pehlivan (2007). Utilization of the brown coals for the treatment of aqueous solution containing Cr(VI) ions is gaining attention as a simple, effective and economical means of wastewater treatment. The mechanism of Cr(VI) ion binding to brown coals may include ion exchange, surface adsorption, chemosorption, complexation, and adsorption-complexation. The kinetics of Cr(VI) biosorption by brown coals was fast, reaching 50–90% of the total biosorption capacity in 60 min. The kinetics studies indicated that equilibrium in the adsorption of Cr(VI) on the brown coals was reached in 80 min of contact time between the brown coals and the solution.

Cr(VI) adsorption on brown coals was described only by the Freundlich isotherm model. The adsorption of Cr(VI) increased with an increase in the concentrations of these metals in solution. The substantially lower cost, easily available of the low-rank coal indicates great potential for the removal of Cr(VI) ions from aqueous systems.

The paper of Bansal et al. (2009) reported the feasibility of using pre-consumer processing agricultural waste to remove Cr(VI) from synthetic wastewater under different experimental conditions. For this, rice husk (agricultural waste obtained from the rice mills) has been used after pre-treatments (boiling and formaldehyde treatment). Maximum metal removal was observed at pH 2.0. The efficiencies of boiled and formaldehyde treated rice husk for Cr(VI) removal were 71.0% and 76.5% respectively for dilute

solutions at 20 g L^{-1} adsorbent dose. According to the researchers this data can be used by small scale industries having low concentrations of Cr(VI) in wastewater using batch or stirred-tank flow reactors where standard material such as activated carbon is not available.

Cadmium

Cadmium is a non-essential element and one of the most hazardous trace elements, being considered a *priority metal* from the standpoint of potential hazard to human health (Alvarez-Ayuso and Garcia-Sanchez, 2007). Toxic metal ions such as Cd(II) can eventually reach the top of food chain and thus, become a risk factor for people's health (Katircioglu et al., 2008). Cadmium, which is a widely used metal and extremely toxic in relatively low concentrations, is one of the heavy metals responsible for causing kidney damage, renal disorder, high blood pressure, bone fragility, and destruction of red blood cells (Abia and Asuquo, 2007).

Tan and Xiao (2008) investigated the adsorption behaviour of cadmium on ground wheat stems in aqueous solution to understand the physico-chemical process involved and to explore the potentiality of wheat stems in wastewater treatment. The results showed that 0.1032 mmol of cadmium is adsorbed per gram of ground wheat stems. The results of the study indicated that wheat stems has great potential to remove cadmium ions from aqueous solution through a low-cost and eco-friendly way.

Shin et al. (2007) compared the capacity of sorbents prepared from juniper wood and bark to adsorb cadmium from aqueous solutions at different pH values. Adsorption kinetics, adsorption isotherms, and adsorption edge experiments were used to characterize adsorption behaviour. Results from kinetics and isotherm experiments showed that juniper bark had 3–4 times higher adsorption capacity for Cd than juniper wood. In addition to higher capacity, JB exhibited a higher strength of adsorption (45.3 versus 9.1 L $mmol^{-1}$) and faster uptake kinetics (0.0119 versus 0.0083 g μmol^{-1} min^{-1}) compared to juniper wood. For both these adsorbents, increasing Cd adsorption with increasing solution pH in the range of 2–6 suggests that surface carboxyl groups (RCOOH) might be involved in interaction with Cd.

In the study of Al-Anber and Matouq (2008), olive cake was used as an adsorbent. It was generated during the squeezing of olive. Olive cake is an abundant and a low-cost adsorbent material on a large scale in many Mediterranean countries especially in Jordan. Jordan has a strong agricultural foundation that leaves behind 80,000 tonnes annually of olive cake wastes, with manure possibly being the most problematic one. The goal of this work was to study the capacity of using untreated olive cake to treat wastewater contaminated by the cadmium ion under different operating conditions (temperature, dose and pH). The adsorbent used in the study exhibited as good sorption at approximately pH 6 at temperatures 28, 35 and 45°C, respectively. The removal efficiency was found to be 66% at pH 6 and temperature 28°C.

Researches have also been carried out by using wheat bran as sorbent, which proved to be much economical, effectual and more viable than conventional sorbents, according to Nouri et al. (2007).

The ability of neem oil cake (NOC), a bio-waste material obtained as by-product of neem fruit to remove Cu(II) and Cd(II) ions from aqueous solution was investigated by Rao and Khan (2009). Neem (*Azadirachta indica*) is a fast growing, usually evergreen plant, which reaches a height of 15–20 m and a trunk girth of 1.5–3.5 m. Neem has been widely explored for solving various problems related to agriculture, public health, population control and environmental pollution. Neem has been recognized as a natural air purifier and it has been suggested that the planting of neem trees on roadside is an effective way to regulate traffic related pollution. The increase in initial concentration of Cu(II) and Cd(II) ions results in an increase in the sorption capacity, q_e (mg g^{-1}). The maximum sorption capacities at equilibrium were found to be 1.47, 2.4 and 4.94 mg g^{-1} at 15, 25 and 50 mgL^{-1} initial Cd(II) concentration, respectively. The equilibrium time was found to be 10, 50 and 60 min, respectively, for the initial Cd(II) concentrations of 15, 25 and 50 mgL^{-1} showing that equilibrium time depends upon the initial Cd(II) concentrations.

Bulut and Tez (2007) have investigated the adsorption behaviour of Ni(II), Cd(II) and Pb(II) from aqueous solutions by shells of hazelnut and almond, which are a very cheap and readily available material for the removal of selected heavy metals from aqueous solutions. The shells of hazelnut (SH) and almond (SA) were obtained commercially and used for the preparation of adsorbent. It was washed several times with distilled water to remove surface impurities and then dried. The structural properties and surface chemistry of the shells were characterized using sorption of nitrogen and Boehm titration. The equilibrium adsorption capacity of shells was obtained by using linear Langmuir and Freundlich adsorption isotherms. The best correlation coefficients were obtained for the pseudo second-order kinetic model. Ion exchange is probably one of the major adsorption mechanisms for binding divalent metal ions to the shells of hazelnut and almond. The selectivity order of the adsorbents is Pb(II) > Cd(II) > Ni(II).

Copper

Copper (Cu) is one of toxic metals. In particular, excessive intake of copper over 1.0 mg/L from drinking results in hemochromatosis and gastrointestinal catarrh diseases because it is accumulated in the livers of animals and humans. Since copper is an essential metal in a number of enzymes for all forms of life, problems arise when it is deficient or in excess. However, the carcinogenic character of copper is accepted and epidemiological evidence, such as the higher incidence of cancer among coppersmiths, suggests a primary carcinogenic role for copper. In addition, copper is phytotoxic so that it has been used as an algicide to control algal blooms. It can, therefore, cause plant damage if, for example, it is present at too high a concentration in sewage sludge that is

applied to agricultural land. A principal source of copper in industrial waste streams is metal cleaning and plating baths, and rinses, as brass, boiler pipe, cooking utensils, fertilizers, and from copper metal working, which requires periodic oxide removal by immersing the metal in strong acid baths (Ho and McKay, 2004). In recent years, various biosorbents have been used for removal of Cu(II) (Sari et al., 2007a).

Cotton boll was used as an adsorbent by Duygu Ozsoy and Kumburfor (2006), with the aim of removing of the Cu(II) ions from the aqueous solutions. The adsorption process was carried out in a batch process and the effects of contact time (2–24 h), adsorbent concentration (1–20 g L^{-1}), initial pH (2.0–6.0), initial metal ion concentration (20–160 mg L^{-1}) and temperature (20–45 ^{o}C) on the adsorption were investigated. Experimental results showed that the maximum adsorption capacity was determined at pH 5.0 and adsorbed Cu(II) ion concentration was increased with increasing adsorbent concentration and contact time. The isothermal data of cotton boll could be well described by the Langmuir equations and the Langmuir monolayer capacity had a mean value of 11.40 mg g^{-1}. Experimental results indicated that the pseudo-second order reaction model provided the best description of the data with a correlation coefficient 0.99 for different initial metal concentrations and therefore it was explained that chemical sorption was the basic mechanism in this system. FT-IR results showed that oxygen and nitrogen atoms in structure of cotton boll were involved in Cu(II) ions adsorption.

The study of Yazici et al. (2008) was aimed at determining the effect of chemical pretreatment on copper (II) biosorption by *Marrubium globosum* subsp. *Globosum* leaves. The uptake capacity of the biomass was increased by chemical pretreatment when compared with the raw biomass. The results of biosorption experiments, carried out at the conditions of 50 mg L^{-1} initial metal concentration and pH 5.5, showed that pre-treating the biomass with alkali solutions (laundry detergent, sodium hydroxide and sodium bicarbonate, 0.5 M) improved the biosorption capacity of biomass (45.90, 45.78 and 43.91%, respectively) compared with raw biomass. Pretreatment with sulfuric and nitric acid solutions, 0.5 M, increased the biosorption capacity of biomass by 11.82 and 10.18%, respectively, while there was no considerable change in the biosorption capacity of biomass (0.35%) after pretreatment with formic acid solution, 0.5 M. Furthermore, in sodium chloride and calcium chloride, 0.5 M, pretreatments resulted in the improvement in biosorption capacity of biomass (31.38 and 26.69%, respectively). FT-IR analysis revealed that hydroxyl and carboxyl functional groups were mainly responsible for Cu(II) biosorption.

The litter of natural trembling poplar (*Populus tremula*) forest (LNTPF) was used for the biosorption of Cu(II) ions in a batch adsorption experiment by Dundar et al. (2008). The sorption capacity of LNTPF was investigated as a function of pH, particle size, agitating speed, initial Cu(II) concentration, adsorbent concentration and temperature. The efficiency of copper uptake by the used LNTPF increases with a rise of solution pH, adsorbent concentration,

agitating speed, temperature, and with a decline of particle size and initial Cu(II) concentration. The biosorption process was very fast: 94% of Cu(II) removal occurred within 5 min and equilibrium was reached at around 30 min. These results demonstrate that the LNTPF has great potential and is low-cost heavy metal adsorbent. The LNTPF could be used as an effective, cheap and abundant adsorbent for the treatment of Cu(II) containing wastewaters.

Other metal ions such as zinc, arsenic, mercury and cobalt present in various industrial effluents are of environmental concern due to their toxicity even in low concentrations (Sud et al., 2008).

Arsenic poisoning is a serious health concern worldwide. Arsenic concentration above permissible limits is reported from many countries. Batch isotherms for arsenic sorption on Vindhyan shales were compared by Paikaray et al. (2005), with arsenic sorption on black cotton soil. High sorption was observed on pyrite-rich shales and the Freundlich capacity constant yielded a good correlation with sediment pyrite content. Shales with high organic carbon sorbed more arsenic; however, the organic carbon-rich soil demonstrated significantly lower sorption. This difference may be due to the condensed nature of organic carbon in shale, which may have facilitated formation of organo-arsenic complexes. The pyrite content was also strongly correlated with the organic carbon content, possibly due to microbial synthesis during shale diagenesis.

Arsenic sorption by commercially available carbons and other low-cost adsorbents are surveyed and critically reviewed by Mohan and Pittman (2007) and their sorption efficiencies are compared. Some low-cost adsorbents showed better performances including treated slags, carbons developed from agricultural waste (char carbons and coconut husk carbons), biosorbents (immobilized biomass, orange juice residue), goethite and some commercial adsorbents, which include resins, gels, and silica; treated silica tested for arsenic removal came out to be superior. Immobilized biomass adsorbents offered outstanding performances. Desorption of arsenic followed by regeneration of sorbents has been discussed. Strong acids and bases seem to be the best desorbing agents to produce arsenic concentrates. Arsenic concentrate treatment and disposal obtained is briefly addressed. This issue is very important but much less discussed. This review should help in initially screening various sorbent media for setting up the treatment plants based on the community level or household levels in developed, developing and underdeveloped countries.

The removal of *Zn(II)* from aqueous solution by different adsorbents was also investigated (Bhattacharya et al., 2006). Clarified sludge (a steel industry waste material), rice husk ash, neem bark and a chemical adsorbent activated alumina were used for the adsorption studies. The adsorption of Zn(II) increased with increased concentration of the adsorbents and reached maximum uptake at 10 g/L and pH between 5 and 7. The equilibrium time was achieved after 1 h for clarified sludge, 3 h for rice husk ash and 4 h for activated alumina and neem bark, respectively. The best adsorbent for the Zn(II) removal is the

clarified sludge. The optimum conditions were pH 5, adsorbent dosage level 10 g/L, and equilibrium contact time 1 h.

Pilot tests have shown that coir (fibres from Coco nucifera) is suitable as a metal ion sorbent (Conrad and Hansen, 2007). In the range tested, more than 80% of the metal was sorbed within the first 10 min and the total amount sorbed was 91–97% of the metal initially added. In addition to high affinity and capacity, the two most interesting properties of the coir biosorption are the low pH optima for sorption (pH 4.5 for Zn and 2.5 for Pb) and the low desorption (less than 13% for Zn and 1% for Pb), the low pH optima making it possible to use coir directly in cleaning acidic waste water without a prior pH increase. The low degree of desorption ensures that the metal ions will not desorb in situations with lowering of the metal concentration in the solution, e.g. caused by a periodic drop in the metal concentration level of the waste water. Before applying coir for water cleaning purposes, further investigations are needed, the most important being sorption studied in flow experiments, desorption at low pH and competition between ions. In conclusion, coir has a promising potential for being a metal ion sorbent.

In the paper of Chen and Wang (2008), the removal of four metal ions, Pb^{2+}, Ag^{+}, Sr^{2+} and Cs^{+} by waste biomass of brewery was studied. The experimental results showed that metal uptake is a rapid process, which can be described by pseudo-second order kinetic model. The maximum biosorption capacities for four metal ions were 0.413 mmol Pb^{2+}/g, 0.396 mmol Ag^{+}/g, 0.091 mmol Sr^{2+}/g and 0.076 mmol Cs^{+}/g, respectively. The binding of metals was also discussed in terms of several factors. The order of accumulated metal ions at equilibrium state on the molar basis was as follows: $Pb^{2+} > Ag^{+} > Sr^{2+} > C^{s+}$.

Eucalyptus camaldulensis bark, a forest solid waste, is proposed as a novel material for the removal of *Hg(II)* from aqueous phase (Ghodbane and Hamdaoui, 2008). The maximum sorption capacity was 33.11 mg g^{-1} at 20 ºC and the negative value of free energy change indicated the spontaneous nature of sorption. These results demonstrate that eucalyptus bark is very effective in the removal of Hg(II) from aqueous solutions.

A novel sorbent, *Carica papaya*, was evaluated for sorption of Hg(II) from aqueous solution. Maximum removal was observed at pH 6.5, 70.8 mg g^{-1}. The equilibrium data followed Langmuir isotherm confirming the monolayer coverage of Hg(II) onto papaya wood particles. This work illustrated an alternative solution for the management of unwanted biological material like *C. papaya,* which is a waste from papaya tree, when it completes its fruit bearing life. Therefore the use of *C. papaya* for the removal of heavy metals from contaminated waters may be a novel and cost-effective alternative (Basha et al., 2009).

Fluted pumpkin (*Telfairia occidentalis*) is a creeping vegetative shrub that spread low across the ground with large lobed leaves, and long twisted tendrils in the West African sub-region and therefore creates one of the major agro-waste problems in Nigeria. The purpose of Horsfall Jr. and Spiff (2005)

was to use the waste of fluted pumpkin as adsorbent for metal (Al^{3+}, Co^{2+} and Ag^{+}) removal from aqueous system. The experimental results were analyzed in terms of five two-parameter adsorption isotherm equations—the Langmuir, Frendlich, Temkin, Dubinin-Radushkevich and Flory–Higgins isotherms. According to the evaluation using Langmuir equation, the monolayer sorption capacity obtained was 16.98 mg/g, 10.43 mg/g and 8.03 mg/g for Al^{3+}, Co^{2+} and Ag^{+} respectively. Sorption capacity increases with increase in smaller ionic radius metal ion. The result showed that fluted pumpkin waste could be used for the removal of Al^{3+}, Co^{2+} and Ag^{+} from wastewater. The fluted pumpkin is abundantly available but is scarcely useful.

Agricultural by-products, and in some cases appropriately modified, have shown to have a high capacity for heavy metal adsorption. Toxic heavy metals such as Pb(II), Cd(II), Hg(II), Cu(II), Ni(II), Cr(III), and Cr(VI) have been successfully removed from contaminated industrial and municipal waste waters using different agro-waste materials.

The removal of heavy metal ions from aqueous solution has been taking on great importance in recent years, either for pollution control or for raw material recovery. A large number of biomass types have been investigated for their metal binding capability under various conditions. For the past few decades, the metal biosorption by various biological materials including fungi, algae, bacteria and yeast have received great attention as it offers a low cost biosorbent, high efficiency and can be regenerated (Arica et al., 2001; Akar et al., 2005).

Although many biological materials can bind heavy metals, only those with sufficiently high metal-binding capacity and selectivity for heavy metals are suitable for use in a full-scale biosorption process.

3.5.2 Dyes Biosorption

Water pollution by dyes is one of the major pollution sources. Wastewater containing dye cause water pollution by lowering light penetration and photosynthesis and toxicity from heavy metals associated with pigments (Waranusantigul et al., 2003; Prasad Naveen et al., 2008)

Dyes have been extensively used in industries, such as textile, paper, printing, cosmetics, plastics and rubber, for the colouration of products (Kiran et al., 2006; Atar et al., 2008; Vijayaraghavan and Yun, 2008). Annually, over 7×10^5 tons of dyes are produced worldwide, and 10–15% of them are discharged by the textile industry (Ncibi et al., 2007).

Dyes usually have synthetic origins and complex aromatic molecular structures (Banat et al., 1996; Fu and Viraraghavan, 2003). According to their dissociation in an aqueous solution, dyes can be classified as follows (Mishra and Tripathy, 1993):

- anionic: acid, direct and reactive dyes
- cationic: basic dyes
- nonionic: disperse dyes.

Many different and complicated molecular structures of dyes make dye wastewaters difficult to be treated by conventional biological and physico-chemical processes.

In recent years, biosorption has been considered as a promising technology for the removal of dyes from industrial effluents and natural waters (Robinson et al., 2001; Zhang et al., 2009). Use of microbial biomass for decolourization of textile industry waste water is considered a promising alternative in which some bacteria and fungi are used to replace present treatment process (Jadhav and Govindwar, 2006).

The biosorption of Acid Blue 225 and Acid Blue 062 and Basic Blue 41 from aqueous solution by *Bacillus macerans* was investigated (Atar et al., 2008; Colak et al., 2008).

3.5.3 Organic Pollutants

Persistent organic pollutants (POPs) generate a serious risk to the environment and also to the human health due to direct exposure or through residues in food and drinking water.

The inventory of current POPs concentrations in various environmental compartments and assessment of their trends is essential in the development of effective control measures based on the improved understanding of a current status, relevant pathways, and potential effects of chemical substances in the environment (Holoubek and Klánová, 2008). For most other pollutants, concentrations tend to decrease from the point of release due to dispersion, degradation and dilution. In the world, alarming levels of persistent organic pollutants have been reported in air, water, soil as well as in foods and biological materials (Wania, 2000; Gavrilescu, 2005; Betianu and Gavrilescu, 2006; Dimcheva and Kraptcheva, 2008).

POPs are represented by two important subgroups including both the polycyclic aromatic hydrocarbons and some halogenated hydrocarbons. This latter group includes several organochlorines which, historically, have proved to be most resistant to degradation and which have had wide production, use and release characteristics. These chlorinated derivatives are generally the most persistent of all the halogenated hydrocarbons (Kumar et al., 2007).

Some of POPs are used as pesticides, while others are industrial chemicals. POPs are also generated unintentionally as by-products of combustion and industrial processes. Phenol and substituted phenols, such as chlorophenols are priority pollutants because of their high toxicity to human beings at low concentrations, being recognized as toxic carcinogens (Wu and Yu, 2006; Nadavala et al., 2009).

3.5.4 Radionuclides

Environmental contamination caused by radionuclides, in particular by uranium and its decay products is a serious problem worldwide. The development of

nuclear science and technology has led to increasing nuclear waste containing uranium being released and disposed in the environment (Gavrilescu et al., 2009).

Although biosorptive uptake of some heavy metals was well documented, radionuclide sorption is a research area with several non-elucidated aspects. Various biosorbents were applied for uranium biosorption directly from soil or after it was transferred in water (Wase, 1997). Also, large efforts have been concentrated especially on studying the uranium sorption by different microorganisms such as bacteria (Strandberg et al., 1981; Merroun and Selenska-Pobell, 2001; Tsuruta, 2002; Sar et al., 2004; England, 2006), actinomycetes (Gorab et al., 1991), fungi (White and Gadds, 1990; Akhtar et al., 2007b), yeasts (Akhtar et al., 2007b) or algae (Davis et al., 2003; Aleissa et al., 2004).

3.6 Biosorbents

3.6.1 General

Practically all biological materials have an affinity for metal species and other types of pollutants and a depth of other research exists with macroalgae (seaweeds) as well as plant and animal biomass and derived products (e.g. chitosan) (Gadd, 2009).

While choosing biomaterials for pollutant biosorption, their origin is a major factor to be considered. They can be: microorganisms as a by-product of fermentation industry, organisms naturally available in large quantities in nature and organisms cultivated or propagated for biosorption purposes using inexpensive media (Ahluwalia and Goyal, 2007). The source of the biosorbent has a major impact on the feasibility of the operation of biosorption. Biosorbents (biomass) should always be obtained from the least-expensive source, such as from the effluent of a fermenter, seaweeds from nearby bodies of water, algae etc. The spent biosorbents can be regenerated at very low cost using water, so the material can be reused many times (Gupta et al. 2000; Gavrilescu, 2004, 2005).

Both living and dead biomass as well as cellular products such as polysaccharides can be used for metal removal (Gadd, 1993). It is generally recognized that all biological material has an affinity for various pollutants, so that the kinds of biomass potentially available for biosorption purposes are enormous. All kinds of microbial, plant and animal biomass, and derived products, were investigated in a variety of forms, and in relation to a variety of substances (Volesky, 1990; Volesky, 2001; Gadd, 2009; Gavrilescu et al., 2009). Each sorbent has its specific physical and chemical characteristics such as porosity, surface area and physical strength. In addition, sorption capacities of the sorbents also vary, depending on the experimental conditions.

The tested biosorbents can be basically classified into the following categories (Mohanty et al., 2006; Vijayaraghavan and Yun, 2008; Wang and Chen, 2009):

- bacteria (e.g. *Bacillus subtillis*)
- fungi (e.g. *Rhizopus arrhizus*)
- yeast (e.g., *Saccharomyces cerevisiae*)
- moss peat
- algae
- industrial wastes (e.g., *S. cerevisiae* waste biomass from fermentation and food industry)
- agricultural wastes (e.g. corn core)
- polysaccharide materials
- bark
- aquatic plants
- lignin
- peanut hulls etc.

Sorption capacity is the most important characteristic of an adsorbent. It is defined as the *amount of adsorbate taken up by the adsorbent per unit mass of adsorbent.* This variable is governed by a series of properties, such as pore and particle size distribution, specific surface area, cation exchange capacity, pH, surface functional groups, and also temperature. Most of the adsorption capacity for biosorbents (obtained from Freundlich K_F parameter) summarized in this chapter is quite low in comparison to the commercially available activated carbons. Apart from this fact, different types of biosorbents are still attractive due to its biosorption advantages and cost-effectiveness (Febrianto et al., 2009).

The role of some groups of microorganisms has been well reviewed, such as bacteria, fungal, yeast, algae etc. Volesky and Holan (1995) have presented an exhaustive list of microbes and their metal-binding capacities. It has been well documented that microbial biomass could be used for the removal of heavy metals from aqueous solutions (Tobin et al., 1984; Ondruschka and Bley, 2003; Gavrilescu, 2004). Sorbents for the removal of persistent organic compounds should meet several requirements, such as, efficiency for the removal of a wide variety of compounds and tolerance of a range of parameters.

3.6.2 Natural Vegetable Waste and Products

Research interests into the production of alternative sorbents to replace costly activated carbon and synthetic resins have intensified in recent years. Attention has been focussed on various sorbents, which have sorption capacities and are able to remove unwanted compounds from contaminated water at low cost. The first major challenge for the biosorption field was to select the most promising types of biomass from an extremely large pool of readily available and inexpensive biomaterials (Kratochvil and Volesky, 1998; Wang and Chen, 2009).

Certain waste products, natural materials and bioadsorbents have been tested and proposed for POPs removal. For example, the use of modified tannin resins was tested in relation with heavy metal biosorption from water, in several studies proposed in the literature (Table 3.7).

Table 3.7: Tannin biosorbents

Tannin biosorbent	*Removed metals*	*Maximum adsorption, pH, T*	*Reference*
Tannin sorbent (*Eucaliptus saligna*)	Cr(IV)	47.87 mg g^{-1}, pH 2	Lima et al. (1998)
Tannin sorbent (*Lysiloma latisiliqua*)	Cr(IV)	197.58 mg g^{-1}, pH 2	Lima et al. (1998)
Condensed Mimosa tannin	Cr(IV)	287 mg g^{-1}, pH 2	Nakano et al. (2001)
Condensed Wattle tannin	Pb(II)	114.9 mg g^{-1}, pH 4.2	Zhan and Zhao (2003)
Condensed Wattle tannin	Au(III)	8000 mg g^{-1}	Ogata and Nakano (2005)
Persimmon peer gel	Au(III)	NA	Parajuli et al. (2007)
Bayberry tannin immobilized	Pt(IV)	(Lang) 45.8 mg g^{-1}, pH 3	Ma et al. (2006)
Collagen fibre membrane	Pd(II)	(Lang) 33.4 mg g^{-1}, pH 4	Ma et al. (2006)
Bayberry tannin immobilized on collagen fibre	Bi(III)	0.348 mmol g^{-1} , 303 K	Wang et al. (2006)
Bayberry tannin immobilizedcollagen fibre membrane	UO_2^{2+}	56.8 mg U g^{-1}	Liao et al. (2004a)
Black wattle tannin immobilized collagen fibre membrane	UO_2^{2+}	53.0 mg U g^{-1}	Liao et al. (2004a)
Immobilized persimmon tannin	U	pH 6, $t > 30°$ C	Nakajima and Sakaguchi (1999)
Myrica rubra tannin immobilized onto collagen fibres	Th(IV)	73.67 mg g^{-1}	Liao et al. (2004b)
Larch tannin immobilized ontocollagen fibers	Th(IV)	18.19 mg g^{-1}	Liao et al. (2004b)
Lysiloma Latisiliqua tannin sorbent	Ce, Cu(II), U(VI), Eu, Fe(III), Th, Nd	E.g. Cu removal: 54.2% mg, pH 7	Santana et al. (2002)
Pinus pinaster bark	Cd(II), Hg(II)	pH ≥ 6	Vazquez et al. (2002)

(*Contd.*)

Eucaliptus saligna Sm sorbent	Hg	1.2 mmol g^{-1}, pH 7	Torres et al. (1999)
Lysiloma Latisiliqua sorbent (LTS)	Hg	8.5 mmol g^{-1}, pH 7	Torres et al. (1999)
Persimmon Tannin gel	V (from $VOCl_2$ solution)	0.832 mmol g^{-1}, pH 5-6	Nakajima (2002)
Persimmon Tannin gel	V (from NH_4VO_3 solution)	0.955 mmol g^{-1}, pH 3.75	Nakajima (2002)
Wattle tannin gel ($TANNIX^R$)	Am	1.7 mg Am g^{-1}	Matsumura and Usuda (1998)

Biomaterials of plant origin can interact effectively with pollutants such as heavy metals Some biomaterials have been reported to remove heavy metals, dyes: agricultural byproducts like rice husk, bark and orange peel (Tables 3.8 and 3.9) (Namasivayam et al. 1996; McKay et al., 1999; Inbaraj et al., 2002).

Table 3.8: Plant derived biomass used for metal removal

Biosorbent	*Metals*
Activated baggase carbon	Cr
Alfalfa	Cu, Pb
Artocarpus heterphyllus	Cd
Azolla filiculoides	Pb
Banana pith (*Musacea zingiberales*)	Pb, Cu, Ni, Cr, Zn
Hydrilla verticillata casp. and *Salvinia* sp.	Cu
Carrot pulp	Pb, Ni, Zn, Fe
Ceratophyllum demersum	Cu, Pb, Zn
Cupressus Female Cone	Cr(IV)
Eucalyptus wood powder	Pb, Ni, Zn, Fe
Eucalyptus bark	Cr(IV)
Ground corncobs	Cd, Cu, Pb
	Ni, Zn
Hydrilla verticillata casp.	Cu
Larrea tridenta	Cu
Olive mill solid residue	Hg, Pb, Cu
	Zn, Cd
Paper mill sludge	Pb, Cu, Ag
	Cd
	Pb, Ni, Zn, Fe
Sago processing waste	Cu, Pb

(*Contd.*)

Sawdust	Zn, Ni, Cd, Cu, Pb Cr(IV)
Wheat stem and spent babul bark	Ni
Oryza sativa L. hush	Pb
Waste tea-leaves	Pb, Ni, Zn, Fe
Wolffia globosa	Cd, Cr
Water hyacinth roots	Cr
Helianthus annuus L. (Sunflower)	Cu
Allium sativum L. (Garlic)	Cd
Grape stalk waste	Cu, Ni
Hemidesmus indicus	Pb
Myriophyllum spicatum	Pb, Cu, Cd

Table 3.9: Some biosorbents for dyes

Biosorbent	*Dye*
Corynebacterium glutamicum	Reactive Black 5
Cephalosporium aphidicola cells	Acid Red 57
Biomass of Laminaria sp.	Reactive Black 5
Posidonia oceanica (L.) *fibres*	Methylene blue
Green alga *Chorella vulgaris*	Remazol Black B
Azadirachta indica leaf powder	Congo Red
Orange peel	Congo Red
Rice husk	Safranine
Cotton	Safranine
Bark	Safranine
Hair	Methylene blue
Coal	Methylene blue
Activated carbon from coconut husk	Methylene blue
EPS	Basic Blue 54

Recently, some natural and cheap adsorbents have been developed in the laboratory (Ahmaruzzaman and Sharma, 2005; Ahsan et al., 1994; Batabyal et al., 1995; Gupta et al., 1998, 2001, 2004; Haghseresht and Lu, 1998; Hobday et al., 1994; Jain et al., 2004; Otero et al., 2003; Rio et al., 2005; Sarkar and Acharya, 2006; Srivastava et al., 2006; Tarasevich, 2001; Tor et al., 2006;Wang and Jiang, 2007).

Several sorbents, such as herbaceous peat, fibre, rice husk ash, sawdust and starch have been used for removing heavy metal ions from aqueous solution. It has been well known that chitosan demonstrates the unique adsorption ability towards many metal cations. Now this material attracts growing attention in

view of its utilization for removing heavy metal cations from diluted aqueous solution (Kucherov et al., 2003; Tao et al., 2009).

Several chromium removal studies were carried out using naturally available biomaterials such as, Bengal gramhusk (Ahalya et al., 2005), eucalyptus bark (Sarin and Pant, 2006), saw dust, sugarcane bagasse, sugar beet pulp (Sharma and Foster, 1994), coconut husk fibres (Huang and Wu, 1997), palm pressed fibres (Tan et al., 1993), waste tea (Mahvi et al., 2005) and *Ocimum basilicum* seeds (Melo and D'Souza, 2004). The interesting features of the new adsorbents are their high versatility, metal selectivity and high uptake.

3.6.3 Microorganisms

The search for new and innovative treatment technologies has focussed attention on the effect of heavy metal toxicity on, and uptake by, microorganisms (Hu, 1996; Aksu et al., 1997; Aksu and Tezer, 2005; Zhang et al., 2009).

Various types of microbial biomass, including bacteria (Bueno et al., 2008; Chang et al., 1997; Iyer et al., 2005; Zhou et al., 2007; Trivedi and Patel, 2007), yeast (Volesky et al., 1993; Seki et al., 2005; Göksungur et al., 2005), fungi (Dursun et al., 2003; Pal et al., 2006; Tunali et al., 2005, 2006), have been evaluated with the aim of identifying highly *efficient metal removal biosorbent.*

The application of these biosorbents on a commercial scale, however, meets operational limitations associated with their physical characteristics, such as small particle size with low density, poor mechanical strength and low rigidity, and solid-liquid separation (McHale and McHale, 1994: Iqbal et al., 2007).

In order for biosorption to function efficiently, the microbial biomass should sometimes be immobilized in a particulate form which preserves the biomass biosorptive properties and provides physical characteristics similar to those of conventional adsorbent particles, such as activated carbon or ion exchange resins (Tsezos et al., 1997). Cell immobilization is one of the methods used to overcome the incorporating free suspended cell in industrial operations. It offers several advantages including minimal clogging in continuous systems (Ting and Sun, 2000; Arica et al., 2001; Bayramoglu et al., 2003), easy to separate from the reaction system (Annadurai et al., 2007) and can be regenerated and reused for a few cycles (Arica et al., 2001). Natural polymers mostly used as the matrix for the immobilization of microbial cells are alginate, chitosan, chitin and cellulose derivatives (Arica et al., 2001; Bayramoglu et al., 2003).

Bacterial Biosorbents

Bacteria were used as biosorbents because of their small size, their ubiquity, their ability to grow under controlled conditions, and their resilience to a wide range of environmental situations (Urrutia, 1997). Bacteria species such as *Bacillus, Pseudomonas, Streptomyces, Escherichia, Micrococcus* etc., have been tested for uptake metals or organics (Wang and Chen, 2009).

Bacteria are the most abundant and versatile of microorganisms and constitute a significant fraction of the entire living terrestrial biomass of ~1018 g (Mann, 1990).

Some of the important results of metal biosorption using bacterial biomasses, according to some published references, were summarized in literature (Ahluwalia and Goyal, 2007; Alluri et al., 2007; Vijayaraghavan and Yun, 2008; Wang and Chen, 2009) (Table 3.10).

Table 3.10: Bacterial biomass used for metal removal

Biosorbent	*Metals*
Pseudomonas fluorescens	Th, Cu
Pseudomonas sp.	U
Ochrobactrum anthropi	Cr, Cd, Cu
Thiobacillus ferooxidans	Zn
	Cr
Rhodococcus erythropolis	Cd, Zn
Ocimum basilicium	Cr
Sphingomonas paucimobilis	Cd
Bacillus firmus	Pb, Cu, Zn
B. coagulans	Cr(IV)
B. megaterium	Cr(IV)
Zoogloea ramigera	Cr(IV)
	Cu
Streptomyces noursei	Cd, Cu, Ni
	Pb, Zn, Ag, Co
	Cr, Pb
S. longwoodensis	Pb, U
S. rimosus	Zn

Fungal Biosorbents

Three groups of fungi have major practical importance: *the molds, yeasts* and *mushrooms*.

Filamentous fungi and yeasts have been observed in many instances to bind metallic elements (Kapoor et al., 1999; Mashitah et al., 2008; Wang and Chen, 2009). In the field of biosorption, the molds and yeast are of interests since they are easy to grow, produce high yields of biomass and can be manipulated genetically and morphologically. Fungi is better suitable for the removal of metals from wastewater than other microbes because of their great tolerance towards heavy metals and other adverse conditions such as low pH, high cell wall binding capacity and high intracellular metal uptake capacity (Gadd, 1987). Ganoderma species have been also reported as an efficient biosorbent in copper

removal compared to other macrofungi species (Mashitah et al., 1997). These fungi could remove metal ions from aqueous solution by adsorbing metal ions on their mycelium (Bayramoglu et al., 2003).

Metal binding to the cell walls of *Saccharomyces cerevisiae* has been attributed to coordination with amino, carboxyl and hydroxyl groups (Brady et al., 1994).

Several fungal biosorbents (*Aspergillus niger, Rhizopus* spp., *Saccharomyces* spp., *Mucor rouxii* and *Mucor* spp.) were studied as a potential biosorbent in heavy metals removal from aqueous solution (Kapoor and Viraraghavan, 1997; Aloysius et al., 1999; Kyung Kim et al., 2003; Yan and Viraraghavan, 2003).

The fungus *Phanerochaete chrysosporium* was immobilized in several polymer matrices such as Ca-alginate, Ca-alginate-polyvinyl alcohol, and pectin, and was then used as a biosorbent for removing 2,4-dichlorophenol (2,4-DCP) in wastewater (Juan and Han-Qing, 2007; Nadavala et al., 2009).

Fungal strains belonging to the taxonomic group of *Zigomycetes* are of interest due to the presence of chitin, chitosan and glucan in their cell walls (Bartnickni-García, 1968; Gavrilescu, 2004). These polysaccharides have shown to be efficient metal biosorbents. Fungi have been reported to be useful in the removal of valuable metals, particularly uranium (Rome and Gadd, 1991; Mullen et al., 1992). The protective action of mycorrhizal fungi may be based on the adsorptive properties of fungal surfaces (Rome and Gadd, 1991; Mullen et al., 1992; Brady et al., 1994).

For metal removal and recovery from aqueous solutions, dead fungal biomass seems to offer several advantages the biomass may be obtained inexpensively as a by-product of some industrial processes; it is not subject to metal toxicity since there is no metabolic activity; the biomass needs no nutrient supply; and recovery of surface-bound metals is not a detrimental process for the biomass (Volesky and Holan, 1995).

3.6.4 Algae

Algae biomass was also tested as biosorbent (Yu et al., 1999; Lodeiro et al., 2005; Hansen et al., 2006). Algae available in large quantities in many regions are promising biological resources.

Many studies found that alga had a high biosorption capacity numerous heavy metals (Gupta et al., 2001; Mohanty et al., 2006; Brinza et al., 2009). Table 3.11 gives some information about algal biomass applied for heavy metals removal from water and wastewater (Ahluwalia and Goyal, 2007; Alluri et al., 2007).

Some researchers found that Cr(VI) removal from aqueous solutions was achieved partly through reduction (Kratochvil et al., 1998; Park et al., 2004, 2005; Yang and Chen, 2008; Shen et al., 2007).

Table 3.11: Algal biomass for removal of heavy metals from aqueous solution

Biosorbent	*Metals*
Ascophyllum nodosum	Cd
	Co
	Ni, Pb
	Cd
Aphanothece halophytica	Zn
Chorella vulgaris	Ag
	Cd
	Cu
	Cr
	Cr, Cu, Ni
	U
Chorella fusca	Pb
Chorella sorokiniana	Cd
Cladophora crispate	Cr
Caulerpa lentillifera	Cu, Cd, Pb, Zn
Dunaliella sp.	Cr
Fucus vesiculosus	Cd
	Ni, Pb
Fucus spiralis	Cd
Ecklonia maxima	Cd
Laminaria japonica	Cd
Laurencia obtuse	Cr
Lyngbya taylorii	Cd, Pb, Ni, Zn
Phormidium laminosum	Cu, Ni, Zn
Pilayella littoralis	Al, Cd, Co, Cr, Ni, Zn
Pachymeniopis sp.	Cr (IV)
Oscillatoria anguistissima	Zn
	Zn, Cu, Co
Spirogyra sp.	Cr
Scenedesmus quadricula	Cd, Cu, Zn
Scenedesmus obliquus	Cr, Cu, Ni
Scenedesmus abundans	Cd, Cu
Scenedesmus incrassatulus	Cr, Cd, Cu
Sargassum fluiyans	Cu

(*Contd.*)

Sargassum natans	U
	Cd
	Ni, Pb
Sargassum sp.	Zn
Sargassum sp.	Cu
	Cd, Zn, Cu
	Cd
Tetraselmis suecica	Cd
Ulothrix zonata	Cu
Ulva lactuca	Hg

The *blue-green algae Spirulina* sp. genus was found to have a versatile metabolism—it can grow either photoautotrophically, heterotrophically or mixotrophically (Vonshak, 1997; Chojnacka and Noworyta, 2004). The cell wall of algal cells is surrounded by a porous three-dimensional macromolecular network. Important cell wall components are: peptydoglycan, teichuronic acid, teichoic acid, polysaccharides and proteins (Schiewer and Wong, 2000), which display mainly carboxylic, hydroxyl and phosphate groups (Aksu, 2002; Markai et al., 2003). Most of these molecules are polyelectrolytes that carry charged groups, such as carboxyl, phosphate, hydroxyl or amine (Daughney et al., 1998). The presence of anionic and cationic sites gives algal wall amphoteric properties and, depending on the pH, the groups are either protonated or deprotonated (Van der Wal et al., 1997; Esposito et al., 2002). The chemical composition of the cell wall, the presence and availability of metal-binding sites are not only associated with microbial species, but depend also on growth conditions, availability of nutrients, stress etc. (Chojnacka et al, 2005; Lovley, 2000). Blue-green algae *Spirulina* sp. possesses a very high maximum biosorption capacity that depended on biomass growth conditions (Chojnacka et al., 2005).

The green filamentous macro-alga *Cladophora* is widely distributed in eutrophic wastewater, lagoon and inter-tidal zone in many parts of the world. In shrimp breeding ponds, *Cladophora albida* may flourish wildly to form "green blankets" which soon make the pond anoxic (Deng et al., 2009). Özer et al. (1994, 2004) studied the adsorption isotherms of Cu(II), Pb(II) and Cr(VI) on *Cladophora crispate*. Aksu and Kutsal (1998) determined adsorption kinetic parameters of Cu(II) on *Cladophora* sp. in a packed bed column reactor. Deng et al. (2009) reported the utilization of green algae *C. albida* for Cr(VI) removal as a function of pH, algal dosage, initial Cr(VI) concentration, temperature, and co-existing ions. The kinetics was obtained from batch experiments. The removal mechanism was discussed by analyzing the concentration of Cr(VI) and total Cr in the solution. Actual industrial wastewater was used to evaluate the practicality of the biomass *C. albida.*

3.6.5 Peat

Peat is a polar, highly porous, material that could have significant applications as an adsorbent for removal of heavy metals from aqueous solutions. Several studies have established the potential of peat to capture dissolved metals, nutrients, suspended solids, organic matter, oils and odours from domestic and industrial effluents, as well as to adsorb spilled oil or oil from contaminated water (Ho and McKay, 1999a,b; McKay and Porter, 1997; Ringqvist et al., 2002; Ringqvist and Oborn, 2002; Malterer et al., 1996; Gardera-Torresday et al., 1996; Brown et al., 1997).

Basic aspects of uranium adsorption by peat have been investigated during batch experiments, when the influence of different experimental parameters such as final solution pH, adsorbent dosage, sorption time, temperature and various concentrations of uranium on uptake were evaluated (Parab et al., 2005). It was observed that maximum uranium adsorption occurred in the pH range 4.0–6.0.

3.7 Mechanism and Functional Groups Related to the Biosorption

As a generic term, sorption is used for both absorption and adsorption. In an excellent review, Gadd (2008) explains the two processes, relative to various pollutants:

"*Absorption* is the incorporation of a substance in one state into another of a different state (e.g. liquids being absorbed by a solid or gases being absorbed by water), i.e. into a three-dimensional matrix. *Adsorption* is the physical adherence or bonding of ions and molecules onto the surface of another molecule, i.e. onto a two-dimensional surface. In this case, the material accumulated at the interface is the adsorbate and the solid surface is the adsorbent. If adsorption occurs and results in the formation of a stable molecular phase at the interface, this can be described as a surface complex".

According to the dependence on the cells metabolism, biosorption mechanisms can be divided into (Volesky, 1990; Ahalya et al., 2003; Vijayaraghavan and Yun, 2008):

- metabolism dependent and
- non-metabolism dependent.

In addition, according to the location where the metal removed from solution is found, biosorption can be classified as

- extra cellular accumulation/precipitation
- cell surface sorption/precipitation and
- intracellular accumulation.

The distinction between *bioaccumulation* and *biosorption* is important in attempts to use (microbial) biomass for metal sequestering/concentration

purposes. It is important for biosorption process design and often has been poorly understood and described by many researchers. Bioaccumulation and biosorption have often been combined into the single heading of metal uptake because the predominant mechanism in effect is not known. Biosorption possesses certain inherent advantages over bioaccumulation processes, which are listed in Table 3.12 (Vijayaraghavan and Yun, 2008; Hlihor and Gavrilescu, 2009b).

Biosorbents are complex and variable materials. The uptake of contaminants by biological materials occurs via various physico-chemical mechanisms including *ion exchange, sorption, complexation, chelation, microprecipitation* etc. (Volesky et al., 1999; Gadd, 2008). The composition of cell wall, to which metal ions are bound, depends not only on biosorbent species, but also on environmental conditions of its growth.

In fact, cell walls of microorganisms, consisting mainly of *polysaccharide, protein, lipid, teichoic acid, extra-cellular* and *surface polysaccharides*, offer many anionic functional groups such as phosphoryl, carboxyl, amino, sulfhydryl, hydroxyl groups containing ligands (Arief et al., 2008). Deprotonated ligands, e.g. –RCOO-, behave as Lewis bases and adsorption of metal cations can be interpreted as competitive complex formation (Gadd, 2009; Stumm and Morgan, 1996). The mechanism of binding by inactivated biomass may depend on the chemical nature of pollutant (species, size, ionic charge), type of biomass, its preparation and its specific surface properties, and the environmental conditions (pH, temperature, ionic strength, existence of competing organic or inorganic ligands in solution) (Lin and Juang, 2009).

Quantitative structure-activity relationships (QSARs) have been used extensively to predict the bioactivity and toxicity of classes of organic compounds (Zamil et al., 2009). The goal of a QSAR approach is to relate characteristics of a compound to observe toxicity (or bioavailability), and account for variation in toxicity in a related group of chemicals. Once the relationship has been established, one can also predict the additional effects of molecular substitutions. On the other hand, quantitative ion character-activity relationships (QICARs) have been developed by Newman and coworkers as a useful tool to predict, for example, the relative toxicity of metal ions based on metal–ligand binding tendencies. From then, QICARs have been employed successfully to various effects, species, and media for prediction of metal toxicity (Newman et al., 1998; Tatara et al., 1998; Ownby and Newman, 2003).

Mechanisms of Metal Removal

Biosorption is a cost effective way of removing toxic metals from industrial wastewaters in a concentration range below 100 mg/L, where other techniques are ineffective or costly, by passive cation binding by dead or living biomass (Volesky, 1990; Schiewer and Volesky, 1995). Certain types of microbial biomass can retain/bind relatively high quantities of *metal ions* on their cell wall due to its structural characteristics.

Table 3.12: Some features of biosorption/bioaccumulation

Features	*Biosorption*	*Bioaccumulation*
Cost	Usually low. Most biosorbents used were industrial, agricultural and other type of waste biomass. Cost involves mainly transportation and other simple processing charges.	Usually high. The process involves living cells and cell maintenance is cost prone.
pH	The solution pH strongly influences the uptake capacity of biomass. However, the process can be operated under a wide range of pH conditions.	In addition to uptake, the living cells themselves are strongly affected under extreme pH conditions.
Temperature	Since the biomass is inactive, temperature does not influence the process. In fact, several investigators reported uptake enhancement with temperature rise.	Temperature severely affects the process.
Maintenance/ storage	Easy to store and use.	External metabolic energy is needed for maintenance of the culture.
Selectivity	Poor. However, selectivity can be improved by modification/ processing of biomass.	Better than biosorption.
Versatility	Reasonably good. The binding sites can accommodate a variety of ions.	Not very flexible. Prone to be affected by metal/salt conditions.
Degree of uptake	Very high. Some biomasses are reported to accommodate an amount of toxicant nearly as high as their dry weight.	Because living cells are sensitive to high toxicant concentration, uptake is usually low.
Rate of uptake	Usually rapid. Most biosorption mechanisms are rapid.	Usually slower than biosorption. Since intracellular accumulation is time consuming.
Toxificant affinity	High under favourable conditions.	Depends on the toxicity of the pollutant.
Regeneration and reuse	High possibility of biosorbent regeneration, with possible reuse over a number of cycles.	Since most toxicants are intracellularly accumulated, the chances are very limited.
Toxicant recovery	With proper selection of elutant, toxicant recovery is possible. In many instances, acidic or alkaline solutions proved an efficient medium to recover toxicants.	Even if possible, the biomass cannot be utilized for next cycle.

Many studies can be found in literature about the biosorption of *metals* using *living* and *nonliving* biomass. The mechanism of metal biosorption varies according to the metal species and type of biosorbent (Han et al., 2006). Metal ion binding during biosorption processes has been found to involve complex mechanism, such as ion-exchange, complexation, electrostatic attraction and microprecipitation (Volesky and Holan, 1995).

Several research groups have provided good discussions about the metal binding mechanism on functional groups based on FTIR results (Doshi et al., 2007; Yu et al., 2007a; Yu et al., 2007b; Elangovan et al., 2008; Panda et al., 2008; Yazici et al., 2008). Specifically, most biosorption studies which utilize FTIR are dedicated to determine the availability of certain surface functional groups as part of the structure of biosorbents. Arief et al. (2008) have reviewed some surface functional groups biomass using FTIR method.

The biosorption process can be a passive uptake that utilizes cell wall of biomass to sequester the metal ions from aqueous solutions (Vijayaraghavan et al., 2005; Dursun, 2006). The presence of functional groups on biomass cell wall such as carboxyl, hydroxyl, ketones and amino groups will involve a physico-chemical interaction between the metal ions during the biosorption processes (Mashitah et al., 1999).

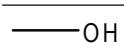
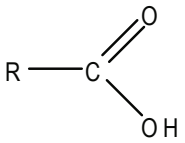
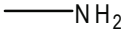
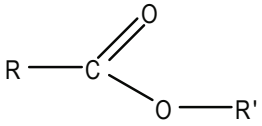
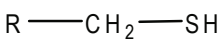
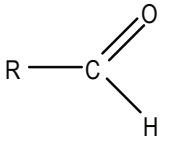
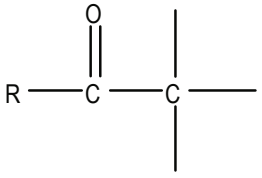
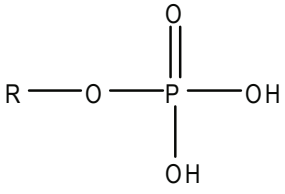
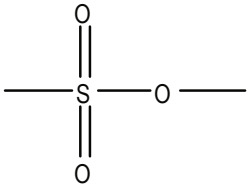
Metal biosorption by biomass mainly depends on the components on the cell, especially through cell surface and the spatial structure of the cell wall. Various *polysaccharides, including cellulose, chitin, alginate, glycan* etc. existed in fungi or algae cell walls, have been proved to play a very important role in metal binding.

The biosorption of metal ions by the *algal biomass* arises from the coordination of the ions to different functional groups on the algal cell. These coordinating groups (provided by proteins, lipids and carbohydrates) include *amino, carboxyl, sulphydryl, phosphate, hydroxyl groups* as well as the *sulphate* (Aksu et al., 1997; Gardea-Torresdey et al., 1990).

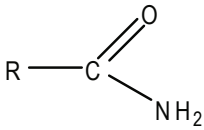
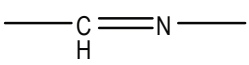
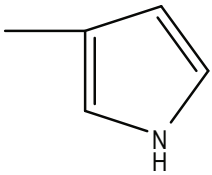
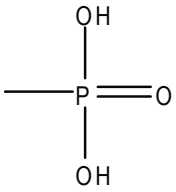
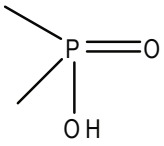
Various proteins are also proved to involve in metal binding for certain kinds of biomasses. Some functional groups have been found to bind metal ions, especially carboxyl group (Table 3.13). There are some evidence to confirm that the O-, N-, S-, or P-containing groups participate directly in binding certain metals.

The way *microbes* interact with metals depends in part on whether the organisms are prokaryotic or eukaryotic (Ehrlich, 1997). Both types of microbes have the ability to bind metal ions present in the external environment at the cell surface or to transport them into the cell for various intracellular functions. On the other hand, only the prokaryotes (eubacteria and archaea) include organisms that are able to oxidize Mn(II), Fe(II), Co(II), Cu(I), AsO^{2-}, Se^0 or SeO_3^{2-}, or reduce Mn(IV), Fe(III), Co(III), AsO_4^{2-}, SeO_4^{2-}, or SeO_3^{2-} on a large scale and conserve energy in these reactions. Some microbes may reduce metal ions such as Hg^{2+} or Ag^+ to Hg^0 and Ag^0 respectively, but do not conserve energy from these reactions (Summers and Sugarman, 1974; Ehrlich, 1997).

Table 3.13: Some functional groups available for pollutants binding during biosorption (Talaro and Talaro, 2002; Volesky et al., 2007; Wang and Chen, 2009)

Formula or functional group	*Name*	*Class of compounds*	*pKa*	*Ligand atom*
	Hydroxyl	Alcohols, carbohydrates	9.5-13	O
	Carboxyl	Fatty acids, proteins, organic acids	1.7-4.7	O
	Amino	Proteins, nucleic acids	8-11	N
	Ester	Lipids		O
	Sulfhydryl	Cysteine, proteins	8.3-10.8	S
	Carbonyl (terminal end)	Aldehydes, polysaccharides		O
	Carbonyl (internal)	Ketones, polysaccharides		O
	Phosphate	DNA, RNA, ATP		P
	Sulfonate	Sulfated polysaccharides	1.3	O

(*Contd.*)

	Thioether	Amino acids		S
	Amide	Amino acids		N
	Imine	Amino acids	11.6-12.6	N
	Imidazole	Amino acids	6.0	N
	Phosphonate	Phospholipids	0.9-21	O
	Phosphodiester	Teichoic acid, lipopolysaccharides	1.5	O

Some prokaryotes and eukaryotes may form metabolic products, such as acids or ligands, that dissolve base metals contained in minerals, such as Fe, Cu, Zn, Ni, Co and others. Others may form anions that precipitate dissolved metal ions. Some prokaryotes may methylate some metal and metalloid compounds, producing corresponding volatile metal derivatives (Summers and Sugarman, 1974; Ehrlich, 1997; Beveridge and Koval, 1981, Gavrilescu, 2004).

Wang and Chen (2009) have elaborated an interesting review dedicated to the microbial structure and function involved in the heavy metals biosorption mechanism considering two fundamentally different types of cells—procaryotic and eukaryotic—distributed among several domains. Considering cell surface as the interface between the microbial cells and its external environment they discussed in detail about three groups of biomass materials in terms of their metal biosorption performance, namely:

- bacteria (Gram-positive and Gram-negative cells)
- fungi (filamentous fungi and yeast)
- algae

During bioremediation process, the microorganisms cannot destroy metals, but they can alter their chemical properties via a surprising array of mechanisms (Fig. 3.4), some of which can be used to treat metal contamination (Lloyd, 2002; Gavrilescu, 2004).

In addition to surface adsorption of metal ions, which is usually reversible, some bacterial cells can accumulate metals intracellular. This metabolism-dependent process is also energy-dependent, requiring active respiration by the cell through a specific transport system. While intracellular accumulation is usually slower than adsorption, the end result in many cases is that more metal is bound to the cells. With some species, both processes may occur. A study of *Pseudomonas aeruginosa*, for instance, found that copper uptake initially resulted from extracellular adsorption of Cu^{2+} ions, which was followed by intracellular accumulation that resulted from the exchange of copper ions with calcium and magnesium ions across the cell wall (Philip et al., 1995; He and Tebo, 1998).

Chromium is one of the most frequent and dangerous environmental pollutant; therefore, numerous studies were devoted to the mechanism of Cr(VI) biosorption. In their studies, Park et al. (2004, 2005) found that the biosorption mechanism included direct and indirect reduction, with an exemplification with chromium, where Cr(VI) was completely reduced to Cr(III) by contact with brown seaweed *Ecklonia* biomass. Yang and Chen (2008) reported that the removal of Cr(VI) by *Sargassum* sp. was a complicated process, in which reduction, surface complex formation and ion exchange were involved. The mechanism of Cr(VI) removed by biomaterials is not ‘‘anionic adsorption” but ‘‘adsorption coupled reduction” (Park et al., 2006).

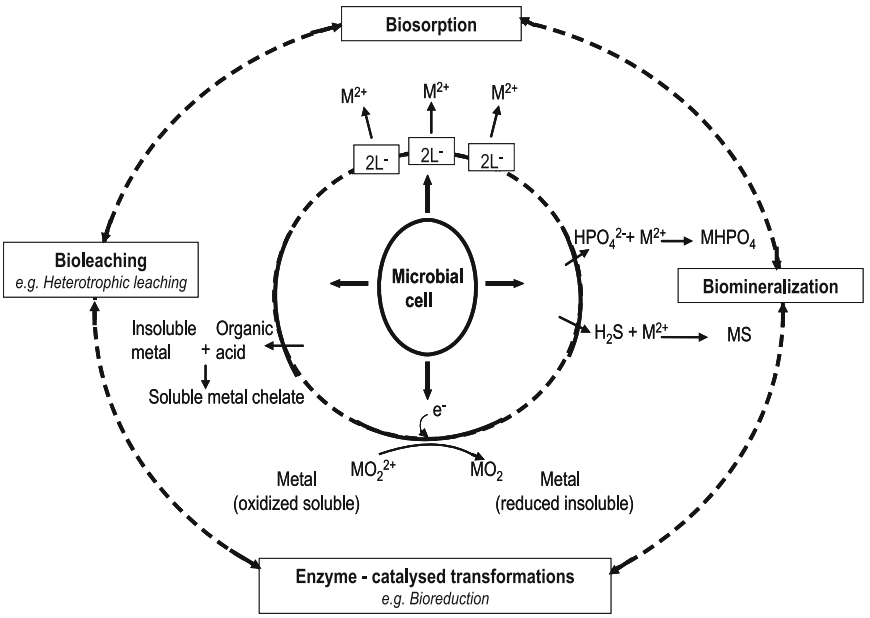


Fig. 3.4: Mechanisms of metal-microbe interactions that can be harnessed for bioremediation applications (Lloyd, 2002; Gavrilescu, 2004).

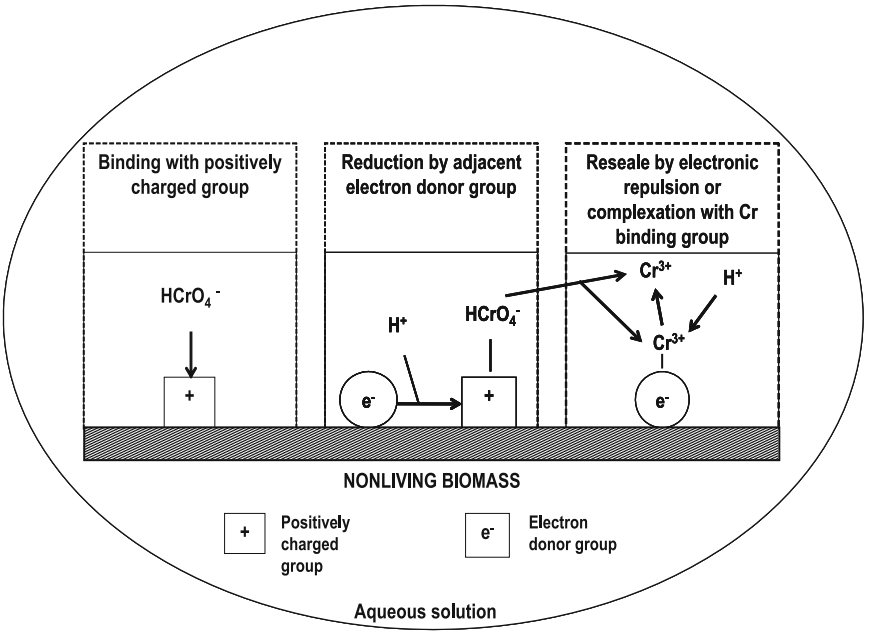


Fig. 3.5: Feasible mechanism for Cr(VI) biosorption by nonliving biomass (Deng et al., 2009; Park et al., 2004, 2005, 2006).

The biosorption process of Cr(VI) is described considering three steps (Fig. 3.5.) (Deng et al., 2009; Park et al., 2004, 2005, 2006):

(1) The binding of anionic Cr(VI) ions with the positively charged groups present on the biomass surface;
(2) the reduction of Cr(VI) to Cr(III) by adjacent electron-donor groups; and
(3) the release of the Cr(III) ions into the aqueous phase due to electronic repulsion between the positively charged groups and the Cr(III) ions, or the formation of complexes of the Cr(III) with adjacent groups capable of Cr-binding.

Scanning Electron Microscopy (SEM) was one of several methods used by Han et al. (2006) to probe the surface complexation mechanism in Cr(III) biosorption by *Chlorella miniata*. The observations indicate that the primary Cr(III) sequestering sites are on the cell wall surface instead of the intracellular sites.

Das and Guha (2007) conducted another study using chromium as a heavy metal model. A cage-like structure was discovered as being formed during the interaction of Cr(VI) ions with several functional groups on the surface structure of *Termitomyces clypeatus.*

The X-ray Photoelectron Spectroscopy (XPS) spectra resulted in the mechanism investigation of Cd(II), Ni(II), and Pb(II) biosorption by the brown

marine macroalgae *Sargassum vulgaris* which showed that heavy metal binding was accompanied by changes in sulfur, nitrogen, oxygen, and carbon binding (Raize et al., 2004).

Liu and Xu (2007) studied the mechanisms of Ni^{2+} biosorption by aerobic granules. Energy dispersive X-ray spectroscopy (EDX) analysis indicated that Ni^{2+} ions could penetrate into the aerobic granule core, and additionally on element mapping, the adsorbed Ni^{2+} distribution within the aerobic granule was seen to be uniform.

Table 3.13 offers a representative functional groups and classes of organic compounds in biomass, able to bind heavy metals. The symbol R is shorthand for residue, and its placement in a formula indicates that what is attached at that site varies from one compound to another (Talaro and Talaro, 2002; Wang and Chen, 2009).

Volesky et al. (2000) gave some indications and details that ion-exchange plays an important role in metal sorption by algal biomass.

The sorption with a high-molecular weight sorbent involves more binding-sites, stronger van derWaals forces than in the case of the sorption with a low-molecular weight sorbent (Zhang et al., 2007). For a sorbent, the linear structure can assure more binding-sites to be functional and adsorbing more dye molecules (Jang et al., 2001; Zhang et al., 2006). Carboxyl, hydroxyl and amino groups are the preferred groups for most sorption processes (Guibaud et al., 2005; Comte et al., 2006).

A great number of chitosan derivatives have already been obtained to adsorb metal ions by grafting new functional groups on the chitosan backbone. The new functional groups are incorporated with chitosan to increase the density of adsorption sites, to change the pH range for metal adsorption and to change the adsorption sites in order to increase adsorption selectivity for the target metal (Tao et al., 2009; Jayakumar et al., 2005).

Peat mainly contains lignin and cellulose. Functional groups in lignin that include alcohols, aldehydes, ketones, acids, phenolic hydroxides, and ethers allow it to bind with various metal ions (Sen Gupta et al., 2009).

The physical and chemical characteristics of biosorbents are important for understanding the metal binding mechanism on the biomass surface. When the metal-biomass interaction mechanism(s) are reasonably understood, it opens the possibilities of (Gavrilescu, 2004):

- optimizing the biosorption process on the molecular level;
- manipulating the biosorption properties of biomass when it is growing;
- developing economically attractive analogous sorbent materials;
- simplifying and effectively guiding the screening process;
- 'activating' biomaterials low level biosorbent behaviour.

Depending on the nature of the biosorbents, a variety of techniques are useful for this purpose, e.g., Fourier Transform Infra-Red (FTIR) spectroscopy, X-ray Photo Electron Spectroscopy (XPES), Scanning Electron Microscopy

(SEM), X-ray Diffraction (XRD), Energy Dispersive X-ray (EDX) fluorescence spectrophotometry, nitrogen sorption etc. (Arief et al., 2008).

Mechanisms of Persistent Organic Pollutants Removal

Bell and Tsezos (1988) studied the biosorption of pentachlorophenol, lindane, diazinon and malathion onto two types of inactive microbial biomass: a mixed culture of aerobic activated sludge and a pure culture of *R. arrhizus*. They concluded that biosorption process involves uptake by both the cell walls and other cellular components of the microorganisms.

3.8 Factors Affecting Biosorption

The investigation of the efficacy of the pollutants uptake by the biomass is essential for biosorption application, as it gives information about the equilibrium of the process which is necessary for the design of the equipment. For successful application on a large scale, biosorption needs to be economically viable. The feasibility of a biosorption process depends on such factors as:

- biosorbent uptake performance
- the source of the raw biomass
- biomass granulation and treatment
- the desorption and regeneration processes used.

3.8.1 Factors Affecting Heavy Metals Biosorption

There are numerous factors that can influence biosorption capacity but major three types are: metal ionic characteristics (e.g., atomic weight, ion radius, valence, etc.), the nature of the biosorbents (e.g., cell age), and biosorption conditions (e.g., pH, temperature, contact time etc.).

As with any adsorption process, many environmental factors influence the chemical nature of bacterial surface binding sites (Tobin et al., 1984; Sterritt and Lester, 1996; Zamil et al., 2009). Among those factors are such common characteristics of metal-bearing streams such as:

- a variable flow rate
- variable pH or pH spikes
- high concentrations of harmless metals such as sodium, calcium, and magnesium
- variable temperatures and dissolved oxygen concentrations.

Other characteristics are:

- the presence of additional metals
- mixed, cationic, or anionic ligands
- organic contaminants such as surfactants and oils.

The presence of organic and inorganic ligands that act as chelators is of particular concern. Such ligands may compete with the microorganisms

for metal ions, and once metal-ligand complexes are formed, they may not be adsorbed by the cells. Metal uptake will be most affected if the binding constants for the ligands are greater than those for the biosorbent. In studies using *Thiothrix* species strain to adsorb copper, nickel, and zinc, the bacteria did not bind copper that was chelated to ethylenediaminetetraacetic acid and nitrilotriacetic acid (Shuttleworth and Unz, 1993).

Bioremoval of single species of heavy metal ions using microorganisms is affected by several factors. These factors include the specific surface properties of the microorganism and the physicochemical parameters of the solution such as temperature, pH, initial metal ion concentration and biomass concentration (Acikel et al., 2004; Aksu et al., 1997; Aksu and Akpinar, 2000; Parameswari et al., 2009).

The metal uptake is affected both by the biosorbent and the metal characteristics, as is illustrated in Table 3.14 (Ahalya et al., 2003).

Table 3.14: Comparative data on diverse metal uptake capacities using various macrophytes

Biosorbent	*Metal uptake capacity (mg/g)*			*References*
	Lead	*Zinc*	*Copper*	
M. spicatum	46.69	15.59	10.37	Keskinkan et al. (2003)
M. spicatum	55.6	13.5	12.9	Wang et al. (1996)
P. lucens	141	32.4	40.8	Schneider and Rubio (1999)
S. herzegoi	-	18.1	19.7	Schneider and Rubio (1999)
E. crassipes	-	19.2	23.1	Schneider and Rubio (1999)

The pH value of aqueous solution is an important environmental factor influencing not only site dissociation, but also the solution chemistry of the heavy metals: hydrolysis, complexation by organic and/or inorganic ligands, redox reactions, precipitation are strongly influenced by pH and, on the other hand, strongly influence speciation and biosorption availability of the heavy metals (Deng et al., 2009; Esposito et al., 2002).

The pH dependence of Cr(VI) removal can largely relate with type and ionic state of these functional groups and also the metal chemistry in solution (Matheickal and Yu, 1999). Chromium exhibits different types of pH dependent equilibriums in aqueous solutions (Rollinson, 1973). As pH is shifted, the equilibrium will also shift; at lower pH (pH < 2.0) values, $Cr_3O_{10}^{2-}$ and $Cr_4O_{13}^{2-}$ species are formed; and at a pH range of 2–6, $HCrO_4^-$ and $Cr_2O_7^{2-}$ ions are in equilibrium. When pH > 8.0, CrO_4^{2-} is the predominant species in the solution (Rollinson, 1973; Bai and Abraham, 2001; Mor et al., 2006). At lower pH, the negatively charged chromium species bind through electrostatic attraction to positively charged functional groups on the surface of biomass cell wall because more functional groups carrying positive charges would be exposed. As pH increased, the overall surface charge on cell walls became negative and

biosorption decreased. On the other hand, the reduction process of hexavalent to trivalent chromium requires a large amount of proton (Barrera et al., 2006).

Deng et al. (2009) found that the decrease of total Cr and Cr(VI) in the solution at pH 2.0, when the alga biomass *C. albida* was used as sorbent, is time dependent as depicted in Fig. 3.6. So the Cr(VI) can be completely removed as long as the contact time is sufficient.

The biosorption characteristics of Pb(II) and Cd(II) ions from aqueous solution using the macrofungus (*Amanita rubescens*) biomass were investigated by Sari and Tuzen (2009) as a function of pH, biomass dosage, contact time, and temperature. The maximum biosorption capacity of *A. rubescens* for Pb(II) and Cd(II) was found to be 38.4 and 27.3 mg/g, respectively, at optimum conditions of pH 5.0, contact time of 30 min, biomass dosage of 4 g/L, and temperature of 20 °C. The metal ions were desorbed from *A. rubescens* using both 1 M HCl and 1 M HNO_3. The recovery for both metal ions was found to be higher than 90%. The high stability of *A. rubescens* permitted ten times of adsorption-elution process along the studies without a decrease of about 10% in recovery of both metal ions.

Khambhaty et al. (2009) carried out the sorption of Cr(VI) in a batch system using dead biomass of marine *Aspergillus niger*. The removal rate of Cr(VI) was increased with a decrease in pH and an increase in Cr(VI) and biomass concentration. *A. niger* exhibited the highest Cr(VI) uptake of 117.33 mg g^{-1} of biomass at pH 1.0 in the presence of 400 mg L^{-1} Cr at 50 °C.

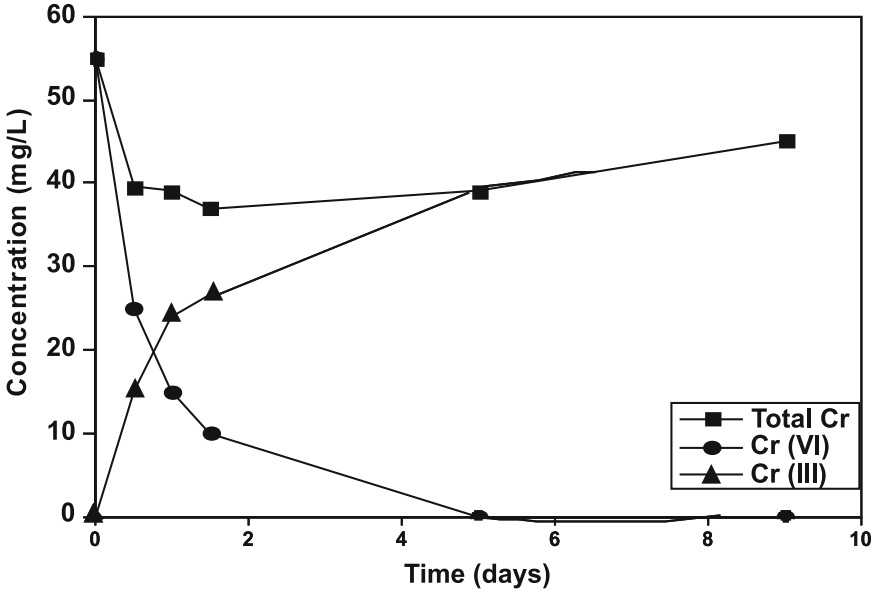


Fig. 3.6: Variation of Cr(VI), Cr(III) and total Cr concentrations with the contact time (pH 2, T = 25°C, 2g/L biomass) (Deng et al., 2009).

3.9.2 Factors Affecting Dyes Biosorption

The *initial pH* of the dye solution is one of the most important parameter controlling the adsorption process. It does not only affect the biosorbent site dissociation, but also the solution chemistry of dyes (Atar et al., 2008) (Fig. 3.7).

The pellets of *Penicillium oxalicum* exhibited a high dye adsorption capacity (80–180 mgg^{-1}) for three dyes (Reactive Blue 19, Reactive Red 241 and Reactive Yellow 145) over a wide pH range (pH 2–10), and the maximum adsorption was obtained at pH 2. The adsorption capacity was mildly increased by increasing salinity.

The uptake of the dye increases with increasing *initial dye concentration*. This may be attributed to the extent of a driving force of concentration gradients with the increase in the initial dye concentration (Atar et al., 2008).

A white rot basidiomycete *Phanerochaete chrysosporium* immobilized in loofa sponge was evaluated and characterized as a new biosorbent of dyes. Effects of biosorption process parameters on dye uptake capacity of loofa sponge-immobilized fungal biomass were studied and compared with free fungal biomass. Remazol Brilliant Blue R, a reactive dye, uptake from aqueous solution was found to be influenced by solution pH, temperature and initial dye concentration. Biosorption of dye by both biosorbents increased as the initial dye concentration increased in the medium.

Contact time also influence dyes biosorption efficiency. The removal of Reactive Blue 19 using mycelium pellets of *Penicillium oxalicum* as biosorbent was up to 60% in 10 min and 91% in 80 min (Zhang et al., 2003).

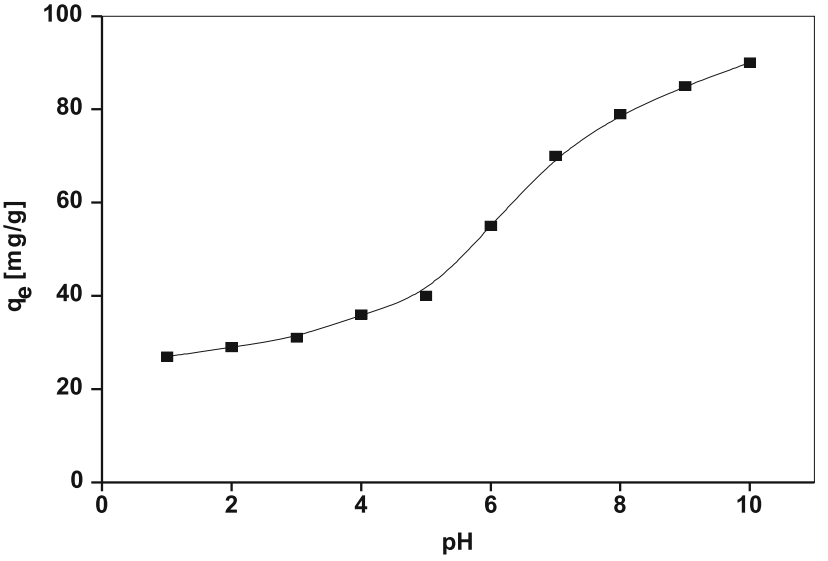


Fig. 3.7: Effect of pH on the removal of Basic Blue 41 by *B. macerans* (contact time: 90 min, initial dye concentration: 100 mg/L, solution temperature: 25 °C, biosorbent concentration: 1 g/L).

As the *mass of the biosorbent* dosage increases, the biosorption capacity of biomass also increases. This may be attributed to the fact that the number of available adsorption sites enhances with a rise in the biosorbent dosage resulting in an increase of adsorbed dye concentration (Atar et al., 2008).

Hong et al. (2000) studied the biosorption of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TCDD) by *Bacillus pumilus* biomass. The results showed that dead biomass of microorganism could remove these molecules from the medium more effectively than live cells and, they explained that extracellular polymeric substances might also be involved in the biosorption process.

Dynamic batch experiments were carried out by Aravindhan et al. (2007) for the biosorption of basic blue dye on to the green macro algae *Caulerpa scalpelliformis*. The factors affecting the sorption process such as the initial concentration of the dye, pH of the solution, the adsorbent dosage and the time of contact were studied. It has been observed that the sorption process was significantly affected by the pH of the initial dye solution.

3.9.3 Factors Affecting Persistent Organic Pollutants Biosorption

POPs biosorption is affected significantly by the amount of sorbent and sorbate, equilibration time between the two phases, pH and temperature. Therefore these parameters have to be optimized one by one, keeping all others constant. For example, one of the most important influencing factor for organic compounds sorption has been referred to as pH. In a certain pH range, most compounds sorption increases with increasing pH up to a certain value and then decreases with further pH increase (Akhtar et al., 2007a).

The removal of aqueous phenanthrene using dead tissue of brown seaweed *Sargassum hemiphyllum* was studied by Chung et al. (2006). The results showed that the removal capacities were 430–460 μg g^{-1} for tests spiked with 1000 μgL^{-1} PHE, in the range of 91.7–98.4%.

Mathialagan and Viraraghavan (2008) studied the sorption of pentachlorophenol from aqueous solutions by a fungal biomass. They used non-viable *Aspergillus niger* biomass, for the biosorption of pentachlorophenol (PCP) from aqueous solutions and the sorption was rapid with an equilibrium time of 2 h.

Wu and Yu (2006) investigated the sorption of 2,4-dichlorophenol from aqueous solution on non-living mycelial pellets of *Phanerochaete chrysosporium*. The study showed that the fungal biomass exhibited the highest sorption capacity of 4.09 mg/g at an initial pH of 5.0 and a pellet size of 1.0–1.5 mm. They found that the sorption capacity increased with the increase of initial concentration of 2,4-DCP and the sorption capacity decreased with the temperature and size of mycelial pellets increasing.

3.10 Advantages vs. Disadvantages

The main attraction of biosorption is its cost effectiveness and the good removal performance. Biosorption is considered to be highly competitive with the presently available technologies like ion exchange, electrodialysis, reverse osmosis etc.

Biosorption has distinct advantages over conventional methods of treatments: the process does not produce chemical sludge; it is more efficient, easy to operate (Volesky, 1990). It is selective, effective, cheap and works well at very low concentrations (Hammaini et al., 2002). In addition, it is eco-friendly, since the biosorption processes do not generate toxic sludge, which offers further possibilities for metal recovery and potential biosorbent regeneration (Gupta et al., 2000; Benguella and Benaissa, 2002; Baran et al., 2007; Arief et al., 2008; Bueno et al., 2008).

Raw materials which are either abundant (sea weeds) or wastes from other industrial operations (fermentation wastes, activated sludge processing wastes) can be used as the biosorbents presenting good separation performances (Lin and Juang, 2009). A major advantage of biosorption is that it can be used in situ, and with proper design that may not need any industrial process operations and can be integrated with many systems in the most eco-friendly manner (Deng et al., 2009; Tewaria et al., 2005).

The use of dead biomass seems to be more advantageous than the use of living biomass because there are no toxicity concerns, the process is not governed by the physiological constraint of living microbial cells, no requirements of growth media or costly nutrients, so that the problems of disposal of surplus nutrients or metabolic products are not present. Also, a wider range of operating conditions such as pH, temperature and metal concentration is possible and no aseptic conditions are required. There are easy techniques to desorb metal ions from the biomass and reuse them (Yan and Viraraghavan, 2008).

An important disadvantage is that early saturation can be problem. Also, there is no biological control over characteristic of biosorbent.

3.11 Conclusions

Biosorption is a property of certain types of inactive, dead, microbial biomass to bind and concentrate heavy metals from even very dilute aqueous solutions. This process takes advantage of the ability of certain materials to absorb various pollutants from aqueous solutions by either metabolically mediated or physico-chemical pathways of uptake. The biosorption process involves a solid phase (biosorbent: biological material or biomass) and a liquid phase (solvent: usually water) containing a dissolved species to be sorbed (sorbate: metal ions, dyes, persistent organic pollutants, radionuclides). Biomass exhibits this property, acting just as a chemical substance, as an ion exchanger of biological origin. It is particularly the cell wall structure of certain natural vegetable waste and

products, microorganisms (fungi, bacteria) algae, which was found responsible for this phenomenon.

Biosorption offers several advantages including cost effectiveness, high efficiency, minimization of chemical/biological sludge, and regeneration of biosorbent.

Many scientific studies are currently in progress to provide a deeper understanding of biosorption and to support its effective application.

Great efforts have to be made to improve biosorption process, including immobilization of biomaterials, improvement of regeneration and re-use, optimization of biosorption process etc.

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4

Lignins and Polyphenols in Bioremediation

Valentin I. Popa, Alina Petronela Stingu and Irina Volf

4.1 Introduction

Bioremediation by definition is concerned with the fate and, if necessary, removal of unwanted organic chemicals from soil and/or water. At present, bioremediation is considered as a less expensive alternative to physical and chemical means of degradation of organic pollutants. It deals with substances that are anthropogenic, distributed in nature and recalcitrant. At the same time, it is not lacked of interest to involve the bioremediation to improve some properties of the soils. The lignins are biosynthesized in plants to carry out different functions, such as storage of energy, bonding agent between plant cells, protection agent against microorganisms, antioxidant, and hydrophobic agent. Lignin is an important precursor for stabilized soil organic carbon. Terrestrial ecosystems that produce large amounts of lignin are thought to have high potential for sequestering carbon which can decrease global warming by reducing atmospheric CO_2. After the death of the plants, their compounds are transformed at the soil level with humus formation. Based on biological transformation some utilization of lignins could be developed among other things such as crop cultivation and bioremediation (Abaecherli and Popa, 2005).

Nowadays heavy metals, aromatic hydrocarbons, phenolic compounds and their derivatives, catechols, and other different organic compounds are considered priority pollutants because they are harmful to living organisms even at low concentrations. Conventional methods for removal of metal ions from aqueous solutions include chemical precipitation, ion exchangers, chemical oxidation/reduction, reverse osmosis, electrodialysis, ultrafiltration,

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etc. Adsorption process is efficient for the removal of organic pollutants and activated carbon is most widely used in this direction. Biosorption of pollutants by using lignocellulosic residues from wood, grass, agricultural residues, forestry waste material, is a relatively new process that has shown significant contribution for the removal of contaminants from aqueous effluents. Lignocellulosic residues constitute a renewable resource from which many useful biological and chemical products can be derived. Several novel markets for lignocellulosic residues have been identified. The use of fungi in low cost bioremediation projects might be attractive given their highly efficient lignocellulose hydrolysis enzymes system (Sanchez, 2008). By using wastes resulted from wood processing (spruce wood bark) and other grades of biomass (wine stems, grape seeds, *Asclepias syriaca* herb) the polyphenolic compounds have been separated and a number of important utilizations have been established (Popa and Volf, 2006).

Phenols along with other organic compounds and heavy metal remediation of aqueous streams are one of a special concern due to recalcitrant and their persistency in environment. Adsorbent materials derived from low cost agricultural wastes, modified lignin and natural polyphenols can be used for the effective removal of heavy metal ions and organic compounds from waste water. The aim of this chapter is to provide the important role of the lignin, polyphenols along with other lignocellulosics in the soil structure, carbon cycle, plant growth and seed germination, soil conditioning and fertilizers, biosorption process and composting.

4.2 Formation of Soil Organic Matter

The ultimate model cycling of the carbon moiety of chemicals in nature is terrestrial carbon cycle. Carbon dioxide and water are used for the biosynthesis of phytomass which, in turn, is mineralized by microorganisms and animals to carbon dioxide and water again, the whole cycle being driven by energy from sun light (Fig. 4.1).

In this process the polysaccharides from phytomass are used by microorganisms as carbon sources, while it is known that by the microbiological action, lignin from vegetal wastes is transformed at soil level in organic products with physiological activity on plants development. Thus, lignin may be considered as an initial source for humic acids formation which represents a main component of the soil humus (Fig. 4.2) (Abaecherli and Popa, 2005).

On the other hand, some low molecular mass compounds resulting from plant wastes decomposition, along with polyphenols coming from extraction of plant residues, could be inhibitors or promoters of plants growth. Therefore, the knowledge of complex processes including soil, plants and microorganisms consortium allow us to use the lignin and its derivatives to control both soil fertility and plants development with a view to increase soil productivity and to perform soil bioremediation.

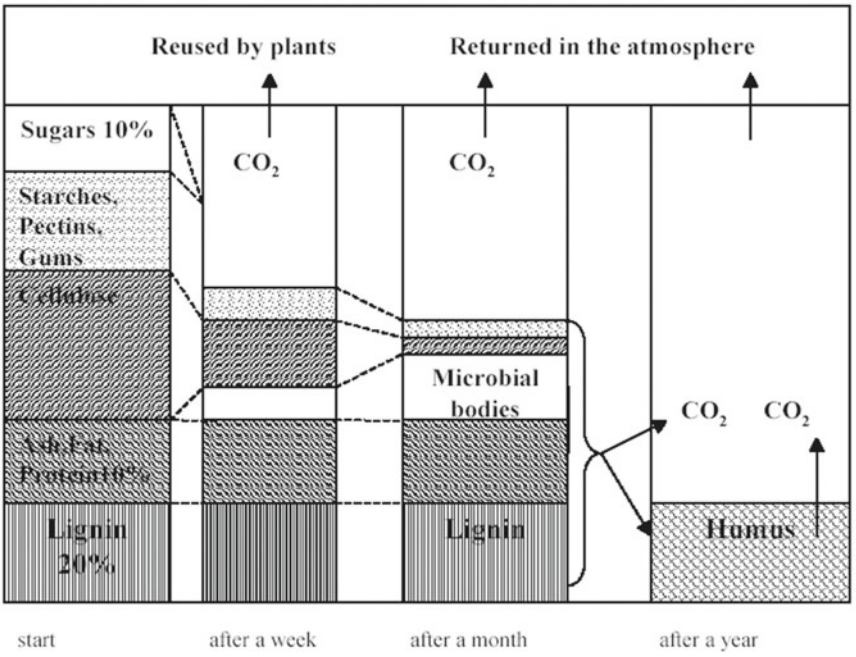

Fig. 4.1: The progress of humus formation resulting from microbial degradation of plants (Abaecherli and Popa, 2005).

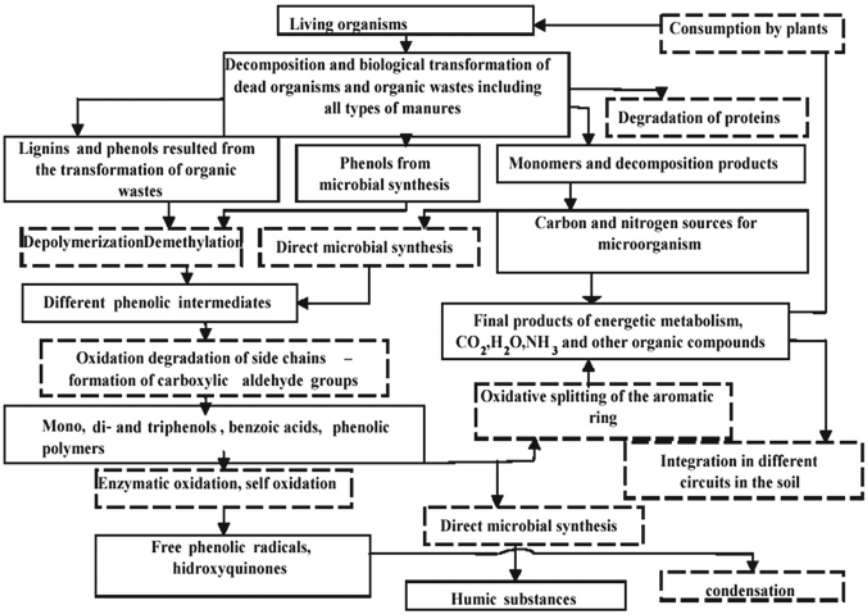

Fig. 4.2: The pathway of humus formation (Abaecherli and Popa, 2005).

For a long time it was thought that humic substances were derived from lignin. According to this theory, lignins are incompletely utilized by microorganisms and the residue becomes part of the soil humus. Modifications in lignin include loss of methoxyl (OCH_3) groups with generation of hydroxyphenol and oxidation of aliphatic side chains to form COOH groups. The modified material is subject to further unknown changes to yield first humic acids and then fulvic acids (Fig. 4.3).

Lignin plays an important role on humus formation and in these transformations some products are involved in redox plant cell reactions. One can assess that this natural aromatic polymer along with polyphenols might represent a possible source of valuable compounds for plant growth (Popa and Volf, 2006).

Lignins and their derivatives as non-conventional additives in the crops cultivation and bioremediation, have a very important role in (Abaecherli and Popa, 2005):

- the energy management and distribution (regulation of carbon cycle);
- the management of chemical nutrients;
- maintaining the trophic web;
- the soil structuring; and
- the sequestration of pollutants (bind heavy metal ions, solubilize petroleum hydrocarbons etc.).

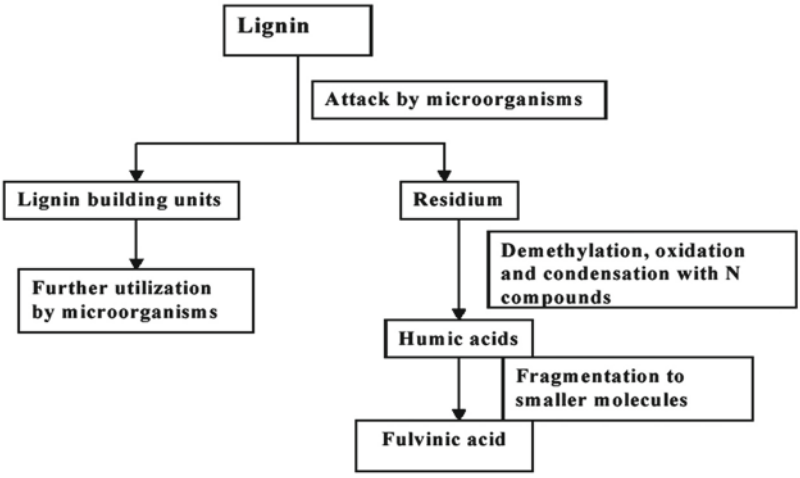

Fig. 4.3: Mechanism proposed for lignin participation in humus formation (Abaecherli and Popa, 2005).

4.3 Biomass

The term biomass (Greek, bio, life + maza or mass) refers to wood, short-rotation woody crops, agricultural wastes, short rotation herbaceous species, wood wastes, bagasse, industrial residues, waste paper, municipal solid waste,

sawdust, biosolids, grass, food processing waste, aquatic plants, algae animal wastes, and a host of other materials (Demirbas, 2000).

Lignocellulose is the major component of biomass, comprising around half of the plant matter produced by photosynthesis (also called phytomass) and representing the most abundant renewable organic resource in soil. It consists of three types of polymers, cellulose, hemicelluloses and lignin that are strongly intermeshed and chemically bonded by non-covalent forces and by covalent cross linkages (Pérez et al., 2002).

Studies carried out in the last decade lead to the conclusion that biomass could represent a convenient resource of chemical compounds and energy, if the processing of raw materials, having in view their different sources and different compositions, had taken place following a technology to separate each compound as a function of the accessible resource. Possibilities of biomass complex processing, experimented, using different kinds of biomass are related in Fig. 4.4 (Popa and Volf, 2006).

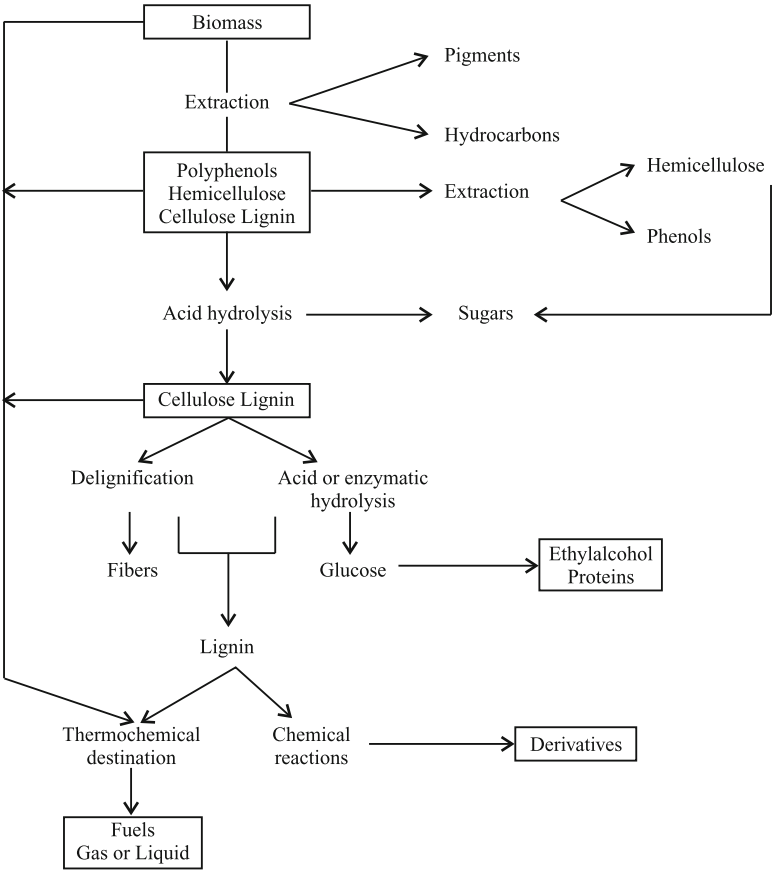

Fig. 4.4: Scheme of complex processing of vegetable biomass (Popa and Volf, 2006).

4.3.1 Composition of Lignocellulosic Residues: Chemical Properties

The major component of lignocellulosic materials is cellulose, followed by hemicelluloses and lignin (Fig. 4.5).

Cellulose and hemicelluloses are macromolecules constructed from different sugars, whereas lignin is an aromatic polymer synthesized from phenylpropanoid precursors. The composition and proportions of these compounds vary between plants (Table 4.1) (Prassad et al., 2007; Howard et al., 2003; Rowell, 1992; John et al., 2006; Hon, 2000; Pérez-Díaz et al., 2005).

Cellulose is a linear pure organic polymer, consisting solely of units of anhydroglucose held together in a giant straight chain molecule (Demirbas, 2000). Cellulose usually is present as a crystalline form and a small amount of nonorganized cellulose chains forms amorphous cellulose. In the latter conformation, cellulose is more susceptible to enzymatic degradation (Pérez et al., 2002).

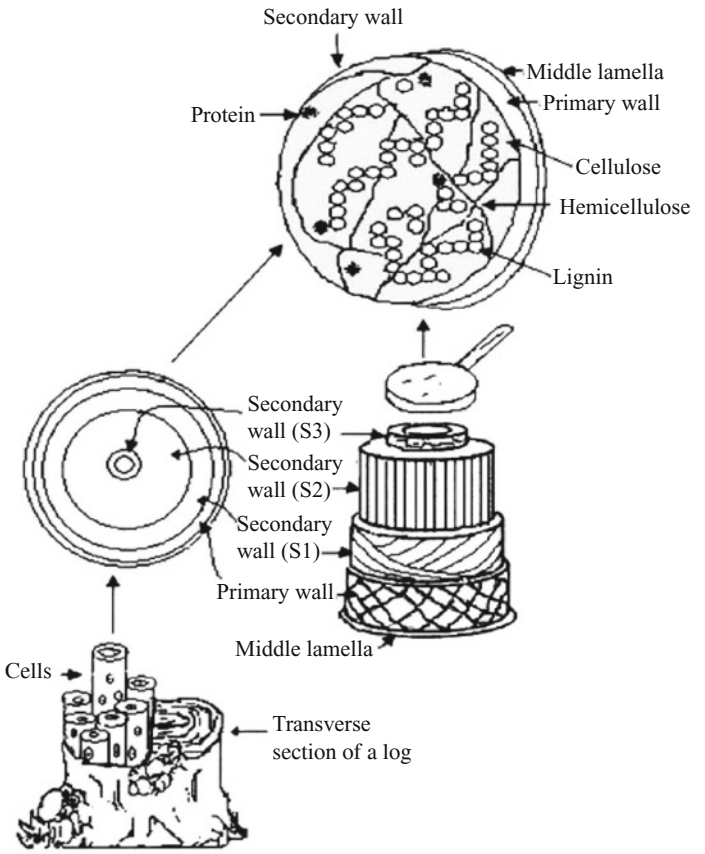

Fig. 4.5: Composition of lignocellulosic residues: Cellulose, hemicellulose and lignin (Sanchez, 2008).

Table 4.1: Composition of some lignocellulosic materials

Lignocellulosic residue	*Lignin (%)*	*Hemicelluloses (%)*	*Cellulose (%)*	*Ash (%)*	*Reference*
Hardwood stems	18-25	24-40	40-55	NA	Howard et al. (2003)
Softwood stems	25-35	25-35	45-50	NA	Howard et al. (2003)
Nut shells	30-40	25-30	25-30	NA	Howard et al. (2003)
Corn cobs	15	35	45	1.36	Prassad et al. (2007)
Paper	0-15	0	85-99	1.1-3.9	Howard et al. (2003)
Rice straw	18	24	32.1	NA	Howard et al. (2003)
Sorted refuse	20	20	60	NA	Howard et al. (2003)
Leaves	0	80-85	15-20	NA	Howard et al. (2003)
Cotton seeds hairs	0	5-20	80-95	NA	Howard et al. (2003)
Newspaper	18-30	25-40	40-55	8.8-1.8	Howard et al. (2003)
Waste paper from chemical	5-10	10-20	60-70	NA	Howard et al. (2003)
Pulps	24-29	NA	8-15	NA	Howard et al. (2003)
Primary wastewater solids	NA	28	6	NA	Howard et al. (2003)
Swine waste	6.4	35.7	25	NA	Howard et al. (2003)
Coastal Bermuda grass	12	31.4	45	NA	Howard et al. (2003)
Switch grass	2.7	15.8	21.3	NA	Howard et al. (2003)
S32 rye grass (early leaf)	7.3	25.7	26.7	NA	Howard et al. (2003)
Orchad grass (medium maturity)	10-30	25-50	25-40	1.5	Howard et al. (2003)
Grasses	19-24	27-32	32-44	4.5-9	Rowell (1992)
Sugar cane bagasse	16-21	26-32	29-35	NA	Prassad et al. (2007)
Wheat straw	14-15	24-29	31-34	5-7	Rowell (1992)
Barley straw	16-19	27-38	31-37	6-8	Rowel (1992)
Oat straw	16-19	27-30	33-35	2-5	Hon (2000)
Rye straw	21-31	15-26	26-43	1.7-5	Hon (2000)
Bamboo	17-19	27-32	33-38	6-8	Hon (2000)
Grass Esparto	22	23.9	NA	6	Hon (2000)
Grass Sabai	23.9	24	22	6	Hon (2000)
Grass Elephant	23	25	47	5	Hon (2000)
Bast fiber Seed flax	15-19	22-23	31-39	2-5	Hon (2000)
Bast fiber Kenaf	21-26	18-21	45-53	0.5-2	Hon (2000)

(*Contd.*)

Bast fiber Jute	8.8	17.3	60.8	1.1	Hon (2000)
Leaf Fiber Abaca (Manila)	7-9	21-24	43-56	0.6-1.1	Hon (2000)
Leaf Fiber Sisal (agaye)	13.1	4-8	77.6	0.6-1	Hon (2000)
Leaf Fiber Henequen	18.8	46.3	35	8.2	Pérez-Díaz et al. (2005)
Coffee pulp	14	14.8	13.2	11.4	John et al. (2006)
Banana waste	NA	NA	NA	4.2	John et al. (2006)
Yucca waste					John et al. (2006)

Cellulose appears in nature to be associated with other plant compounds and this association may affect its biodegradation. Cellulose is insoluble in most solvents and has a low accessibility to acid and enzymatic hydrolysis and it must be hydrolyzed to glucose before fermentation to ethanol (Demirbas, 2008).

Hemicelluloses are polysaccharides with a lower molecular mass than cellulose. They are formed from D-xylose, D-mannose, D-galactose, D-glucose, L-arabinose, 4-O-methyl-glucuronic, D-galacturonic and D-glucuronic acids. Among the most important sugars of the hemicelluloses component is xylose (Hashem, 2007). Hemicelluloses are related to plant gums in composition, and occur in much shorter molecule chains than cellulose. Hemicelluloses are derived mainly from chains of pentose sugars, and act as the cement material holding together the cellulose micells and fibre. Hemicelluloses are largely soluble in alkali and, as such, are more easily hydrolysed (Demirbas, 2008).

The main difference between cellulose and hemicelluloses is that hemicelluloses have branches with short side chains consisting of different sugars and cellulose consists of easily hydrolyzable oligomers (Hashem, 2007).

Lignin is a macromolecule, which consists of alkylphenols and has a complex three-dimensional structure (Demirbas, 2008). Lignin is linked to both hemicelluloses and cellulose, forming a physical seal that is an impenetrable barrier in the plant cell wall. It is present in the cell wall to give structural support, impermeability and resistance against microbial attack and oxidative stress. It is an amorphous heteropolymer, non-water soluble and optically inactive that is formed from phenylpropane units joined together by non-hydrolyzable linkages. The basic chemical phenylpropane units of lignin (primarily syringyl, guaiacyl and *p-hydroxy* phenol) are bonded together by a set of linkages to form a very complex matrix. This matrix comprises a variety of functional groups, such as hydroxyl, methoxyl and carbonyl, which impart a high polarity to the lignin macromolecule (Hashem, 2007; Demirbas, 1993).

4.3.2 Generation of Lignocellulosic Residues

The increasing expansion of agro-industrial activity has led to the accumulation of a large quantity of lignocellulosic residues from wood (e.g. poplar trees),

herbaceous (e.g. switch grass), agricultural (corn stover, and wheat straw), forestry (e.g. sawdust, thinnings, and mill waste), municipal solid wastes (e.g. waste paper) and various industrial wastes all over the world. Table 4.2 summarizes the worldwide generation of lignocellulosic residues (Sanchez, 2008).

4.3.3 Biodegradation of Lignocellulosic Residues

Only a small amount of the cellulose, hemicelluloses and lignin produced as by-products in agriculture or forestry is used, the rest being considered waste. Many microorganisms are capable of degrading and utilizing cellulose and hemicelluloses as carbon and energy sources. However, a much smaller group of filamentous fungi has evolved with the ability to break down lignin, the most recalcitrant component of plant cell walls. These are known as white-rot fungi, which possess the unique ability of efficiently degrading lignin to CO_2. Other lignocellulose degrading fungi are brown-rot fungi that rapidly depolymerize cellulosic materials while only modifying lignin. Collectively, these wood and litter-degrading fungi play an important role in the carbon cycle.

Table 4.2: Lignocellulosic residues generated from different agricultural sources

Lignocellulosic residues	*Ton × 10^6 / year*
Sugar cane bagasse	317-380
Maize straw	159-191
Rice shell	157-188
Wheat straw	154-185
Soja straw	54-65
Yuca straw	40-48
Barley straw	35-42
Cotton fibre	17-20
Sorghum straw	15-18
Banana waste	13-15
Mani shell	9.2-11.1
Sun flower straw	7.5-9
Bean straw	4.9-5.9
Rye straw	4.3-5.2
Pine waste	3.8-4.6
Coffee straw	1.6-1.9
Almond straw	0.4-0.49
Hazelnut husk	0.2-0.24
Sisal and henequen straw	0.077-0.093

In addition to lignin, white-rot fungi are able to degrade a variety of persistent environmental pollutants, such as chlorinated aromatic compounds, heterocyclic aromatic hydrocarbons, various dyes and synthetic high polymers (Bennett et al., 2002).

The ability of fungi to degrade lignocellulosic materials is due to their highly efficient enzymatic system. Fungi have two types of extracellular enzymatic systems: the hydrolytic system, which produces hydrolases that are responsible for polysaccharide degradation and a unique oxidative and extracellular ligninolytic system, which degrades lignin and opens phenyl rings (Sanchez, 2008).

Lignin biodegradation by white-rot fungi is an oxidative process and phenol oxidases are the key enzymes (Rabinovich et al., 2004). Of these, lignin peroxidases (LiP), manganese peroxidases (MnP) and laccases from white-rot fungi (especially *Botrytis cinerea, P. chrysosporium, Stropharia coronilla*, *P. ostreatus* and *Trametes versicolor*) have been studied (Martinez et al., 2004).

The lignin contains no bonds that can be cleaved hydrolytically under conditions prevalent in cells. The strategy for its degradation, therefore, is based on free radicals reactions which are catalyzed by extracellular enzymes. The transformation of polyphenolic substances and lignins to humic acids at soil level is possible at least by two pathways: the abiotic oxidation catalyzed by minerals or metal salts and the enzymatic oxidation by laccase and other oxidative enzymes. The abiotic polymerization of phenolic compounds has been shown by demonstration of the catalytic effects of certain oxides of Mn, Fe, Al, and Si to polymerize phenols to polymers. Also, the phenol oxidases (peroxidases or polyphenoloxidases – laccases) have an important function in the structural assembly of the humic acid complex. The origin of the aromates may either be the aromatic moiety of the litter, root organic substances, or metabolites from litter decomposing microorganisms. From the chemical background of this reaction it is obvious that a free phenolic group is needed for this polymerization, a fact which is very important in the case of bioremediation of the area polluted with aromatic/phenol compounds (Abaecherli and Popa, 2005).

Cellulolytic microorganisms can establish synergistic relationships with non-cellulolytic species in cellulosic wastes; the interaction leads to complete degradation of cellulose. Microorganisms capable of degrading cellulose produce a battery of enzymes with different specificities, working together. Cellulases responsible for the hydrolysis of cellulose, are composed of a complex mixture of enzyme proteins with different specificities to hydrolyze the β-1,4-glycosidic linkages bonds. Cellulases can be divided into three major enzyme activity classes (Rabinovich et al., 2004). These are endoglucanases (carboxymethylcellulases), cellobiohydrolase (exoglucanase), and β-glucosidase (Sanchez, 2008).

Hemicelluloses biodegradation requires more enzymes for a complete degradation because of its greater heterogeneity compared with cellulose

(Malherbe and Cloete, 2002). Hemicelluloses are biodegraded to monomeric sugars and acetic acid. Xylan is the main carbohydrate found in hemicelluloses. Its complete degradation requires the cooperative action of a variety of hydrolytic enzymes. Hemicellulose degradation needs accessory enzymes such as xylan esterases, ferulic and *p*-coumaric esterases, α-1-arabinofuranosidases, and α-4-O-methyl glucuronosidases, acting synergistically to efficiently hydrolyze wood xylans and mannans (Sanchez, 2008).

This degradative ability of white-rot fungi is because of the strong oxidative activity and low substrate specificity of their ligninolytic enzymes. Little is known about the degradation mechanisms of lignocellulose by soft-rot fungi, in contrast to white and brown-rot fungi. Nevertheless, it is clear that some soft-rot fungi can degrade lignin, because they erode the secondary cell wall and decrease the content of acid-insoluble material (Klason lignin) in angiosperm wood. Soft-rot fungi typically attack higher moisture, and lower lignin content materials (Shary et al., 2007).

4.4 Lignin and Polyphenols in Soil

Polyphenols are the most widely distributed class of plant secondary metabolites and several thousand different compounds have been identified. Polyphenols play many different roles in plant biology and human life, including UV protective agents, defensive compounds against herbivores and pathogens, contributors to plant colours, to the taste of food and drink, and pharmaceuticals.

Polyphenols could inhibit the activity of digestive enzymes and/or precipitate nutritional proteins. Polyphenols have also been recognized as regulators of soil processes, where it has been suggested that they inhibit nitrification, as well as decomposition and nutrient recycling, as a by-product on their antiherbivore activity (Hattenschwiler and Vitousek, 2000).

Polyphenols are known to affect litter quality, at times having a larger effect on decomposition rates than more frequently measured parameters, such as nitrogen or lignin (Palm and Sanchez, 1990).

Phenolic compounds can directly affect the composition and activity of decomposer communities, thus influencing the rate of decomposition and nutrient cycling. Different types of soluble polyphenols, such as feluric acid, galic acid or flavonoids, have been found to either stimulate or inhibit spore germination and hyptal growth of saprotrophic fungi (Kuiters, 1990).

Lignin and polyphenols improve the soil fertility and can be useful in bioremediation of polluted area (with heavy metals, hydrocarbons etc.). In these conditions an effect of rhizosphere is expected. The rhizosphere is a unique and dynamic zone of interaction between roots of plants and soil microorganisms. This region is characterized by the presence of a biomass and an increased soil activity. The community of rhisosphere is composed from microbios (bacteria, fungi and algae) and micro- and mezofauna (protozoa, nematodes and insects). The abundance of the microorganisms in the rhizosphere is of 5 to 20 or even 100

times higher than in the uncultivated soils. The symbiosis between plants and microorganisms can assure the protection of the plants against toxic compounds along with their biodegradation (Popa and Beleca, 1994).

The fixation of nitrogen by plants becomes possible along with the formation of polyphenolic compounds from biodegradation of lignin. The polyphenols could represent signal molecules which can induce phytohormonal exchanges, influencing some modifications at the level of root cortical cells. In this way the division and differentiation of cell are starting.

It is known that soil microorganisms degrade the lignin and their derivatives by cleaving off units consisting of one, two or at the most three phenolic acid moieties. These transformations are accelerated when the modified lignins are used, especially in the case of those with a high degree of degradation. On the other hand some chemicals from plants may be released from living leaves as volatiles or leachates, or from roots through exudation or sloughing of dead tissue. They also may leach from litter on the soil surface. Most of them are of polyphenolic nature. These chemical compounds influence the biological processes in a complex manner both at the soil level (in the humus formation) and plant development (by their intervention in the hormonal chain). Thus, in the last case it was possible to identify products with inhibitory (herbicides or pesticides) or with stimulatory action on different stages of plants propagation (seeds germination, plant grafting, plant developments). Also, among them the compounds that can act on the expression of heredity programme in another plant were identified.

In the soil, soluble polyphenols face four different fates. They might be degraded and mineralized as carbon source by heterotrophic microorganisms; they can be transformed into insoluble and recalcitrant humic substances by polymerization and condensation reactions (with the contribution of soil microorganisms); they might adsorb to clay minerals or form chelates with Al or Fe ions; or they might remain in dissolved form, leached by percolating water, and finally leave the ecosystem as part of dissolved organic carbon.

There is evidence for both negative and positive effects of phenolic compounds on the activity of soil organisms and hence on decomposition and nutrients mineralization. The regulation of nutrient mineralization through soil microbial activity is only one way that polyphenols can influence nutrient cycling. Complexation with inorganic sesquioxides, cations and proteins might also allow sustained P and base cation availability in highly weathered soils, and an advantage to the plant-mycorrhiza symbiosis over free-living microorganisms in the competition for N (Abaecherli and Popa, 2005).

4.5 Utilization of Lignins and their Derivates in the Crops Cultivation and Bioremediation

The lignin and their derivatives could carry out the following functions (Abaecherli and Popa, 2005):

- soil conditioners (in natural state as fertilizer; for the improvement of salty soil);
- mineral nutrient sources used in a non-conventional manner (low rate; as ammonized/oxyammonolyzed lignin, as lignostimulator complex, as support for nitrogen containing fertilizers);
- wetting agents or surfactants;
- biological inoculants and activators (as compost based on lignin);
- plant stimulants and growth regulators (modified by oxidative nitration—nitroquinone polycarboxylic acids);
- non-conventional fertility concepts or programmes; and
- as soil structuring agent and bioremediation of polluted soils.

The literature data evidenced some possibilities to use non- or modified lignins in the directions which are presented in Table 4.3.

Table 4.3: Possibilities of lignins and polyphenols utilization in the fields of crops cultivation and bioremediation (Abaecherli and Popa, 2005)

Utilizations	*Lignins/lignin derivatives*
Non-conventional fertilizers	Lignosulfonates, ammoniated lignosulfonates and hydrolysis lignin, oxiammonolyzed lignosulfonates and hydrolysis lignin [fertilizers with N slow release, granulation (bonding agents), micronutrients, fromulations, chelating agents (microelements)], oxidated lignins: lignosulfonates, hydrolysis lignin, kraft lignin [using O_2 (air)/ammonia with/without catalysts, H_2O_2, persulfates]
Slow release for pesticides, insecticides, herbicides	Crosslinked lignin with epichlorhydrine, kraft lignin, Alcell lignin, lignosulfonates (non-or modified), sulfonated kraft lignin, alkali lignin
Growth stimulators	Aqueous soluble lignin, oxidated lignins (oxyammonolysis of lignosulfonates, hydrolysis lignin), nitration or oxidative nitration of hydrolysis lignin, lignocellulose, ammoniated nitrolignin, condensed lignosulfonates with urea, polyphenols
Seed coatings	Lignosulfonates (Na, Ca), lignosulfonates sugar free, desulfonated lignosulfonates
Sequestering agents	Hydrolysis lignin, lignin and lignin-derivatives
Soil conditioners	Kraft lignin, oxidized kraft lignin, grafted lignosulfonates with acrylic/methacrylic acids or acrylonitrile, composted hydrolysis lignin (HL) or amonized HL, lignosulfonates (NH_4^+, Na^+), alkali treated lignin followed by activation with CuO

(*Contd.*)

Wetting and surfacting agents	Kraft lignin highly sulfonated (Kraftsperse 25 M; Reax 88 A, 88 B, and 100 M) kraft lignin low-to mid-level sulfonated (Reax 83 A and 85 B), lignosulfonates from softwood
Compost activators	Hydrolysis lignin, wastes from pulp and paper industry
Bioremediation	Hydrolysis lignin, lignosulfonates, alkali lignin, polyphenols
Plant immunology	Polyphenols

4.5.1 Slow Release Agents for Pesticides, Insecticides and Herbicides

On large scale non-modified lignosulfonates (Na^+, NH_4^+), unsulfonated and sulfonated kraft lignin are used in the field of agrochemicals and bioremediation. Thus, the following directions could be mentioned: dispersants, agents for controlled release of pesticides, insecticides or herbicides, fertilizers formulation (granulation of nutrients—urea, inorganic fertilizers, chelation of micronutrients—boron, chlorine, cobalt, copper, iron, manganese, molybdenum, sodium and zinc, in composition along with compounds which stimulates different stages in plant propagation).

In agricultural lands it might not be appreciated if a pesticide added to soil disappears too soon. To avoid this phenomenon, either crop rotation can be applied, implying the use of different pesticides with each different crop or special extenders (based on lignin derivatives) applied that protect the pesticides against degradation.

Other field trials were reported in former Soviet Union for hydrolysis lignin using large quantities of non-modified (as chelating agent for micronutrients, as mixture with inorganic fertilizers or soil conditioner) or in modified form by composting, ammonization and oxyammonolysis (Abaecherli and Popa, 2005).

4.5.2 Growth Stimulators

The literature information provides the utilization of lignosulfonates, lignin and polyphenols as growth stimulators to improve the soil fertility and for bioremediation of polluted area (with heavy metals, hydrocarbons).

Polyphenols play many different roles in plant biology including defensive compounds against herbivores and pathogens, disease resistance, associated with adventitious root production, contributors to plant colours, etc.

The polyphenols (from spruce bark, oak bark) and lignins (lignosulfonates, alkali lignins), non-modified or modified, have been used in experiments of germination, growth stimulators and plant cultivation.

In plant seeds germination a lot of variants combination has been experimented. The results show that the germination is improved both by

polyphenols and lignin samples. The influence on this property depends on the kind of products and their degree of modification (Popa and Volf, 2006).

The data presented by Popa et al. (1996) reflect that polyphenolic compounds influence especially the germination capacity of wheat seeds while the ligninic products influence the germination capacity of bean seeds. Such an observation should be correlated both with different chemical composition (origin, separation, method) and nature of the biological material (Popa et al., 1996).

Polyphenolic fractions, obtained by extraction with NaOH 1% solution as an extracting agent and in another variant using acetone, were chemically modified by hydrogen peroxide treatment. Both types of polyphenolic fractions, unmodified and modified, were used in experiments of plants cultivation studying their stimulating or inhibitory effects. The best stimulating effect on wheat seeds germination was obtained with unoxidized polyphenolic fractions at concentrations of 0.25-0.5 g/L. The same favourable effect on the yielded plants percentage was obtained with oxidized vegetal polyphenolic fractions at a concentration of 0.25 g/L. The largest plants height was attained by growth stimulation with oxidized polyphenolic II fraction at a concentration of 0.5-1.0 g/L. Plants treatment with oxidized polyphenolic I and II fractions led to high speed of plants growth (Popa et al., 2002).

Polyphenols and lignin derivatives participate as bioregulators in biosynthesis process from plants (Fig. 4.6). The action of these compounds depends on their nature, composition and concentration (Popa et al., 2000; Popa et al., 2005; Tudose and Popa, 2000).

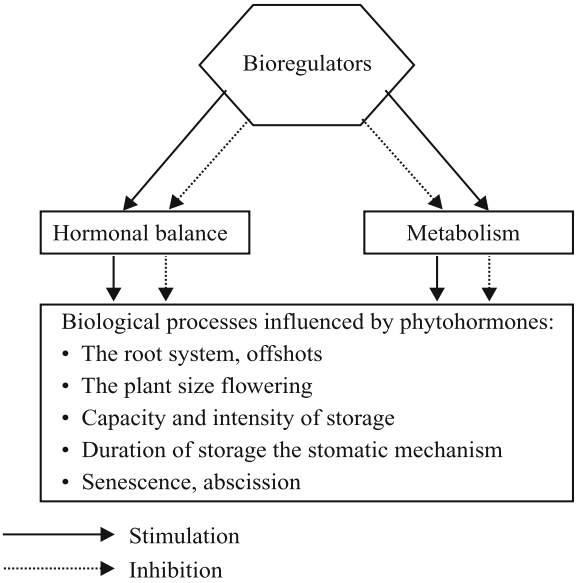

Fig. 4.6: Influence of bioregulators on essential physiological processes in plants (Popa and Volf, 2006).

Ligninic products and their derivativess along with polyphenols can act as allelochemicals which influence patterns in vegetation communities, plant succession, seed preservation, germination of fungal spores, the nitrogen cycle mutuality association, crop productivity and plant defense (Popa et al., 2005).

Exciting goals are to adopt allelochemicals as herbicides, pesticides and growth stimulants, modify crop genomes and manipulate alleochemicals production and better elucidate chemical communications between the components of ecosystem.

4.5.3 Soil Conditioning and Fertilizers

The lignin and its derivatives can be used to obtain soil conditioners. Thus, hydrolysis lignin was applied as conditioning agent (30 t/ha) with the aim to ameliorate acid steppe soils. The modification of the equilibrium of sodium and magnesium ions was observed along with an increasing of crop productivity. In other experiments soluble fractions of lignin obtained by treatment with nitric acid (10%) or nitric acid together with KOH, ammoniated nitrolignin or sulfolignin (Ahmaruzzaman, 2008), activated lignin by alkali and CuO and lignosulfonates were used. These products improve the structure of the soil and its fertility. Kraft lignin was modified with sodium hydroxide and urotropine and a soil conditioner was obtained, which can be used on a large variety of soils (Abaecherli and Popa, 2005).

Soil conditioners were prepared by the modification of water soluble lignin derivatives (lignosulfonates and nitrolignin) with acrylic monomers. The obtained preparations increase the content of water stable aggregates with the size more than 0.25 mm to 90%, as well as they improve water and gas exchange in soil. The soil cultivated in the presence of these preparations (75-150 kg/ha) resists to water and air erosion. As a result, the conditions of plants growth are improved (Kislenko, 1998).

Lignin and its derivatives are non-toxic by-products of the pulp and paper industry and are well known for their ability to bind heavy metal ions. With respect to fertilizers, lignins are capable of keeping micronutrients such as Cu, Co, Mn, and Zn in an available form under pH conditions that might normally cause insolubilization. Since the mentioned ions are more weakly bound than those made with other complexing agents, such as EDTA, they are more readily available to the plants (Tisch et al., 2003).

The addition of lignin derivates to an acidic, metal contaminated feed solution increased the quantity of shoot biomass produced by three of six species tested by Tisch et al. (2003) and photosynthetic efficiency was also improved.

The modified kraft lignin (KL) was also used to mimic the chemical structures of humic substances and evaluating them as new soil conditioning agents. A commercial softwood kraft lignin was modified by oxidation and sulfonation and the interaction between the chemically modified lignins and

Al ions was studied. The obtained data indicate that the modified lignin not only can remove the toxic effect of Al, but it can also have a positive effect on the root elongation. The radish growth test was conducted on culture solution at an exact pH and Al concentration. It is interesting to note that the growth of the radish root was practically prohibited in the absence of a lignin and in the presence of only 5 ppm of Al. With an addition at a concentration of ~350 ppm of kraft lignin, root elongation slowly increased at the beginning and finally reached to the same level as that without Al. The kraft lignin itself had a positive effect on the growth of the radish root. When 70 ppm of KL was added to the culture solution, root elongation was about twice as much as that of the control conditions (Abaecherli and Popa, 2005).

Soil conditioners prepared by the modification of water soluble lignin derivatives (wastes of wood processing that do not have the whole utilization) with acrylic monomers increase the content of water stable soil aggregates with the size more than 0.25mm to 90%, as well as they improve the water and gas exchange in soil. The soil, cultivated in the presence of these preparations (75-150 kg/ha), resists to the water and air erosion and as a result, the conditions of growth of plants are improved (Kislenko, 1998).

4.5.4 Lignin in Biosorption Process

Heavy metal ions are reported as priority pollutants, due to their mobility in natural water ecosystems and due to their toxicity. Heavy metal removal from aqueous solution has been traditionally carried out by chemical precipitation.

The removal of metal ions from aqueous streams using agricultural materials is based upon metal biosorption (Volesky and Holan, 1995). The process of biosorption involves a solid phase (sorbent) and a liquid phase (solvent) containing a dissolved species to be sorbed.

In general, raw lignocellulosic biosorbents were modified by various methods to increase their sorption capacities because metal ion binding by lignocellulosic biosorbents is believed to take place through chemical functional group such as carboxyl, amino, or phenolics (Demirbas, 2008).

4.5.5 Mechanism of Metal Biosorption

Agricultural waste materials are usually composed of lignin and cellulose as the main constituents. Other components are hemicelluloses, extractives, lipids, proteins, simple sugars, starches, water, hydrocarbons, ash and many more compounds that contain a variety of functional groups.

Due to high affinity of the sorbent for the metal ion species, the latter is attracted and bound by rather complex process affected by several mechanisms involving chemisorption, complexation, adsorption on surface and pores, ion exchange, chelation, adsorption by physical forces, entrapment in inter- and intra-fibrillar capillaries and spaces of the structural polysaccharides network as a result of the concentration gradient and diffusion through cell wall and membrane (Basso et al., 2002; Qaiser et al., 2007) (Fig. 4.7).

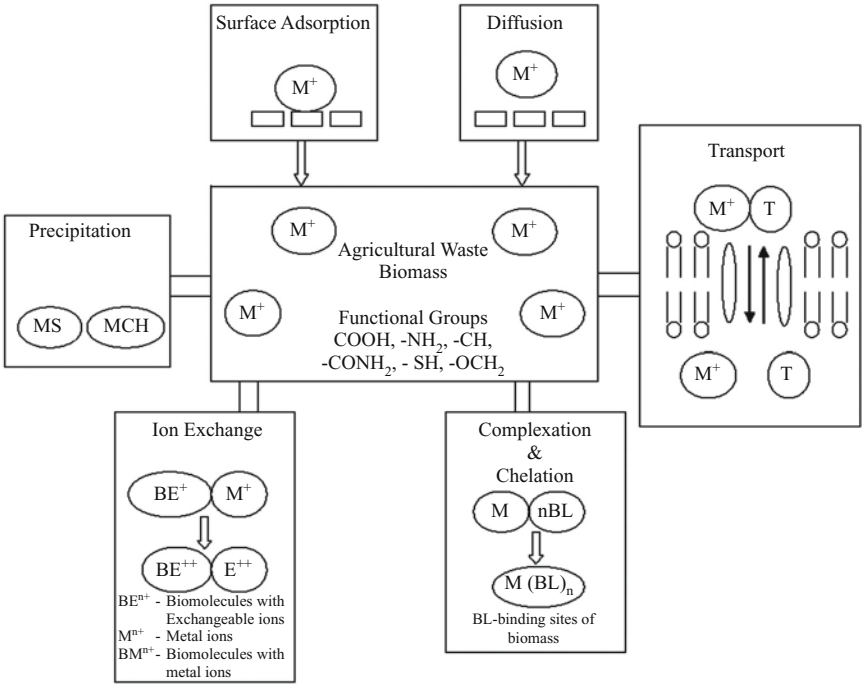

Fig. 4.7: Mechanism of biosorption (Sud et al., 2008).

Laszlo and Dintzis (1994) have shown that lignocellulosics have sorption capacity, which are derived from their constituent polymers and structure. These are adsorbents for a wide range of solutes, particularly divalent metal cations.

Lignocellulosics are hygroscopic and have an affinity for water. Water is able to penetrate the non-crystalline portion of cellulose and all of the hemicelluloses and lignin. Thus, through absorption and adsorption, aqueous solution comes into contact with a very large surface area of different cell wall components. Because of its disordered structure, amorphous cellulose should be much more accessible to reagents than highly structured crystalline cellulose. Cellulose can also retain heavy metals from solution (Acemioglu and Alma, 2002).

The molecular and supramolecular structures have a strong influence on sorption properties. Water adsorption of fibres, orientated in one particular direction, invariably causes swelling. The higher the amount of water adsorption, the higher is the swelling. Swelling also depends on the fibre's structure, degree of crystallinity and amorphous and void regions (Rowell, 2006). Swelling occurs when polar solvents such as water and alcohols are contacted with wood. Wood swelled extremely fast at high temperatures. The polar solvent molecules are attracted to the dry solid matrix and held by hydrogen bonding forces between the –OH or –COOH groups in the wood structure.

To understand how metals bind to the biomass, it is essential to identify the functional groups responsible for metal binding. Most of the functional groups involved in the binding process are found in cell walls. The functional groups present in molecules of different grades of biomass, generally are: acetamido groups, carbonyl, phenolic, structural polysaccharides, amido-, amino-, sulphydryl, carboxyl groups, alcohols and esters (Gupta and Ali, 2000). These groups have the affinity for metal complexation. Some biosorbents are non-selective and bind a wide range of heavy metals with no specific priority, whereas others are specific for certain types of metals depending upon their chemical composition (Sud, 2008).

4.5.6 Agricultural Waste Materials as Biosorbents for the Removal of Heavy Metals

Removal of heavy metal ions from the aqueous streams by agricultural waste materials is an innovative and promising technology. The efficiency of the waste material depends upon the capacity, affinity, and specificity, including also physico-chemical nature of it. Scattered research has already been done on the variety of biosorbents for the removal of metal ions as Cr, Cu, Ni, Pb, Cd, As, Hg etc. The adsorbents are taken either in the natural form, or modified by chemical and thermal treatment for increasing their sorption capabilities (Sud et al., 2008).

Biomass chemical modifications include delignification, esterification of carboxyl and phosphate groups, methylation of amino groups, and hydrolysis of carboxylate groups.

Removal of Chromium

Chromium is a toxic heavy metal being released in the environment by applications like tanning, wood preservation and pigments, dyes for plastic, paints, and textiles. Chromium occurs in a number of oxidation states, but chromium(VI) and chromium(III) are of main environmental concern (Yu et al., 2000).

A number of agricultural wastes, like hazelnut shells, orange peels, maize cobs, peanut shells, jack fruit, and soy bean hulls in natural or modified forms, has been explored and significant removal efficiency was reported (Kurniawan et al., 2006). Diverse plant parts such as coconut fibre pith, coconut shell fibre, plant bark (*Acacia arabica, Eucalyptus*), pine needles, cactus leaves, and neem leave powder have also been tried for chromium removal showing efficiency more than 90-100% at optimum pH (Dakiky et al., 2002; Manju and Anirudhan, 1997; Mohan et al., 2006; Sarin and Pant, 2006; Venkateswarlu et al., 2007). Gardea-Torresdey et al. (2000) reported that *Avena monida* (whole plant biomass) showed 90% removal efficiency of Cr(VI) at optimum pH 6.0. Rice husk in natural form as well as activated rice husk carbon were used for the removal of chromium(VI) and results were also compared with commercial activated carbon and other adsorbents (Bishnoi et al., 2004). Saw dust of Indian

rose wood prepared by treatment with formaldehyde and sulphuric acid showed efficient removal of chromium(VI) (Garg et al., 2004). Utilization of mustard oil cake has been reported with significant removal efficiency and the results of activated carbon of sugar industry waste and commercial granular activated carbon for sequestering of heavy metal ions from aqueous solutions were compared (Ajmal et al., 2005; Fahim et al., 2006).

Most of the studies showed that the chromium biosorption by agricultural waste materials is quite high and varies from 50 to 100%. Mostly biosorption occurs in acidic range particularly at pH 2. Thus chromium speciation plays the dominant role in deciding the removal efficiency as at pH 2 chromium is present as chromium(III) (Sud et al., 2008). Table 4.4 summarizes the results reported in literature for the removal of chromium by using agricultural waste materials.

Removal of Lead

The major source of lead in the environment is from plastics, finishing tools, cathode ray tubes, ceramics, solders, pieces of lead flashing and other minor product, steel and cable reclamation. Lead can result in the wide range of biological effects depending upon the level and duration of exposure. In the environment lead binds strongly to particles such as oil, sediments and sewage sludge so its removal is of great concern (Sud et al., 2008). Different agricultural wastes were studied for the possibility of being used in removal of lead. Summary of works done by various researches using variety of agricultural waste materials for removal of lead is presented in Table 4.5. Literature information revealed that optimized value of biosorption of lead is found around pH 5-6. Gupta et al. (2004) used bagasse fly ash for removal of lead with 65% removal efficiency. Saw dust of *Pinus sylvestries* and rubber wood have shown a removal efficiency of 85-90%, but results indicated that modification did not enhance the removal efficiency for lead (Taty-Costodes et al., 2003).

Removal of Cadmium

Cadmium and cadmium compounds as compared to other heavy metals are relatively water soluble, therefore mobile in soil, and tends to bioaccumulate. The long life time PVC-window frames, plastics and plating on steel are the basic sources of cadmium in the environment. Cadmium accumulates in the human body especially in kidneys, thus leading to disfunction of the kidney (Sud et al., 2008).

Potential use of rice bran and wheat bran was tried for sequestering cadmium, and significant removal efficiency was reported (Montanher et al., 2005; Farajzadeh and Monji, 2004; Singh et al., 2005). Studies were also performed on use of rice polish, rice husk and black gram husk in their natural as well as modified form for the removal of cadmium and their relative efficiency was reported (Kumar and Bandyopadhyay, 2006).

Table 4.4: Summary of results obtained by various researchers using low cost agricultural waste materials for the removal of chromium

Agricultural waste	*Metal ion*	*Results*	*Reference*
Oat biomass	Cr(III), Cr(VI)	> 80%	Gardea-Torresdey et al. (2000)
Formaldehyde treated saw dust Indian rosewood	Cr(VI)	62-86%	Garg et al. (2004)
Beech saw dust	Cr(VI)	100%	Acar and Malkoc (2004)
Chemically treated bagasse	Cr(VI)	50-60%	Krishanani et al. (2004)
Formaldehyde treated rice husk	Cr(VI)	88.88%	Bishnoi et al. (2004)
Bagasse fly ash	Cr(VI)	96-98%	Gupta and Ali (2004)
Wheat bran	Cr(VI)	> 82%	Faraizadeh and Monji (2004)
Coconut shell fibres	Cr(VI)	> 80%	Mohan et al. (2006)
Commercial granular activated carbon (C2 & C3) and AC of waste from sugar industry (C1)	Cr(VI)	93-98% C1 > C2 > C3	Fahim et al. (2006)
Eucalyptus bark	Cr(VI)	~ 100%	Sarin and Pant (2006)
Neem leaf powder	Cr(VI)	> 96%	Venkateswarlu et al. (2007)
Rubber wood saw dust	Cr(VI)	60-70%	Karthikeyan et al. (2005)
Pretreated bagasse with NaOH and CH_3COOH	Cr(VI), Ni(II)	90%, 67%	Rao et al. (2002)
Modified bagasse fly ash	Cr(VI)	67%	Gupta et al. (1999)
Activated carbon from bagasse (carbonization and gasification)	Cr(VI)	Significant metal uptake	Valix et al. (2006)
Sugarcane bagasse, maize corn cob, jatropha oil cake	Cr(III)	Up to 97%	Garg et al. (2007)
Raw rice bran	Cr(VI), Ni(II)	40-50%	Oliveira et al. (2005)

Table 4.5: Summary of works carried out by various researchers using variety of agricultural waste materials for the removal of lead

Agricultural waste	*Metal ion*	*Results*	*Reference*
Orisa sativa husk	Pb(II)	98%	Zulkali et al. (2006)
Agricultural by product *Humulus lupulus*	Pb(II)	75%	Gardea-Torresdey et al. (2002)
Chemical modified apple residue waste	Pb(II)	Upto 80%	Lee et al. (1999)
Agro waste of black gram husk	Pb(II)	Upto 93%	Saeed et al. (2005b)
Febrifuga bark	Pb(II)	100%	Sud et al. (2008)
Chemically modified saw dust of rubber wood	Pb(II)	85%	Raji and Anirudhan (1998)
Coconut char based activated carbon	Pb(II)	100%	Sud et al. (2008) Nasir et al. (2007)
Rose biomass pretreated with NaOH	Pb(II), Zn(II)	75%	Montanher et al. (2005)
Rice bran	Pb(II), Cd(II), Cu(II), Zn(II)	> 80%	Taty-Costodes et al. (2003)
Saw dust of *Pinus sylvestris*	Pb(II), Cd(II)	96%, 98%	Yu et al. (2001)
Maple saw dust	Pb(II), Cu(II)	80-90%, 60-90%	Bailey et al. (1999)
Low cost sorbents (bark, dead biomass, chitin, sea weed, algae, peat moss, leaf, mould, moss, zeolite, modified cotton)	Pb(II), Cd(II), Cr(VI), Hg(II)	Good results	
Waste tea leaves	Pb(II), Fe(II), Ni(II), Zn(II)	92%, 84%, 73%	(Ahluwalia and Goyal 2005b)

(*Contd.*)

Activated carbon from coir pith	Pb(II), Hg(II), Cd(II), Ni(II), Cu(II)	Hg-100% Pb-100% Cu-73% Ni-92% Cd-100%	Kadirvelue et al. (2001)
Rice straw, soybean hulls, sugarcane bagasse, peanut shells, pecan and walnut shells	Pb(II), Cu(II), Cd(II), Zn(II), Ni(II)	Pb > Cu > Cd > Zn > Ni	Sud et al. (2008)
PFP (petiolar felt sheath palm)-peelings from trunk of palm tree	Pb(II), Cd(II), Cu(II), Zn(II), Ni(II), Cr(VI)	> 70% Pb > Cd > Cu > Zn > Ni > Cr	Iqbal et al. (2008)
Activated carbon of peanut shells	Pb(II), Cd(II), Cu(II), Ni(II), Zn(II)	Upto 75%	Wilson et al. (2006)

Use of other parts of the plants such as peels of peas, fig leaves, broad beans, orange peels, medlar peels and jack fruits as adsorbents have been reported to show high removal efficiency at acidic pH (Benaissa, 2006). Adsorption experiments conducted on hazelnut shells, peanut hulls, walnut shells, and green coconut shells gave significant results for removal of cadmium (Kurniawan et al., 2006).

Research has also been carried out by using chemically treated agricultural waste materials like base treated rice husk, treated juniper fibres, and corncob modified with citric acid, modified peanut shells, succinic anhydride treated sugarcane etc. (Karnitz et al., 2007).

Most of the studies showed that agricultural waste either in natural form or modified form is highly efficient for the removal of cadmium metal ions. Summary of research work done is presented in Table 4.6.

Table 4.6: Summary of works performed by various researchers using variety of agricultural waste materials for the removal of cadmium

Agricultural waste	*Metal ion*	*Results*	*Reference*
Peels of peas, fig leaves, broad beans, medlar peel	Cd(II)	70-80%	Benaissa (2006)
Wheat bran	Cd(II)	87.15%	Singh et al. (2005)
Three kinds treated rice husk	Cd(II)	80-97%	Kumar and Bandyopadhya
Rice polish	Cd(II)	> 90%	(2006)

(*Contd.*)

Steam activated sulphurised carbon (SA-S-C) from bagasse pith	Cd(II)	98.8%	Singh et al. (2005)
Base treated juniper fibre	Cd(II)	High removal	Krishna and Anirudhar (2003)
Husk of black gram	Cd(II)	99%	Min et al. (2004)
Straw, saw dust, datesnut	Cd(II)	> 70%	Saeed and Iqbal (2003)
Dried pathenium powder	Cd(II)	> 99%	Ajmal et al. (2006)
Bagasee fly ash	Cd(II), Ni(II)	65%, 42%	Srivastava et al. (2007)
Bagasse	Cd(II), Zn(II)	90-95%	Mohan and Singh (2002)
Rice bran	Cd(II), Hg(II), Pb(II), Cr(II), Cu(II), Nil(II)	> 80%	Montanher et al. (2005)
Wheat bran	Cd(II), Hg(II), Pb(II), Cr(II), Cu(II), Ni(II)	> 82% except Ni	Farajzadeh and Monji 2004
Hazelnut shell, orange peel, maize cob, peanut hulls, soybean hulls treated with NaOH and jack fruits	Cd(II), Cr(II), Cu(II), Ni(II), Zn(II)	High metal adsorption	Kurniawan et al. (2006)
Papaya wood	Cd(II), Cu(II), Zn(II)	98%, 95%, 67%	Saeed et al. (2005a)
Rice straw, soybean hulls, sugarcane bagasse, peanut shells, pecan and walnut shells	Cd(II), Pb(II), Cu(II), Zn(II), Ni(II)	Pb > Cu > Cd > Zn > Ni	Sud et al. (2008)
Poplarwood saw dust	Cd(II), Cu(II), Zn(II)	Cu > Zn > Cd	Sciban et al. (2007)
Chemically modified sugarcane with succinic anhydride	Cd(II), Cu(II), Pb(II)	> 80%	Karnitz et al. (2007)
Powder of green coconut shell	Cd(II), Cr(II), As(II)	98%	Pino et al. (2006)
Bark of *Abies sachalinensis* and *Pecia glehnii*	Cd(II), Cu(II), Zn(II), Ag(II), Mn(II), Ni(II)	Upto 63%	Sud et al. (2008)

(Contd.)

Removal of Nickel

Nickel and its compounds have no characteristic odour or taste. The sources of nickel to the environment are nickel plating, coloured ceramics, batteries, furnaces used to make alloys or from power plants and trash incinerators. The most harmful health effect of nickel is the allergic reactions (Sud et al., 2008).

Experiments on removal of nickel were conducted on *Cassia fistula* biomass in its natural form and results show 99-100% removal efficiency (Hanif et al., 2007). Saw dust of maple, oak and black locust have been reported as promising biosorbent for removal of nickel (Sciban et al., 2006; Shukla et al., 2005). Agricultural wastes such as peanut, pecan, walnut, hazelnut and groundnut shells in natural or modified form were also utilized for biosorption (Kurniawan et al., 2006). Sugar cane bagasse in its natural form showed more than 80% removal efficiency (Garg et al., 2007). Table 4.7 is a compilation of researches performed on the removal of nickel.

Table 4.7: Summary of results obtained by various researchers using a variety of agricultural waste materials for the removal of nickel

Agricultural waste	*Metal ion*	*Results*	*Reference*
Hazelnut shell activated carbon	Ni(II)	Effective normal	Demirbas et al. (2002)
Casia fistula biomass	Ni(II)	100%	Hanif et al. (2007)
Maple saw dust	Ni(II)	75%	Shukla et al. (2005)
Sugarcane bagasse	Ni(II)	> 80%	Garg et al. (2007)
Tea waste	Ni(II)	86%	Malkoc and Nuhoglu (2005)
Defatted rice bran, chemically treated soybean and cotton seed hulls	Ni(II), Zn(II), Cu(II)	57%, 87%	Sud et al. (2008)
Waste tea leaves	Ni(II), Pb(II), Fe(II), Zn(II)	92%, 84%, 73%	Ahluwalia (2005a)
Saw dust of oak and black locust hard wood (modified and unmodified)	Ni(II), Cu(II), Zn(II)	70-90%	Sciban et al. (2006)
Hazelnut shell, orange peel, maize cob, peanut hulls, soybean hulls treated with NaOH and jack fruits	Ni(II), Cr(II), Cu(II), Cd(II), Zn(II)	High metal adsorption	Kurniawan et al. (2006)

(*Contd.*)

Mustard oil cake	Ni(II), Cu(II), Zn(II), Cr(II), Mn(II), Cd(II), Pb(II)	Up to 94%	Ajmal et al. (2005)
Coir fibre chemically modified with hydrogen peroxide	Ni(II), Zn(II), Fe(II)	> 70%	Shukla et al. (2005)
Dye loaded groundnut shells and saw dust	Ni(II), Cu(II), Zn(II)	Up to 90%	Shukla et al. (2005)
PFP (petiolar felt sheath palm-peelings from trunk of palm tree)	Ni(II), Pb(II), Cd(II), Cu(II), Zn(II), Cr(II)	> 70%	Iqbal et al. (2002)
Agro waste of black gram husk	Ni(II), Pb(II), Cd(II), Cu(II), Zn(II)	Pb>Cd>Cu> Zn>Ni>Cr> Up to 93%	Saeed et al. (2005b)
Modified and unmodified kenaf core, kenaf bast, sugarcane bagasse, cotton, coconut coir, spruce	Ni(II), Cu(II), Zn(II)	Up to 88%	Sciban et al. (2007)

Removal of Other Metal Ions

Other metal ions such as copper, zinc, arsenic, mercury and cobalt present in various industrial effluents are of environmental concern due to their toxicity even in low concentrations. Discharge of these metal ions into the aquatic systems is due to various human and industrial applications (Sud et al., 2008). Rice husk and water hyacinth along with other low cost adsorbents were studied for the removal of arsenic and the efficiency varies between 71 and 96% (Mohan and Pittman, 2007). Copper impregnated and chemically modified saw dust were also tried for arsenic removal with significant efficiency (Nag et al., 1998). Utilization of saw dust also played significant role in removal of copper metal ions (Ajmal et al., 2005). Mustard oil cakes and modified bark of *Pinus radiata* have also been proved as potential biosorbent (Palma et al., 2003). All results of these researches are summarized in Table 4.8.

4.5.3 Removal of Phenolic Compounds by Agricultural Solid Wastes

Phenols are generally considered to be one of the important organic pollutants discharged into the environment causing unpleasant taste and odour of drinking water. The major sources of phenol pollution in the aquatic environment are wastewaters from paint, pesticide, coal conversion, polymeric resin, petroleum and petrochemical industries.

Table 4.8: Summary of works carried out by various researchers using different agricultural waste materials for the removal of other metal ions

Agricultural waste	*Metal ion*	*Results*	*Reference*
Chemically treated charred saw dust	As(III), Cr(VI)	80%, 95%	Nag et al. (1998)
Copper impregnated sawdust	As(III)	>99%	Raji and Anirudhan (1998)
Rice husk	As(III)	71-96%	Mohan and Pittman (2007)
Wheat shell	Cu(II)	99%	Basci et al. (2003)
Carbonized corn pith	Cu(II)	90%	
Pinus radiata bark	Multiple metals	>50%	Palma et al. (2003)
Mango saw dust	Cu(II)	60%	Ajmal et al. (2005)
Activated parthenium carbon	Hg(II), Cr(VI), Fe(II)	Significant removal	Rajeshwarisivaraj and Subburam (2002)

Activated carbons are the most widely used adsorbents due to their excellent adsorption abilities for organic pollutants. The high adsorption capacities of activated carbons are usually related to their high-surface-area, pore volume, and porosity. Its high initial cost and the need for a costly regeneration system make it less economically viable as an adsorbent. Taking these criteria into consideration, the search for a low cost and easily available adsorbent has led many investigators to search more economic and efficient techniques to use agricultural waste origin (Ahmaruzzaman, 2008).

Raw agricultural solid wastes and waste materials from forest industries such as sawdust, rice husk, and bark etc. have been used as adsorbents.

Sawdust, an abundant by-product of the wood industry, with various organic compounds (lignin, cellulose, hemicelluloses, polyphenolic groups which are useful for binding phenolic compounds through different mechanism), has proved to be a promising material for the removal of phenols from wastewater. Nenkova and Redev (2004) showed that wood sawdust, and barks and technical hydrolysis lignin (THL) can be used as good adsorbents for the removal of dissolved phenols from waters. The adsorption ability of investigated adsorbents decreases in the following order: sawdust >wood > barks >THL. The adsorption of phenol on sawdust, polymerized sawdust and sawdust carbon was also studied by Jadhav and Vanjara (2004).

Bark, a polyphenol-rich material from the timber industry has been found to be effective in removing phenols from water solutions. Vasquez et al. (2007) have studied the adsorption of phenols on *Pinus pinaster* bark. The influence of variables such as solid/liquid ratio, pH and initial concentration of phenol

in the solution on the adsorption capacity of the bark has been analyzed. Brás et al. (2005) studied the adsorption of pentachlorophenol on the pine bark. The PCP uptake by pine bark was found to be faster in the initial phase followed by a slower process. The neutral PCP species showed to have higher binding capacity to pine bark than the anionic PCP, which was reflected in a decrease in the distribution coefficient (K_d) of the linear adsorption isotherm with the increase of solution pH from 2 to 7.

Rice husk is another agricultural waste product which has the potential to be used as an adsorbent, since the main components of it are carbon and silica (15–22% SiO_2 in hydrated amorphous form like silica gel) (Khalid, 2000). The adsorption potential of chemically and thermally treated rice husk (RHT) for the removal of 2,4-dichlorophenol (DCP) from aqueous solutions has been investigated (Akhtar, 2006). Maximum adsorption was achieved for RHT. The adsorption capacity of RHT was found to be significantly higher than chemically treated rice husk (RHCT) and rice husk untreated (RHUT). Mahvi et al. (2004) in a comparative study showed that rice husk ash is more efficient than rice husk for phenol removal.

Other industrial waste products such as pith, date pits, wheat straw, and wood chips etc. have also been successfully employed for the removal of phenolic compounds from wastewater (Ahmaruzzaman, 2008).

4.5.5 Composting

The degradation of organic matter during composting can be viewed as a quick-motion film of the events leading to the mineralization of organic matter during humus formation and the humus cycle. During composting, both classic biochemical pathways of decomposition of organic molecules and the radical mediated reactions are operating. Owing to the high organic matter content of the compost, the elevated temperatures during its operation, and the high C/N ratio at the beginning of composting (which leads to autolysis cycles with liberation of intracellular peroxidases), a milieu is created in which at least part of the radical reactions induced by white-rot fungi are also operating. Even lignin, when artificially added to the composting process, is also degraded there (Hutterman and Majcherczyk, 1998). In these conditions along with the possibility to obtain the humus precursors, an alternative approach for bioremediation is the use of free radical-mediated reactions that are used by white-rot fungi and are in operation during composting, lignin biodegradation and in the humus dynamic of soils. Ligninolytic enzymes and mediators are active extracellularly, thus white-rot fungi may serve as better candidates for the bioremediation of highly apolar pollutants. If the conditions are not favourable for the spontaneous degradation of the pollutants, measures can be taken for the activation of the existing microflora. If the soil cannot be removed, the percolation with the oxygen and inorganic nutrient (nitrogen and phosphorus) or an electron acceptor (nitrate) can greatly accelerate the rate of toxicant

degradation. If the soil can be taken from the site, the simplest method available for activation of the indigenous microflora is the process of landfarming. Here the soil is formed in mounds that are turned over at certain intervals. During this process, the soil is aerated and necessary substrates can be added.

It must be considered, however, that with this treatment volatile compounds will be released into the air and any measured remediation may be only the result of transfer of this compound into the air. Another approach, using organisms that grow on the contaminants, is the use of specially cultivated bacteria. Thus, pentachlorophenol (PCP)-contaminated soil was remediated with a bacterial strain (*Pseudomonas*) that mineralizes approximately 75% of the PCP to CO_2, what means a relatively little residual toxicity in the soil as measured by seed germination and root elongation of some plant species and by toxicity to earthworms. These direct toxicity measures showed the bioremediated soil to be indistinguishable from clean reference soil. There was, however, a change in lignocellulose based respiration. The change in ability of the soil to process lignocellulose may possibly reflect a change in community composition of the fungi, which are the predominant organisms involved in lignocellulose degradation. It was observed that plant-microbe symbioses may be sensitive indicators of soil ecological disruption. *Rhizobium* nodule formation and vesicular-arbuscular mycorrhizal (VAM) fungal infection were affected in the remediated soil even though several other plant related toxicity measures (germination and root elongation) were unaffected. Rhizobial and VAM fungi plant/microbe symbioses are ecologically important partnerships; particularly in natural or low agronomic input ecosystems where they represent respectively, the major means of nitrogen and phosphorous inputs to vegetation in terrestrial ecosystems (Abaecherli and Popa, 2005).

The plant-microbes symbiosis may also be useful as indicators for the ecological effects and environmental safety of biotechnology products such as bioremediating agents.

Suitable bacterial strains are isolated by a series of enrichment cultures, grown in a cheap food base, such as wheat bran, and added to contaminated soils. All bacterial processes have in common that the organisms use the xenobiotic substances as a substrate for the generation of energy and biomass production. A consortium of microorganisms involved in the soil formation, commercially available could be composed of:

Fungi:

Brown rot fungi – e.g. *Trichoderma reesei, Trichoderma harzianum* (fungi decompose plant material to glucose-carbon source)

White rot fungi – e.g. *Phanerochaete chrysosporium* (fungi decompose very quickly the organic matter and are specific for lignin)

Mycorrhizae fungi – soil may be improved with these fungi which prevent soil compactness, enable water to percolate in the soil, absorb water near the

soil's surface, stabilize soil and inhibit erosion. Mycorrhizae fungi help plants to acquire their nutrients

Bacteria:

Athrobacter globuliformis, Streptomycetes baduiis (these bacteria live in close proximity with fungi and they utilize the decomposition by-products and convert them to carbon and nitrogen compounds)

Nitrogen fixing bacteria: Azotobacter vinelandii, Rhizobium sp. (They use the gaseous nitrogen from the atmosphere and convert it to ammonia compounds. These bacteria produce growth promoting hormones that increase root hair development, enabling the plants to accumulate a greater amount of nutrients).

Pseudomonas fluorescens which produce antibiotics that attack and kill pathogen agents.

4.6 Conclusions

The bioremediation processes could be developed using lignins and polyphenols existing in biomass or in isolated and modified form taking into account their participation in the humus formation. The reactions involved in natural systems of biomass transformation could have an important role in the transformation and sequestration of pollutants. It is possible to obtain some synergetic effects by a combination between biodegradation of components of biomass with plant cultivation.

The understanding of mechanisms participating in humus formation, microorganisms and plants metabolism can offer optimal conditions for bioremediation according to the nature of pollutants. Therefore, the study of the influence of chemical and physical structures of biomass along with biological transformation of its components (especially lignins and polyphenols) under soil conditions on bioremediation requires to be further investigated.

Cellulosic agricultural waste materials are an abundant source of significant metal biosorption. The functional groups are present in agricultural waste biomass due to their compositions or could be introduced by chemical modification (acetamido, alcoholic, carbonyl, phenolic, amido, amino, sulphydryl groups etc.). These groups have the ability to some extent to bind heavy metals by donation of an electron pair from these groups to form complexes with the metal ions in solution or chelates.

Biosorption is a relatively new process that has proven very promising in the removal of contaminants from aqueous effluents. The use of these low cost biosorbents is promoted since they are relatively cheap or of no cost, easily available, renewable and show high affinity for heavy metals.

Chemical modification improves the adsorption capacity and stability of biosorbents.

The process of biosorption requires further investigation in the direction of modelling, regeneration of biosorbent and recovery of metal ions and immobilization of the waste material for enhanced efficiency and recovery.

Toxic heavy metals such as Pb(II), Cd(II), Hg(II), Cu(II), Ni(II), Cr(III), and Cr(VI), as well as some elements from lanthanide and actinides groups, but also phenolic compounds have been successfully removed from contaminated industrial and municipal waste waters using different agro waste materials.

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5

Bioremediation Technology for Hazardous Wastes – Recent Advances

M.H. Fulekar

5.1 Introduction

Hazardous waste: A substance which has no further economic use and is disposed off on to land, water or air which might be potentially harmful to man and his environment, by reason of its physico-chemical, biological properties. Environment (Protection) Act 1986 under the Hazardous Waste (Management and Handling) Rule (1989) describes about the hazardous waste and state to ensure that hazardous waste are managed in a manner which will protect human health and environment against the adverse effects which may result from such wastes.

The global society in the twenty-first century is facing the challenge of improving the quality of air, water and soil environment and maintaining the ecological balance. India today enjoys the honour of being among the 10 most industrialized country in the world. The rapid growth of industrialization, urbanization, modern agricultural development and energy generation resulted into exploitation of natural resources for fulfilling the human desires and need which have contributed in disturbing the ecological balance on which depends the quality of our environment. Human beings in true sense are the product of their environment. Man-environment relationship indicates that pollution has a social origin. The modern technological advancement in chemical processes has increased the pollution level above the self-cleaning capacities of the environment. One of the major issues in recent times is the threat to human life caused due to the progressive deterioration of the environment. Technological

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revolution has brought new changes in products and processes in industries. The waste generated from the development of products and processes are of concern to the environmentalists. The present treatment technologies are not sufficient and/or effective to treat the contaminants to the acceptable level as per the Environmental Protection Acts. The accelerated growth of industrial activities has increased the hazardous wastes. The main sources of the hazardous contaminants are generated by the chemical industries. Chemical industry plays a vital role in economic growth and development of manufacturing sector of a developing country (Fulekar, 2009).

Rapid industrialization and urbanization over the past many decades have resulted in contamination of all the components of the environment i.e. the air, water, soils and even our food. The contamination of groundwater, surface water, soil and air with hazardous and toxic chemicals is one of the major problems the industrialized world faces today. As far as the solution to this problem is concerned, there is urgent need to develop technologies that consume fewer resources, less time and would be environment-friendly. Therefore, biological approaches received great deal of attention in the recent years. One among the effective biological approaches to deal with the environmental contamination is bioremediation. As is clear from the word itself 'bioremediation' (bio + remediation) should involve two components: the bio i.e. the live component and remediation i.e. the treatment of the contaminant. Bioremediation can be defined as any process that uses microorganisms, fungi, green plants or their enzymes to return the natural environment altered by contaminants to its original condition. Around the world bioremediation technologies are categorized as the "innovative technologies". However, this is not a new phenomenon. Actually bioremediation has been going on since life began on this planet. It is a relatively slow process, but eventually nature has healed itself of all the disturbances.

As a contaminant is introduced into the environment, the microbes of the surrounding area get gradually adapted to this changed environment. They begin to elaborate the process of degrading the contaminant by evolving the ability to use it as a carbon or energy source. During this natural process, the nature fulfills the nutritional and physiological needs of the bacteria and the overall process is thus quite slow. With the fast industrialization and urbanization of the world, we have introduced the contaminants at very high rate and now we need instant cleanup solution that is not within the scope of the natural processes. Bioremediation is an attractive and potential alternative for treatment of contaminated environment. Bioremediation has been proved effective for treating soil and groundwater contamination at numerous sites throughout the world, and is accepted as a viable remediation technology by the United States Environmental Protection Agency, Environment Canada, and other regulatory agencies worldwide. Bioremediation has numerous applications including clean-up of ground water, soils, lagoons, sludges, and process-waste streams. Bioremediation is more cost-effective than conventional physical and chemical

processes. The principal advantages include the ability to eliminate pollutants, to harness natural biogeochemical processes, cost-effective alternative, less energy requirement when compared with other technologies, less manual supervision and often transforming them into innocuous by-products such as CO_2, nutrient and biomass.

The innovative techniques for bioremediation of hazardous waste have been developed. The pilot scale research studies incorporating use of a novel source of microbial consortium for bioremediation of hazardous waste compounds, both organics and inorganics in the designed and developed bioreactors, have been carried out.

5.2 Bioremediation of Pesticides in Surface Soil Treatment Bioreactor

The manufacture and use of pesticide has been rising with increasing pest problems in agriculture. The World Health Organization (WHO) data show that only 2-3% of applied chemical pesticides are effectively used for preventing, controlling and mitigating the pests while the rest remains in the soil (EPA, 2005). As a result, pesticide residue remains in the soil-water environment causing toxicity to the biota and thereby entering into the food chain (CFTRI, 2003).

The waste generated in the pesticide industry has become an environmental problem due to the present insufficient and ineffective waste treatment technology involving physico-chemical and biological treatment. The recent advances in bioremediation technology using microbial consortium have been found effective for treatment of pesticides in soil-water environment (Geetha and Fulekar, 2008). A surface soil treatment bioreactor has been designed and developed (Fig. 5.1) by author wherein bioremediation of commonly used pesticides namely chlorpyrifos, cypermethrin, fenvalerate and trichlopyr, butoxy ethyl ester at varying concentrations viz. 20, 50 and 100 mg/kg have been carried out using cow-dung microbial consortia under simulated environmental conditions.

The bioremediation conditions have been monitored and maintained during the study. The investigation has been extended till the parent compounds are converted into intermediates/less harmful compounds and/or environment-friendly compounds on long term acclimatization. The results presented here highlight the potential of cow-dung microbial consortia for bioremediation of soil contaminated with pesticides in surface soil treatment bioreactor.

The cow-dung was characterized for physico-chemical and microbial parameters. The data indicates the presence of organic carbon, nitrogen, phosphorus, sulphate, calcium chloride, sodium, potassium and magnesium in sufficient quantities that provide macro and micro nutrients for proliferation of microbial consortium. The microbial consortium assessed in cow-dung include: *Pseudomonas pseudoalcaligenes* strain MHF ENV 11, *Owenweeksia*

hongkongensis strain MHF ENV 10, *Alcaligenes* sp. MHF ENV 9, *Proteobacterium* MHF ENV 7, *Flavobacterium* sp. MHF ENV 5, *Pseudomonas plecoglossicida* strain MHF ENV-1, *Pseudomonas putida* strain MHF 7109, *Bacillus acidicola* MHF ENV 8, *Alcaligenes faecalis* subsp. *Parafaecalis* MHF ENV 8, *Xanthomonas theicola* MHF ENV 10, and *Providenciastuarti* MHF ENV 10 (Table 5.1).

The presence of nutrients as well as micro-organisms in cow-dung and soil has been found to have great influence on the bioremediation of pesticides.

Table 5.1: Microbial composition of cow dung and their genbank accession number

No.	*Bacterial strain*	*Genbank accession number*
1	*Alcaligenes* sp. MHF ENV 9	GU055763
2	*Proteobacterium* MHF ENV 7	GU055762
3	*Flavobacterium* sp. MHF ENV 5	GU055761
4	*Pseudomonas pseudoalcaligenes* strain MHF ENV 11	GU055765
5	*Owenweeksia hongkongensis* strain MHF ENV 10	GU055764
6	*Pseudomonas plecoglossicida* strain MHF ENV-1	GQ301534
7	*Pseudomonas putida* strain MHF 7109	FJ975149
8	*Alcaligenes faecalis* subsp. *parafaecalis* clone MHF ENV 8	GU183257
9	*Bacillus acidicola* clone MHF ENV 8	GU183258
10	*Providencia stuartii* clone MHF ENV 10	GU183259
11	*Xanthomonas theicola* clone MHF ENV 10	GU183260

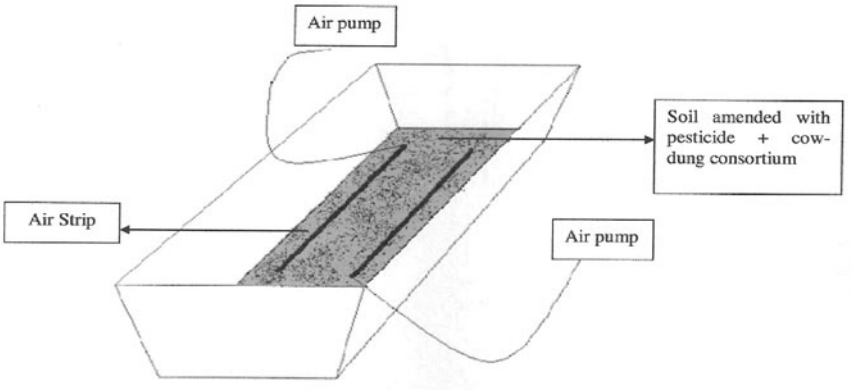


Fig. 5.1: Surface soil treatment bioreactor.

5.2.1 Chlorpyrifos

The concentration of chlorpyrifos and its intermediates during the bioremediation of 25, 50 and 100 mg/kg chlorpyrifos amended soil was estimated and is presented in Fig. 5.2. The analyses carried out on GC-MS showed that chlorpyrifos was rapidly hydrolyzed to 3,5,6 trichloro- 2-pyridinol (TCP) in 25 and 50 mg/kg chlorpyrifos amended soil while in 100 mg/kg chlorpyrifos amended soil it was present till the 3rd day of the experiment. Residue analyses showed that the most persistent intermediates extracted were benzyl pyridine and TCP. In bioreactor containing 25 mg/kg chlorpyrifos spiked soil, during the eight treatment days, TCP was detected in soil for four days and benzyl pyridine for six days and then potentially further metabolized into other simpler compounds. In the case of 50 mg/kg chlorpyrifos amended soil, the study showed that TCP was detected in the soil for a period of six days and very low concentrations of benzyl pyridine were found in the soil till the 5th day. In the case of 100 mg/L chlorpyrifos amended soil, the data indicates that both TCP and benzyl pyridine were present in the soil till the end of the experimental study (Geetha and Fulekar, 2008).

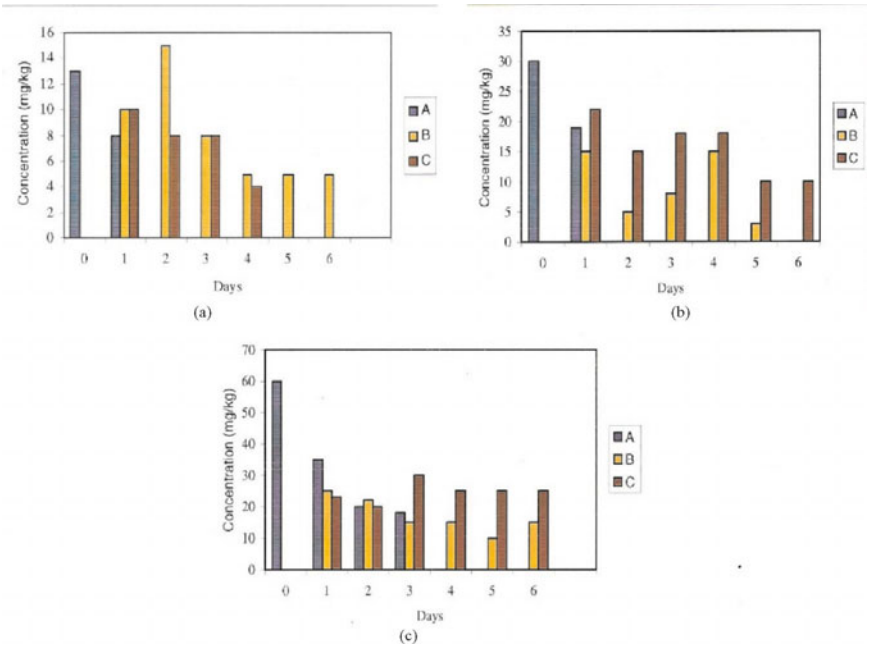


Fig. 5.2: Concentration of intermediates found during the bioremediation of chlorpyrifos amended soil. (a) 25 mg/L chlorpyrifos amended soil, (b) 50 mg/L chlorpyrifos amended soil and (c) 100 mg/L chlorpyrifos amended soil where A = Chlorpyrifos, B = Benzyl pyridine and C = TCP.
Values are expressed as mean ± SD of experiments in triplicate.

5.2.2 Cypermethrin

The bioremediation of Cypermethrin at the three concentrations i.e. 25, 50 and 100 mg/kg in soil treated with activated cow-dung slurry is presented in Fig. 5.3. The quantitative and qualitative analysis carried out on GC-MS showed that cypermethrin was hydrolyzed to 3-phenoxy benzaldehyde and 3-phenoxy-benzyl alcohol during the period of bioremediation in Surface Soil Treatment Bioreactor.

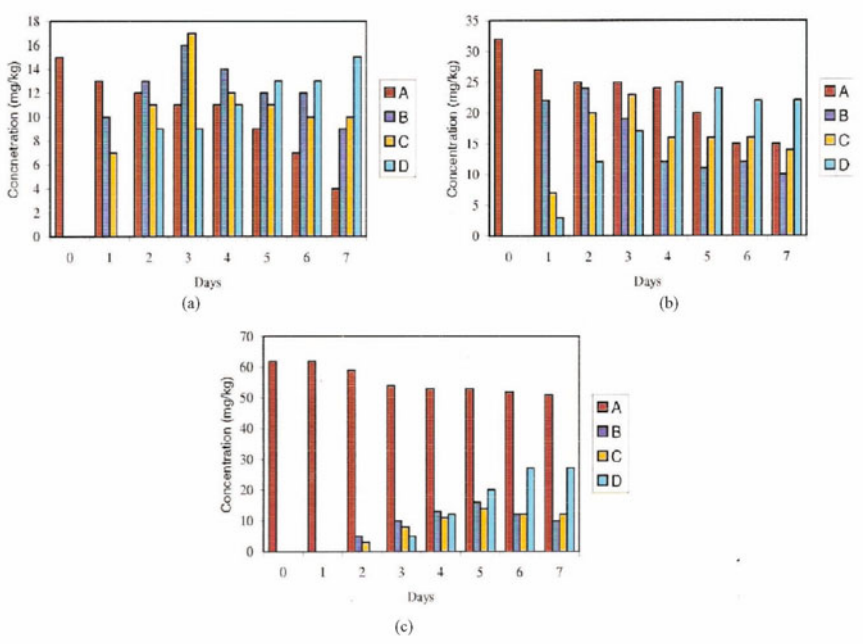


Fig. 5.3: Concentration of intermediates found during the bioremediation of cypermethrin amended soil. (a) 25 mg/L cypermethrin amended soil, (b) 50 mg/L cypermethrin amended soil and (c) 100 mg/L cypermethrin amended soil where A = cypermethrin, B = 3-phenoxy benzaldehyde and C = 3-phenoxy benzyl alcohol and D = 3-phenoxy-benzoic acid. Values are expressed as mean ± SD of experiments in triplicate.

5.2.3 Fenvalerate

The biodegradation of fenvalerate at concentration of 25, 50 and 100 mg/kg amended soil, and its intermediate metabolites are presented in Fig. 5.4. The compounds such as 4-chloro-alpha (1-methylethyl) benzene acetic acid and alpha-cyano-3-phenoxybenzyl alcohol were found to be the principal intermediates of fenvalerate degradation. After duration of one week, at 100 mg/kg concentration, fenvalerate was still detected in the soil. However, at 50 and 25 mg/kg, fenvalerate was found completely metabolised into its intermediates by the action of cow-dung microorganisms.

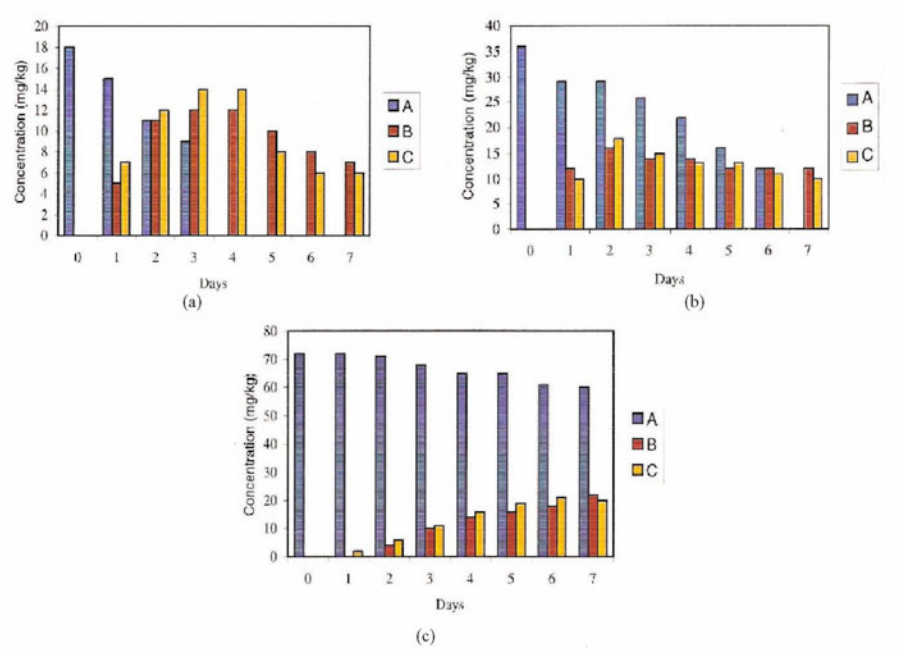


Fig. 5.4: Concentration of intermediates found during the bioremediation of Fenvalerate amended soil. (a) 25 mg/L Fenvalerate amended soil, (b) 50 mg/L Fenvalerate amended soil and (c) 100 mg/L Fenvalerate amended soil where A = fenvalerate, B = 4 chloro-alpha (t-methylethyl) benzene acetic acid and C = alpha-cyano-3-phenoxybenzyl alcohol.
Values are expressed as mean ± SD of experiments in triplicate.

5.2.4 Trichlopyr Butoxyethyl Ester (TBEE)

The concentration of TBEE and its intermediates during the course of bioremediation of TBEE contaminated surface soil at 25, 50 and 100 mg/kg were studied. It is evident from the GC-MS data that TBEE was rapidly broken down into trichlopyr acid via hydrolysis of the ester functional moiety (Fig. 5.5). The compounds trichlopyr acid and 3,5,6-trichloro pyridinol were found to be the principal metabolites of TBEE biodegradation. In the bioreactor containing 25, 50 and 100 mg/kg TBEE contaminated soil respectively, results suggest that; TBEE has been converted into trichlopyr acid within 24 hours. In 100 mg/kg TBEE contaminated soil trichlopyr acid and 3,5,6 trichloropyridinol (TCP) were found throughout the eight days of the experiment.

The bioremediation conditions—pH (6.5-8.0), C:N:P ratio (100:10:1), DO (10-12 mg/L), moisture (60-80%) and temperature (25-28°C)—have been monitored and maintained during the bioremediation of each pesticide at varying concentrations. The higher nutrient availability and larger microbial population of the cow-dung and soil was found to influence bioremediation of pesticides under controlled environmental conditions. Research studies

compiled and demonstrated showed that adaptability of micro-organisms during bioremediation releases enzymes which metabolizes wide spectrum of toxic chemicals (Fulekar, 2005). The present surface soil treatment technique used for bioremediation of pesticide using activated cow-dung and soil micro-flora would be an effective treatment technology for pesticides.

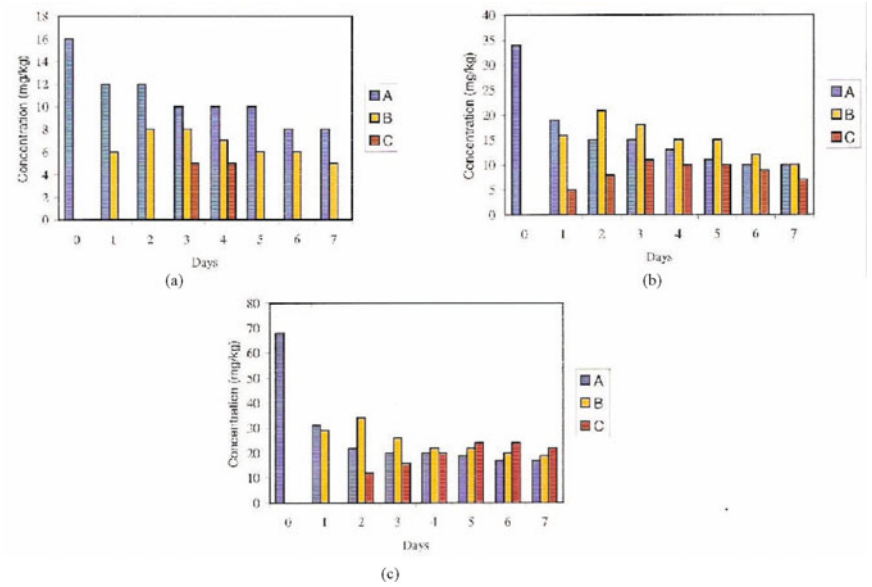


Fig. 5.5: Concentration of intermediates found during the bioremediation of TBEE amended soil. (a) 25 mg/L TBEE amended soil, (b) 50 mg/L TBEE amended soil and (c) 100 mg/L TBEE amended soil where A = TBEE, B = Trichlopyr acid and C = TCP. Values are expressed as mean ± SD of experiments in triplicate.

5.3 Bioremediation of Pesticide using Novel Cow-dung Microbial Consortium

5.3.1 Sequence Biological Reactor

Sequence Biological Reactor (SBR) has been designed and fabricated to study the bioremediation of simulated chlorpyrifos effluent. The SBR consists of an activation reactor, soil reactor and immobilized biomass reactor. The activation reactor (1st reactor) consists of a glass vessel (8″L × 6″B × 7″H) wherein the simulated effluent 500 mg/L of chlorpyrifos was taken and cow-dung slurry as a source of microbial consortium was added for bioremediation. The first reactor was connected to the soil reactor i.e. 2nd reactor (9″L × 7″B × 13″H) wherein gravel, sand and soil were layered one above the other on the perforated plate at the bottom. The air-dried soil was ground and passed through a 2 mm pore size sieve, used for the experiment. Gravels initially washed were packed at the bottom above the perforated plate in the soil reactor, which attained 5″ height from the bottom. Similarly sand, initially washed several times, was packed

above the gravel, which also attained a 5″ height above the gravel layer. Four kg of measured soil sample was packed above sand-gravel layer which attained a 12″ height above the sand layer. Initially, water was introduced from the top of the soil reactor; as soon as the soil surface became slightly saturated, the inflow was stopped. The soil was allowed to equilibrate for two days. The soil reactor (2nd reactor) was further connected to the immobilized biomass reactor i.e 3rd reactor (9″L × 7″B × 4″H); wherein the effluent was trickled down from soil-sand-gravel layer at the bottom of the collector. A photograph of Sequence Biological Reactor (SBR) is shown in Fig. 5.6.

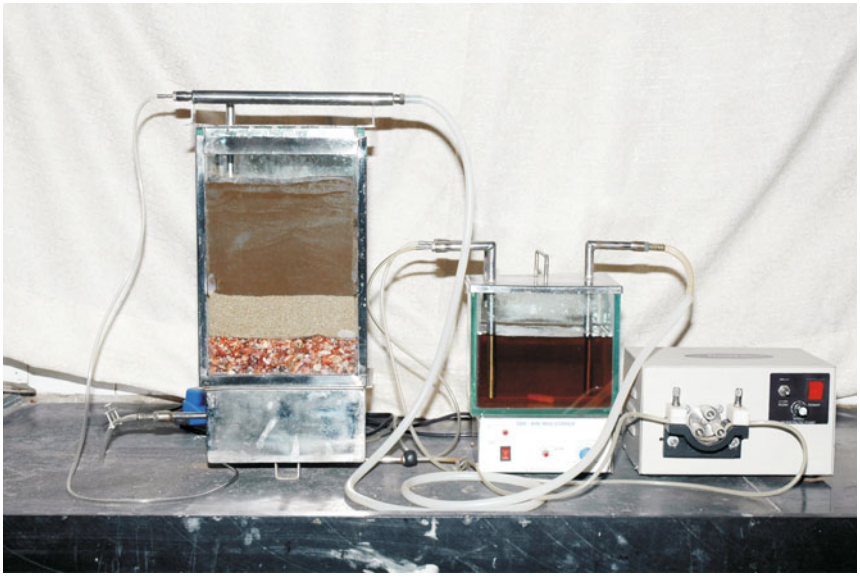


Fig. 5.6: Sequence Biological Reactor in the laboratory (A) Activation reactor, (B) Soil reactor, (C) Immobilized biomass reactor and (D) Peristaltic pump.

5.3.2 Experimental Set Up

Bioremediation of simulated effluent with 500 mg/L chlorpyrifos was studied in Sequence Biological Reactor at three stages viz. activation reactor (first reactor), soil reactor (second reactor) and immobilized biomass reactor (third reactor). In the first reactor simulated chlorpyrifos effluent was mixed with cow dung slurry in the ratio of 1:4. The slurry was allowed to react with chlorpyrifos effluent for a period of 12 hrs for initial bioremediation. The aerobic condition was maintained by supplying symmetric air 0.9-1.2 m^3/h and frequent mechanical stirring. The effluent slurry was then transferred to soil reactor (second reactor) using peristaltic pump. The effluent was found remaining in contact with soil media for a period of 12 hrs during trickling. The pesticide effluent was biodegraded in soil media under the influence of cow dung-soil microflora like trickling filter technique. The infiltrate was collected

in the third reactor i.e. immobilized biomass reactor where the bioremediation was further carried out using immobilized cow dung biomass. After completion of one cycle, the influent was again recycled using peristaltic pump to the first reactor for further treatment till chlorpyrifos effluent was biodegraded into its less toxic or harmless compounds. The bioremediation study was carried out for a total of 10 cycles over a period 21 days in SBR. The samples were drawn from activation reactor, soil reactor and immobilized biomass reactor after every 12 hrs. Samples were stored in glass vials under refrigeration. The extraction of chlorpyrifos and its intermediates from effluent samples was done by liquid partition method. Effluent extracts were analyzed using gas chromatography/mass spectroscopy (GC-MS) (Hewlett Packard GC-MS instrument Model No. G1800A) for chlorpyrifos and its intermediates.

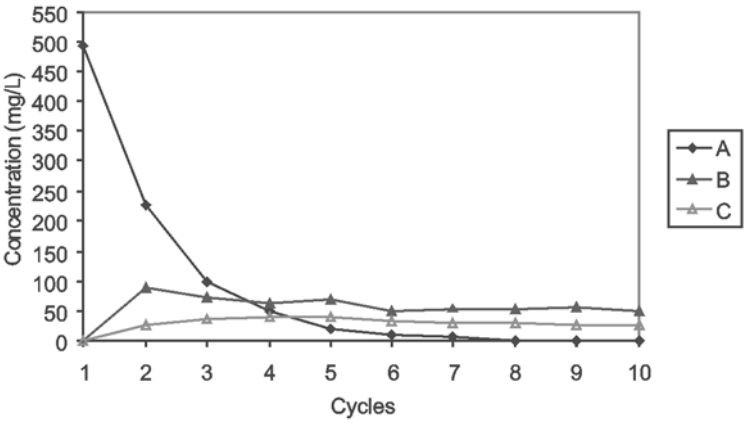
5.3.3 Bioremediation of Chlorpyrifos in SBR: Research Findings

5.3.3.1 *Simulated Effluent in SBR*

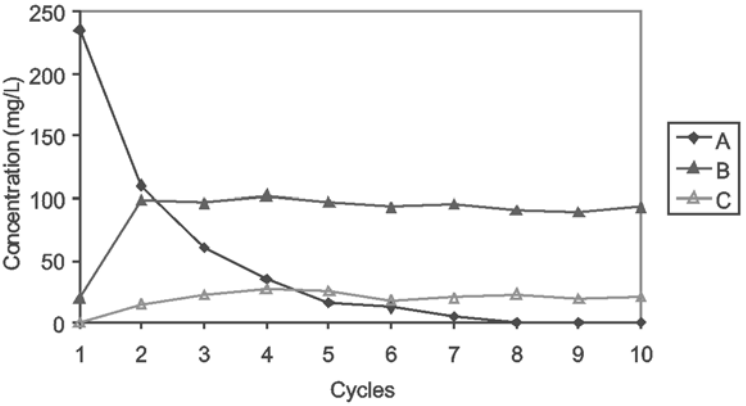
As stated earlier a sequence biological reactor (SBR), designed and developed to carry out the bioremediation of simulated chlorpyrifos effluent, has three stages viz. interaction of chlorpyrifos effluent with cow-dung slurry microbial consortium in the activation reactor (first reactor); followed by effluent infiltration through soil, sand and gravel like a trickling filter, in soil reactor (second reactor) and further interaction with the cow-dung immobilized biomass reactor (third reactor). The interaction of microbial consortium with chlorpyrifos effluent was allowed for a period of 12 hrs in each reactor and thereafter the recycling of the effluent was continued over a period of 12 days. A total of 10 treatment cycles were carried out during the period of bioremediation study in SBR.

5.3.3.2 *Activation Reactor*

Figure 5.7 shows that chlorpyrifos simulated effluent was degraded from 494 mg/L to 98 mg/L during subsequent treatment cycles and was further degraded to 10 mg/L on the sixth effluent recycling. Thereafter, the concentration of the chlorpyrifos was found negligible in activation reactor over a period of 21 days. The compounds such as TCP and 2,4-BIS (1,1-dimethylethyl) phenol were found as the prominent intermediates during the bioremediation of simulated chlorpyrifos effluent. (Fig. 5.7). The intermediate TCP was found at a concentration of 88 mg/L during the second treatment cycle, which subsequently decreased to a concentration of 50 mg/L during the course of experiment over a period of 21 days i.e. within 10 cycles. Another intermediate 2,4-bis (1,1-dimethylethyl) phenol was detected at a concentration of 27 mg/L initially, which gradually decreased during the bioremediation study over a period of 10 cycles (Fig. 5.7).


Concentration (mg/L)
550
500
450
400
350
300
250
200
150
100
50
0
1 2 3 4 5 6 7 8 9 10
Cycles
A
B
C

(a)


Concentration (mg/L)
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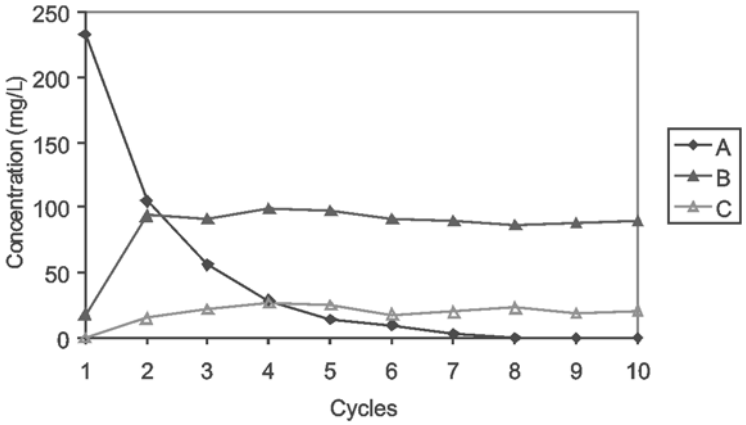
Concentration (mg/L)
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1 2 3 4 5 6 7 8 9 10
Cycles
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C

(c)

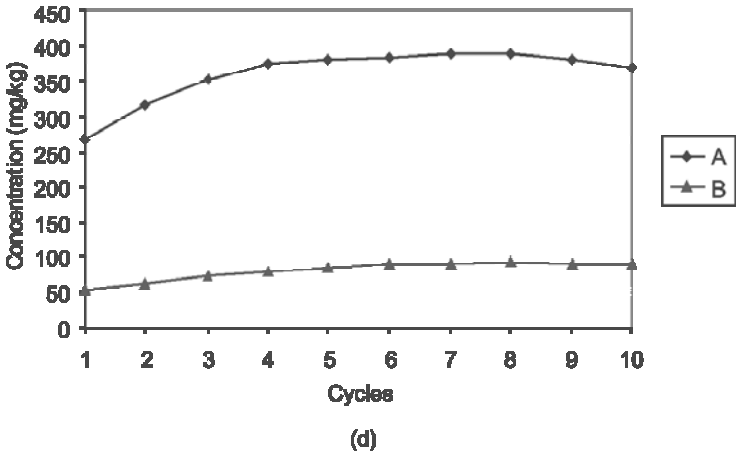

Fig. 5.7: Bioremediation of chlorpyrifos in Sequence Biological Reactor: (a) Activation reactor, (b) Soil infiltrate, (c) Immobilized biomass reactor and (d) Soil. A = Chlorpyrifos, B = TCP and C = 2, 4-bis (1, 1-dimethylethyl) phenol.

5.3.3.3 *Soil Infiltrate*

The GC-MS quantitative and qualitative analysis of soil infiltrate illustrates presence of parent compound chlorpyrifos during the bioremediation study. The concentration of chlorpyrifos recorded at 235 mg/L in the soil infiltrate shows the presence of parent compound chlorpyrifos. The concentration of chlorpyrifos was recorded at 235 mg/L in the soil infiltrate during the first cycle of the study, which subsequently decreased to a concentration of 5 mg/L over a period of seven treatment cycles. During the later stages of the effluent bioremediation, chlorpyrifos was not detected in the soil infiltrate. In comparison to activation reactor, intermediates such as TCP were recorded at a higher concentration in the soil infiltrate (Fig. 5.7). The initial concentration of TCP was found to be 20 mg/L in the soil infiltrate, which eventually increased to a concentration of 102 mg/L in the fourth cycle of the bioremediation study and then gradually decreased to a concentration of 93 mg/L over a period of 10 treatment cycles. The intermediate 2,4-bis (1,1-dimethylethyl) phenol was found varying during the course of experiment carried over a period of 21 days till the completion of 10 cycles, within 21 days.

5.3.3.4 *Immobilized Biomass Reactor*

In immobilized biomass reactor, the soil infiltrated from the second reactor was collected and treated using immobilized activated cow-dung slurry biomass for a period of 12 hrs during each treatment cycle. Figure 5.7 shows that the concentration of chlorpyrifos was persisting over a period of seven cycles, after which the compound was unobserved. However, slight variations in the concentration of TCP and 2, 4-BIS (1,1-dimethylethyl) phenol were observed during the study in immobilized biomass reactor.

5.3.3.5 *Soil Reactor*

After the treatment for 12 hrs in activation reactor, the effluent was sprinkled onto soil-sand-gravel layer in soil reactor like a trickling filter. The concentration of chlorpyrifos and its intermediates in soil reactor were analyzed using GC-MS and presented in Fig. 5.8. The concentration of chlorpyrifos was found as 267 mg/kg in soil during the eighth cycle and thereafter persisted till the end of the study over a period of 10 treatment cycles within a period of 21 days. The compound TCP was found as the only intermediate of chlorpyrifos effluent degradation in soil (Fig. 5.8). The concentration of TCP was ranging from a concentration of 53 mg/kg to 96 mg/kg during the effluent bioremediation in the soil reactor of SBR. After the completion of bioremediation experiment in SBR, the experimental soil was kept aside in a separate bioreactor and was analyzed for further biodegradation of chlorpyrifos over a period of five weeks. Bioremediation conditions like moisture, aeration and microbial action was monitored and maintained in the experimental soil. The GC-MS data depicts considerable amount of decrease in chlorpyrifos concentration. The concentration of chlorpyrifos was observed to be 326 mg/kg during the first week, which rapidly decreased to a concentration of 81 mg/kg on the fifth week of chlorpyrifos effluent soil bioremediation. The intermediate TCP also decreased from a concentration of 110 mg/kg to a concentration of 64 mg/kg over a period of five weeks in the experimental soil.

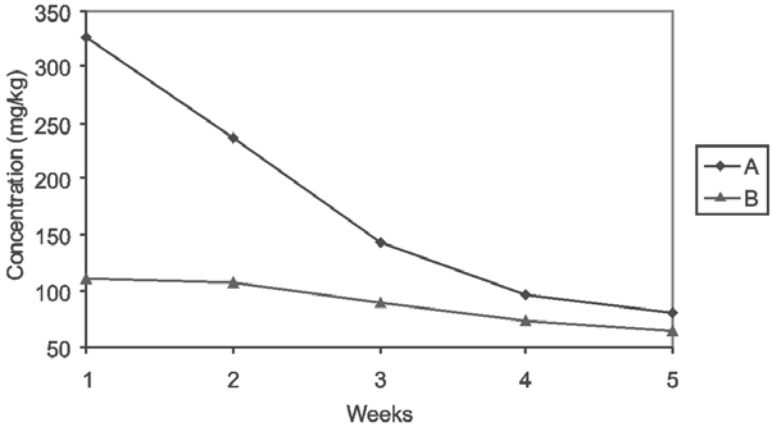

Fig. 5.8: Bioremediation of chlorpyrifos in the experimental soil:
A = Chlorpyrifos, B = TCP.

5.3.4 Bioremediation of Chlorpyrifos in SBR – Data Interpretation

The GC-MS analysis of the effluent sample from the activation reactor (first reactor) of the SBR shows reduction in the concentration of chlorpyrifos with increasing number of treatment cycles. The GC-MS analytical data illustrate

that chlorpyrifos has converted into intermediates such as TCP and 2,4-bis (1,1-dimethylethyl) phenol during the second cycle of bioremediation. Presence of TCP and 2,4-bis (1,1-dimethylethyl) phenol were observed throughout the study in activation reactor over a period of 10 cycles. However, percentage decrease in TCP in the activation reactor, during the period of study, was found to be 47.5% whereas percentage reduction in 2,4-bis(1,1-dimethylethyl) phenol was 14.8%.

The chlorpyrifos effluent passing through the soil reactor (second reactor) was collected at the bottom and analyzed for biodegradation of chlorpyrifos. The results show that, chlorpyrifos was persistent in the soil infiltrate over a period of seven cycles and then was subsequently biodegraded. Concentration of intermediates such as TCP was found at a much higher concentration in the soil infiltrate as compared to that of activation reactor. The soil infiltrate was further allowed to react with immobilized cow-dung consortium in immobilized biomass reactor. The concentrations of chlorpyrifos and its intermediates, after immobilized biomass treatment, were found to be more or less the same as that observed in the soil infiltrate. Significant biodegradation has not been observed during immobilized cow-dung biomass treatment, which is attributed to increasing concentration of primary metabolite TCP in the effluent.

The soil samples from the soil reactor of the SBR were also analyzed for chlorpyrifos biodegradation by the cumulative effect of cowdung-soil microbial consortium. Composite soil sampling was made and analyzed for chlorpyrifos and its intermediates using GC-MS. Microorganisms present in the cow-dung and soil have acted as a consortium for the bioremediation of chlorpyrifos effluent in the soil reactor of the SBR. The GC-MS data show that the concentration of chlorpyrifos was increasing along with the number of treatment cycles over a period of research study, which is attributed to the adsorption ability of the soil particles towards chlorpyrifos. Mechanism of adsorption of chlorpyrifos on soil particles depends on variety of variables such as cation exchange capacity (CEC) of soil, soil texture and organic carbon content of the soil (Christophersen et al., 1980). The primary intermediate TCP was found persisting in the soil during the course of experiment. However, the adsorption potential of soil particles towards TCP was low and the intermediate TCP was comparatively mobile in soil (US EPA, 1992). The percentage concentration increase of TCP in the experimental soil of the soil reactor was observed to be 73.5% over a period of 10 cycles.

Since the chlorpyrifos was found adsorbed to soil particles at a substantial concentration in soil reactor, the experimental soil was monitored separately in another bioreactor for further chlorpyrifos biodegradation using cowdung-soil microbial consortium over a period of five weeks, after the completion of SBR experiment. The GC-MS analytical data show reduction in the chlorpyrifos concentration during a period of five weeks. During this period, microbial conditions, aeration, mechanical stirring and other bioremediation conditions were monitored and maintained. The percentage reduction in chlorpyrifos was

found to be 75.1%, while percentage decrease of 41.8% was recorded in the case of TCP over a period of five weeks. The intermediates will eventually convert into nutrient, biomass and inorganic on sufficient acclimatization with microbial consortium.

The pilot scale research study carried out for bioremediation of pesticide effluent using Sequence Biological Reactor by Fulekar (2008) will be effectively applicable for treatment of pesticide waste/effluent generated by pesticide manufacturing industries.

5.4 Bioremediation of Petrochemical Waste-Phenol by Novel Partitioning Bioreactor

5.4.1 Petrochemical Industries Spectrum

The petrochemical waste generated from refineries and petrochemical industries contains complex organic and inorganic compounds. This industrial waste commonly comprises phenol, benzene, xylene, toluene, cresols, ethylene, naphthalene, paraffin, propylene etc. Phenol present in petrochemical waste is taken as a representative toxic contaminant for bioremediation. Bioremediation has become powerful tool for dealing with the high degree of environmental pollution. Bioremediation is receiving a great deal of attention due to its environmental friendly approach, low-cost and its ability to complete mineralization of toxic contaminants (Fulekar, 2005).

Bioremediation of phenol has been studied using activated cow-dung microbial consortium as a novel source of biomass in designed and developed bioreactor. In the Single Phase Bioreactor (SPB) phenol was bioremediated using activated cow-dung microbial consortium under controlled environmental conditions. In SPB, cow-dung microbial consortium remains under direct contact with phenol. The difficulty associated with direct contact of microorganisms and contaminant is that the initial substrate concentration must be lower than the value at which the organisms are inhibited. In this system, increasing the initial substrate concentration supply prolong the bioremediation process by increasing the duration of lag phase. To overcome this difficulty another experimental set up was using the same bioreactor as a Two Phase Partitioning Bioreactor (TPPB) in which organic waste such as phenol was taken into the organic phase (2-undecanone) which forms the upper layer and the aqueous phase at the bottom (Fig. 5.9). Cow-dung consortium was added to the aqueous phase served as a source of microbial biomass. The contaminants in the TPPB distribute between organic and aqueous phase and the microbial consortium can react at a sub-inhibitory level in aqueous phase and biodegrade the contaminant at higher concentration (Danghlis, 2005). The cow-dung microbial consortium as identified (Sr No. 5.2) contains a number of micro-organisms being used for phenol biodegradation in TPPB.

5.4.2 Design and Development of Bioreactor

Bioreactor has been designed and developed (glass and stainless steel; dimension: 20 cm × 20 cm × 25 cm) with a provision for supply of air (20 mg O_2/L) to maintain the aerobic condition. A stirrer was provided for agitation of biomass and to ensure constant contact between the micro-organism and contaminant. The bioreactor has a sampling port, both at aqueous and organic phase. In the bioreactor system vapourization can take place simultaneously with bioremediation. To avoid vapourization, condenser and cooling systems were installed to condense the vapour of the organic phase in TPPB.

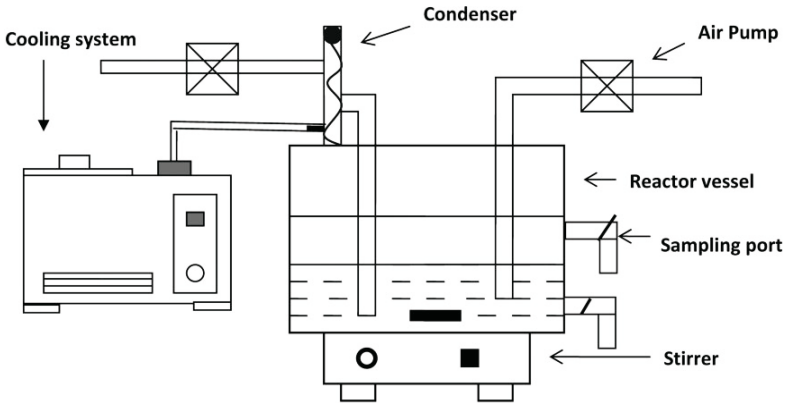

Fig. 5.9: Diagrammatic representation of the bioreactor (single phase and two-phase partitioning bioreactor).

5.4.3 Experimental setup in SPB and TPPB

Bioremediation of phenol was carried out in designed and developed Single Phase Bioreactor (SPB) and Two-Phase Partitioning Bioreactor (TPPB) using cow-dung microbial consortium as given below.

The physicochemical characteristics of activated cow-dung biomass (Table 5.2) show the presence of macro- and micro-nutrient such as carbon, nitrogen, phosphorus, sulfate, calcium, magnesium, and sodium. Initially, the potential of the cow-dung microbial consortium was assessed for degrading phenol using the SPB. In the SPB, the cow-dung microbial consortium remained in direct contact with the phenol (at varying concentration) and degraded phenol up to a concentration of 500 mg/L. In the bioremediation experiment, initial concentrations 100, 250, 500, 1000 mg/L phenol were used. The findings indicated that for 100 mg/L phenol concentration degradation started immediately, i.e., no lag phase was observed, and that 98.59% degradation occurred over a period of 24 h. Similarly, 250 and 500 mg/L were degraded up to 99.4% and 99.6% within 72 and 96 h, respectively. At these concentrations biodegradation started 2–3 h after the experimental setup. The concentration

of 1000 mg/L phenol was found to be inhibitory for the cow-dung microbial consortium, as it was not degraded within the 168 hrs of experimental period.

Table 5.2: Physicochemical characterization of activated cow-dung slurry

Physicochemical parameters	*Quantity*
pH	7.4
Dissolved oxygen	6.3 mg/L
Temperature	27.4°C
Organic carbon	0.31%
Phosphorus	0.13 mg/L
Kjeldahl nitrogen	14 mg/L
Sulfate	34 mg/L
Calcium	9.8 mg/L
Magnesium	127 mg/L
Potassium	159 mg/L
Sodium	90 mg/L
BOD	9.50 mg/L
COD	184 mg/L
Total CFU/mL	1.4×10^7

The degradation pattern of phenol with time in SPB by the cow-dung microbial consortium is presented in Fig. 5.10. The essential environmental parameters responsible for bioremediation were monitored throughout the experiment. The data show an increase in temperature from 25° to 28°C, indicating a bioremediation process. Decreases in pH (from 7.2 to 6.5) and DO (from 12 to 9 mg O_2/L) were observed during the bioremediation process. During bioremediation, the COD and BOD were monitored as indicator for bioremediation and microbial growth (Figs. 5.11 and 5.12). Figure 5.11 demonstrates the decrease in COD levels over a period of bioremediation, which indicates the degradation of phenol by microorganisms present in the cow-dung consortium. The decrease in BOD values (Fig 5.12) indicates the growth of microorganisms in the various concentrations of phenol. The present bioremediation study of phenol in SPB shows that the cow-dung microbial consortium is able to degrade the toxic contaminant below 1000 mg/L. Thus, this system is effective for degradation of lower concentrations of contaminant. To overcome this limitation in biodegrading higher concentration of contaminants, a TPPB was designed and developed in which 5000 mg/L phenol was dissolved into the organic phase, providing an initial concentration of 105 mg/L in the aqueous phase. 2-undecanone was selected as organic solvent as it has biocompatible properties. The biodegradability of 2-undecanone by cow-dung consortium was checked.

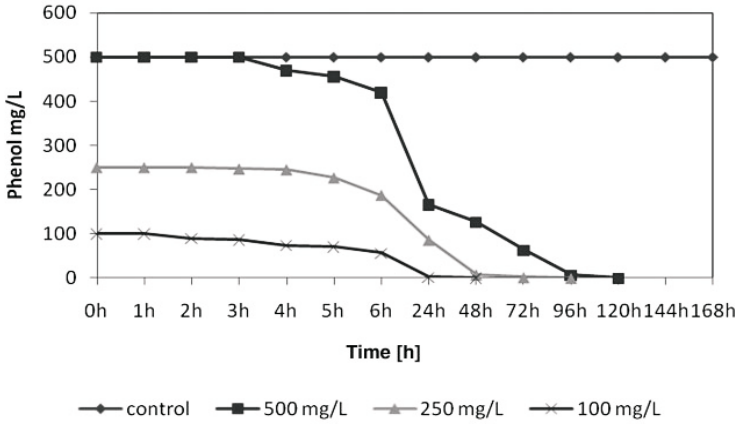

Fig. 5.10: Time course plot of phenol concentration during bioremediation by cow-dung consortium in the SPB.

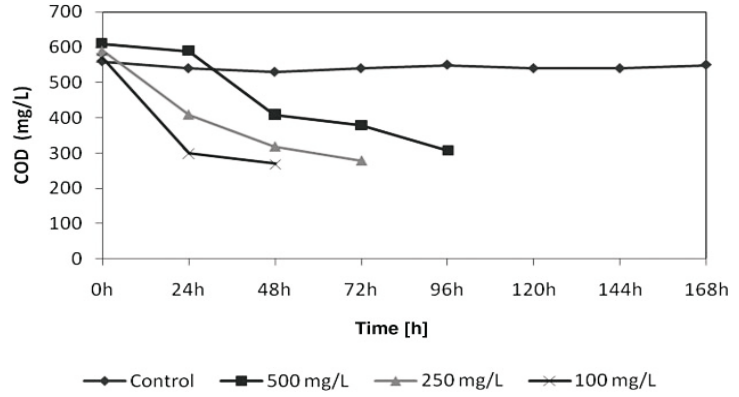

Fig. 5.11: Chemical oxygen demand (COD) variation during phenol bioremediation by cow-dung consortium in the SPB.

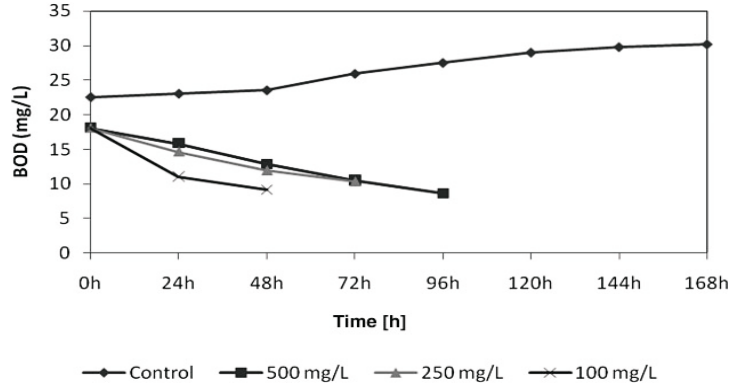

Fig. 5.12: Biological oxygen demand (BOD) variations during phenol bioremediation by cow-dung consortium in the single phase partitioning bioreactor.

It was found that the CFU/mL of cow-dung consortium did not increase as compared to positive control in presence of 2-undecanone. The concentration of phenol partitioned into aqueous phase was much below the inhibitory concentration of phenol observed using the SPB. As presented in Figs. 5.13 and 5.14, the system experienced a lag phase of 48 h after which biodegradation started. Over the 168-h period 54.18% of the phenol was degraded. CFU measurements (Fig. 5.15) indicate that growth and multiplication of microorganism started after 48 h. The number of microorganism increased until the end of the experiment. Thus, this concentration of phenol (5000 mg/L) was not inhibitory for microorganisms in the TPPB.

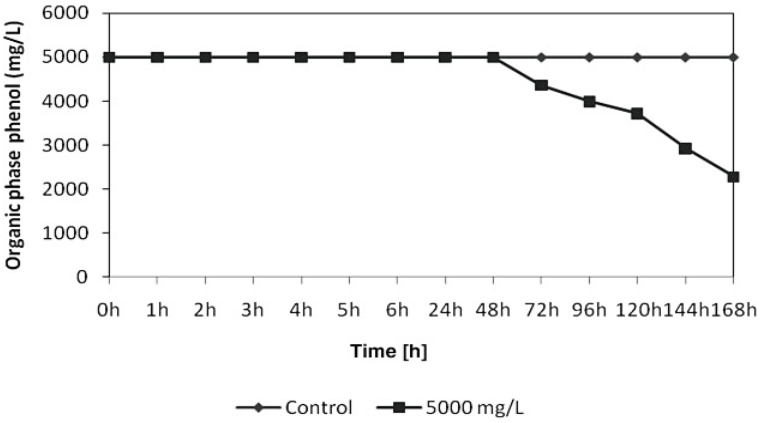

Fig. 5.13: Time course plot of phenol concentration in the organic phase during bioremediation by cow-dung consortium in the two-phase partitioning bioreactor.

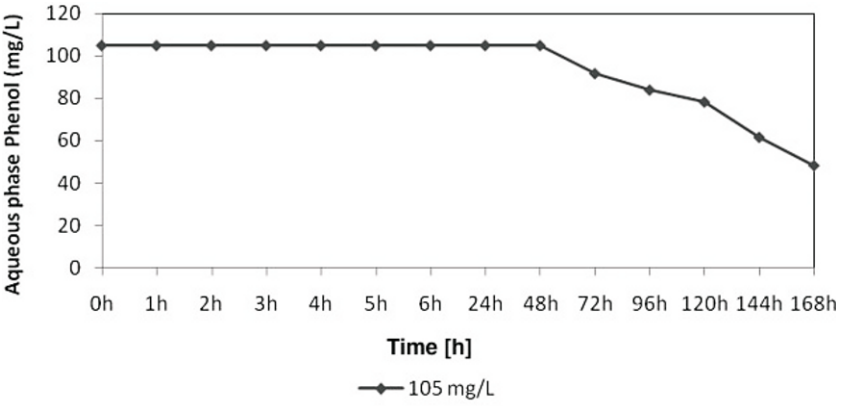

Fig. 5.14: Time course plot of phenol concentration in the aqueous phase during bioremediation by cow-dung consortium in the two-phase partitioning bioreactor.

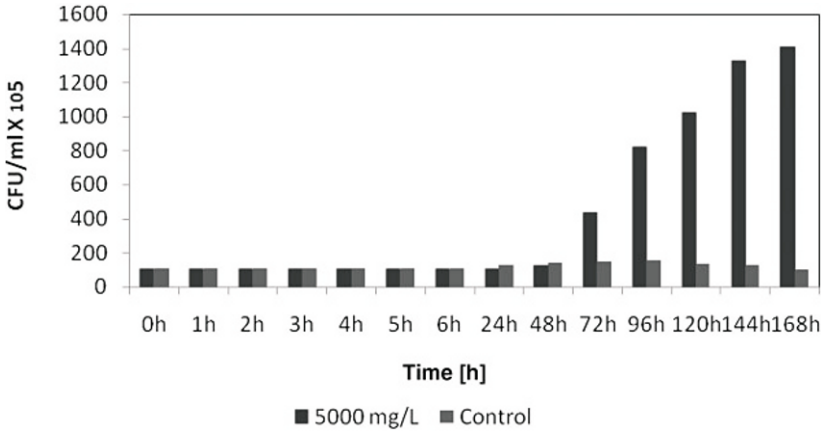

Fig. 5.15: Number of colony forming units (CFU/mL × 105) during bioremediation by cow-dung consortium in the two-phase partitioning bioreactor.

During bioremediation of phenol in the TPPB, the temperature increased from 26° to 28°C, indicating bioremediation. The pH was decreased from 7.4 to 6.1 and the DO from 12 to 7 mg O_2/L. The potential of cow-dung as a microbial consortium for biodegradation of phenol was assessed in both the SPB and the TPPB.

5.5 Bioremediation by identified Phenol Degrader – *Pseudomonas putida* IFO 14671

The most active and potent phenol degraders from cow-dung microbial consortium was isolated, based on bacterial colony that survived at higher concentration of phenol. The genomic DNA of these bacteria was isolated, and the 16S rDNA was amplified using universal primers. A 1.4-kb PCR product was obtained from the 16s rDNA. This product was sequenced and BLAST analysis was performed to identify the bacterium and its closest neighbours. To examine the phylogenetic relationships, a phylogenetic tree was drawn (Fig. 5.16). The BLAST analysis indicated that the bacteria were most similar to *Pseudomonas putida* IFO 14671. Therefore, the potential of *Pseudomonas putida* IFO 14671 towards the biodegradation of phenol was also evaluated. A bioremediation study of phenol (100, 250, 500 mg/L) by *Pseudomonas putida* IFO 14671 was carried out. The *Pseudomonas putida* IFO 14671 was found to biodegrade phenol at faster rate. The isolated *Pseudomonas putida* IFO 14671 strain started biodegradation of 100 mg/L phenol immediately and degraded it completely within 7 h. For 250 mg/L phenol, a lag phase of 30 min was observed, and 81.7% was degraded within 24 h. Similarly, 500 mg/L phenol was consumed completely within 48 h by *Pseudomonas putida* IFO 14671. A lag phase of 60 minutes was observed in this case (Fig. 5.17). The OD of the microbial culture at 600 nm was measured from 30 min up to 48 h (Fig. 5.18). The data indicate

the growth and development of the microorganism during the bioremediation of phenol. Table 5.3 shows the viable count of *Pseudomonas putida* IFO 14671 (in CFU/mL) and confirms the growth and proliferation of the microorganisms. The MS analysis of bioremediation samples showed the presence of catechol and cis 2-muconic semialdehyde during the bioremediation.

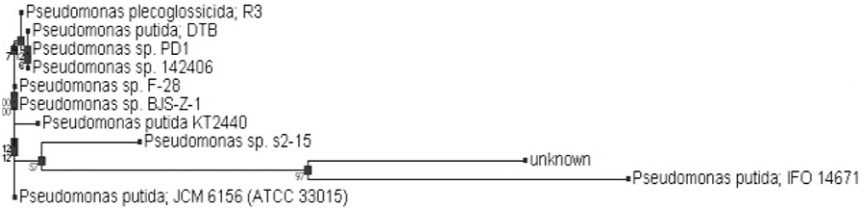

Fig. 5.16: Phylogenetic tree showing the genetic relatedness of bacteria isolated from cow-dung microbial consortium.

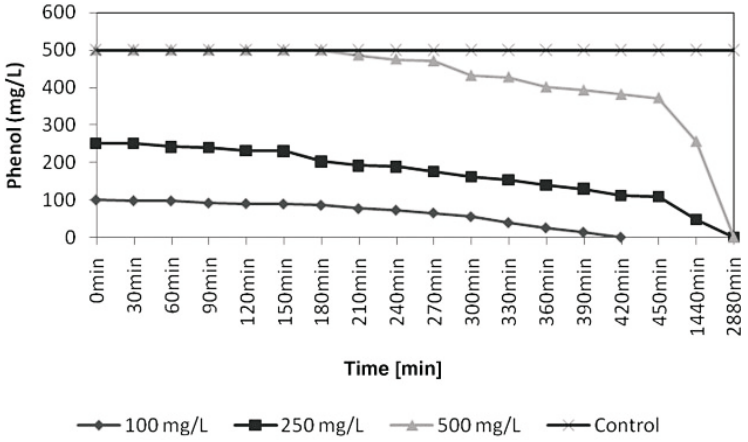

Fig. 5.17: Time course plot of phenol concentration during bioremediation by *Pseudomonas putida* IFO 14671.

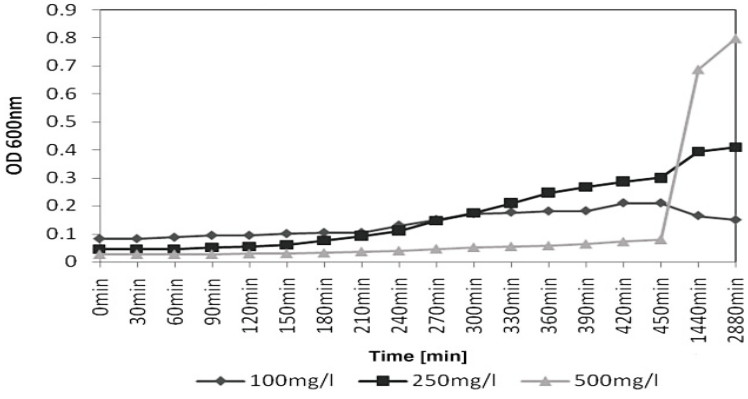

Fig. 5.18: Variation in optical density of the bacterial culture during bioremediation of phenol by *Pseudomonas putida* IFO 14671.

Table 5.3: Viable count (CFU/mL) during phenol degradation by *Pseudomonas putida* IFO 14671

Time (h) 100 mg/L	*250 mg/L*	*500 mg/L*
0 1.8×10^5	1×10^5	0.6×10^5
7 1.3×10^7	1.4×10^8	1.8×10^6
24 2.3×10^8	2.3×10^8	5.2×10^8
48 4.1×10^6	7.1×10^8	7.9×10^9

The present research has shown the advantage of the TPPB over the SPB, in which the microorganism comes into indirect contact with the contaminant, for biodegradation of higher concentrations of a hazardous compound. TPPB designed and developed for bioremediation has been found effective for degradation of hazardous compounds at higher concentration using cow-dung as a novel source of biomass. Further the identified microbial strain *Pseudomonas putida* IFO 14671 has been found to be a potential organism for enhancing bioremediation of hazardous compound generated by petrochemical industries (Singh and Fulekar, 2009). The study of this microorganism, in particular genomics and proteomics, would further provide molecular approaches for bioremediation. Therefore the present research study on bioremediation of hazardous compounds using the cow-dung consortium and *Pseudomonas putida* IFO 14671 as potential organism in TPPB has provided an innovative research in the area of bioremediation.

5.6 Rhizosphere Remediation of Chlorpyrifos in Pot Culture Technique

5.6.1 Ecological Remediation Unit

In the present research study, the rhizosphere of the host plant grown in a mycorrhiza-soil formed a mycorrhizosphere encompassing symbiotic association of mycorrhizal fungi, bacteria, actinomycetes in the immediate vicinity of the mycorrhizal roots (Fig. 5.19). In the plant-assisted bioremediation, rhizosphere helps in increase in soil organic carbon, bacteria and mycorrhizal fungi, all factors that encourage degradation of hazardous compounds such as organic compounds and pesticides in soil.

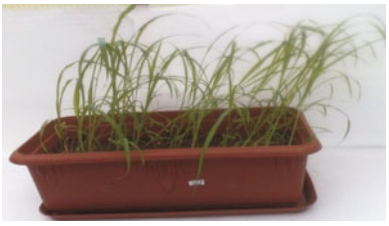

Fig. 5.19: Ecological remediation unit.

The study conducted observed that plants release exudates in soil that helps to stimulate the degradation of organic chemicals by inducing enzyme systems of existing bacterial populations, stimulating growth of the new species that are able to degrade waste, and/or increasing soluble substrate concentrations for all microorganisms. Environmental protection laboratory in Athens, Georgia, examined five plant enzymes systems which are Dehalogenase, Nitroreductase, Laccase, Peroxidase and Nitrilase in sediment and soils in the proximity to the root (1 mm). Dehalogenase enzymes are important in dechlorination reactions of chlorinated hydrocarbons, nitroreductase is needed in the first step for degradation of nitroaromatics, while laccase serves to break aromatic ring structures in organic contaminants. Peroxidase and nitrilase are important in oxidation reactions in rhizosphere soils.

5.6.2 Mycorrhizosphere Remediation of Pesticides

In the present research study, the potential of rye grass for rhizosphere bioremediation of chlorpyrifos in mycorrhizal soil was investigated in the green house using pot culture experiments. The pot cultured soil amended at initial chlorpyrifos concentration of 10 mg/kg was observed to be degraded completely within seven days where rest amended concentrations (25-100 mg/kg) decreased rapidly under the influence of rye grass micro-rhizosphere as incubation progressed till 28 days. The bioremediation of chlorpyrifos in soil is attributed to the micro-organisms associated with the roots in the rye grass rhizosphere. Therefore the micro-organisms surviving in the rhizospheric soil spiked at highest concentration (100 mg/kg) was assessed and used for isolation of chlorpyrifos degrading micro-organism. The potential degrader identified by 16s rDNA analysis using BLAST technique was *Pseudomonas nitroreductase* PS-2. Further bioaugmentation for the enhanced chlorpyrifos biodegradation was performed using PS-2 as an inoculum in the experimental setup similar to the earlier. The heterotrophic bacteria and fungi were also enumerated from the inoculated and non-inoculated rhizospheric soil. In bioaugmentation experiments, the percentage dissipation of chlorpyrifos was 100% in the inoculated rhizospheric soil as compared to 76.24, 50.36 and 90.80% in the non-inoculated soil for initial concentrations of 25, 50 and 100 mg/kg at the 14^{th}, 21^{st} and 28^{th} day intervals respectively.

Bioremediation of Chlorpyrifos in Rye Grass Mycorrhizosphere

Rhizosphere bioremediation of chlorpyrifos are represented in Fig. 5.20. Data indicate that chlorpyrifos rapidly dissipated in rhizosphere soils within first seven days and continued to degrade completely till the end of the experiment over the period of 28 days. The percentage dissipation of the chlorpyrifos in soil amended at varying concentrations (10–100 mg/kg) ranged from 58.73 to 100% at the first harvest period. However, at the end of the experiment, the lower concentrations (10–50 mg/kg) spiked in the soil were totally degraded and percentage degradation at higher concentrations (75 and 100 mg/kg) was

found to be 94.25 and 91.6% respectively. Figure 5.21 demonstrates the mass spectra of chlorpyrifos and its metabolite TCP. The research findings thus show that ryegrass is an able candidate which efficiently degrades chlorpyrifos in its root zone area. The grass varieties are reported to be the most suitable plant species for rhizosphere bioremediation of organic contaminants due to their ability to harbour large number of bacteria on their highly branched root system. In the rhizosphere, an increase in microbial density, diversity and metabolic activity is estimated to be effective due to release of plant root exudates, mucigel and root lysates (enzymes, amino acids, carbohydrates, low molecular mass carboxylic acids, flavonones and phenolics) (Kidd et al., 2008). These rhizo-deposits also stimulate the survival and action of bacteria which subsequently results in efficient degradation of pollutants (Kuiper et al., 2001). In present research study, the chosen plant species, ryegrass, proved competent for rhizosphere bioremediation of chlorpyrifos in the soil which may be credited to its advantageous features of having fibrous root system providing large specific surface area to interact with microorganism (Dzantor et al., 2000) and capacity to release high amount of exudates in the rhizosphere (Meharg and Killham, 1990). The study conducted by Korade and Fulekar (2008) has also found that ryegrass can effectively be used for the remediation of anthracene in the soil.

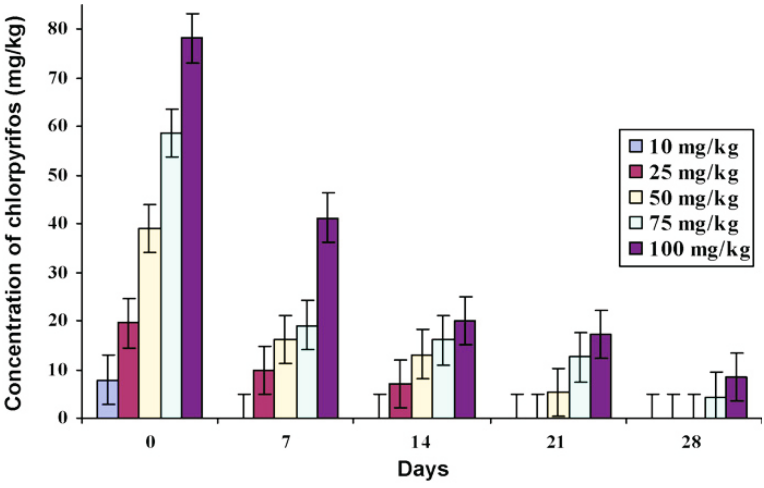

Fig. 5.20: Dissipation of chlorpyrifos in soil (Rhizosphere Bioremediation Expt.).

5.6.3 Isolation and Identification of Chlorpyrifos Degrading Microorganism

The experiment carried out in previous set of experiment proved the effectiveness of the rye grass plant in bioremediation of chlorpyrifos from soil.

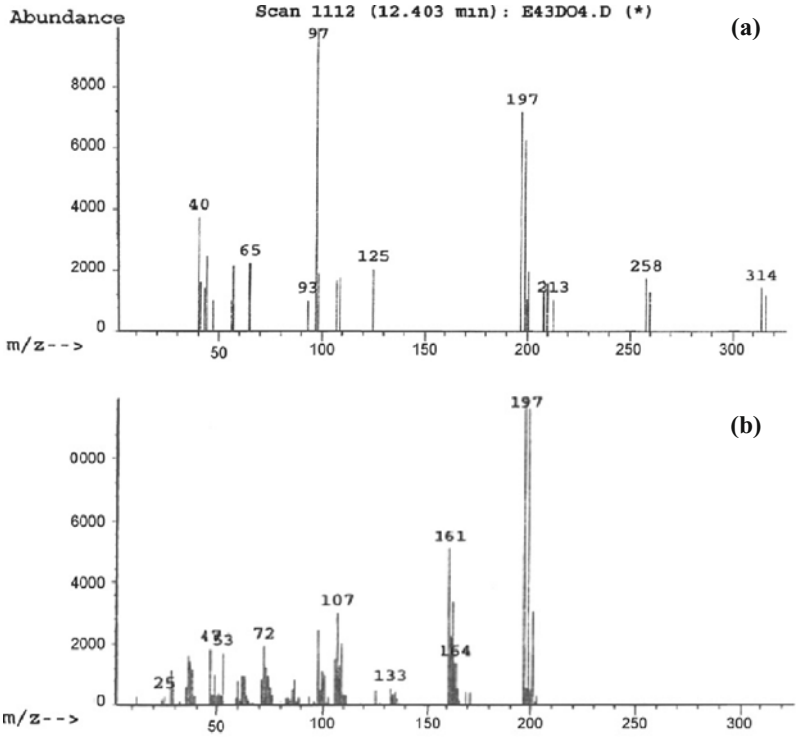

Fig. 5.21: Mass spectra of chlorpyrifos and its metabolite TCP.
(a) Chlorpyrifos (RT-12.403 min; m/z identification ions 97, 197, 314).
(b) TCP (RT-10.835 min; m/z identification ion 161).

Table 5.4: Microbial consortium identified from the remediated Rhizosphere soil

Bacteria	*Fungi*
Pseudomonas spp.	*Penicillum* spp.
Bacillus firmus	*Aspergillus niger*
Clostridium spp.	*Aspergillus fumigatus*
Streptomyse spp.	

The microbial consortium developed in rhizosphere is characterized and presented in Table 5.4. The potential microorganisms existing in rhizospheric soil which have capacity to survive and multiply at higher concentration of chlorpyrifos were selected for bioaugmentation of this hazardous compound. The chlorpyrifos degrader capable of using chlorpyrifos as the only source of carbon in the minimal salt medium was then identified by 16s rDNA analysis. The genomic DNA of the degrader which was found to be a bacterial strain was isolated and the 16s rDNA was amplified using universal primers. A 734 bp PCR product obtained from the 16s rDNA was then sequenced and BLAST

analysis was performed to identify the bacterium and its closest neighbours. To examine the phylogenetic relationships, a phylogenetic tree was drawn (Fig. 5.22). The analysis indicated that the bacterium was most similar to *P. nitroreducens* PS-2 (accession no. FJ588866.1). This bacterium was cultured further into an inoculum for bioaugmentation of chlorpyrifos. Bioaugmentation of chlorpyrifos by inoculation of P. nitroreducens PS-2 into ryegrass rhizosphere soil was performed. Dissipation profile of chlorpyrifos in the inoculated soil in bioaugmentation experimental set up is presented in Fig. 5.23. Along the period of incubation, the degradation of chlorpyrifos was about 1.35 times higher for 25 mg/kg and ranged from 1.38 to 2.02 for 50 mg/kg in bioaugmented soil than the non-bioaugmented soil. Chlorpyrifos dissipation was greater in inoculated soil during the experimentation and ranged from 1.31 to 2.64 times when compared to the non-inoculated soils for 100 mg/kg spiked soil. The concentrations 25 and 50 mg/kg were examined to dissipate completely by 14th day and 21st day as compared to the 21st and 28th day as observed in non-inoculated rhizospheric soil. Chlorpyrifos was completely dissipated at the initial spiked concentration of 100 mg/kg for the inoculated soil in contrast to the non-inoculated soil (6.68 mg/kg) by the end of the bioaugmentation experiments. This data could also be analyzed statistically by defining the regression equations for the time dependent dissipation of chlorpyrifos in the inoculated and non-inoculated soil for the three (25, 50 and 100 mg/kg) chlorpyrifos concentrations initially amended in the soil used in bioaugmentation experiments, where Y is the dependent variable representing time intervals in days, X_1 an independent variable representing the concentration of chlorpyrifos in the inoculated soil and X_2 as an independent variable representing the concentration of chlorpyrifos in the non-inoculated soil. Therefore the regression equations can be given as follows which indicate that this model is good fit:

$$Y = 25.26 + 1.58X_1 - 2.88X_2$$

for 25 mg/kg initially spiked chlorpyrifos concentration;

$$Y = 26.35 + 0.93X_1 - 1.61X_2$$

for 50 mg/kg initially spiked chlorpyrifos concentration;

$$Y = 27.04 + 0.01X_1 - 0.36X_2$$

for 100 mg/kg initially spiked chlorpyrifos concentration.

The differences in percentage dissipation of chlorpyrifos occurring in inoculated and the non-inoculated soil in all the concentrations are shown in Table 5.5. These results indicated that bioaugmentation by adding the isolate PS-2 into the rhizosphere soil of ryegrass significantly improved the degradation of chlorpyrifos ($p < 0.05$) (Mahajan, 1991) and can be used for enhanced degradation of chlorpyrifos from the contaminated soils. The present study has research findings in accordance with Yu et al. (2003), where the degradation of butachlor in wheat rhizosphere soil at the initial concentration of 10 mg/kg was five times improved by bioaugmentation of butachlor degrader.

Table 5.5: Percentage dissipation of chlorpyrifos in the soil inoculated and non-inoculated along the period of time

Chlorpyrifos (mg/kg)	*Inoculated rhizospheric soil*	*Non-inoculated rhizospheric soil*
25	76.24% (14 days)	100% (14 days)
50	90.36% (21 days)	100% (21 days)
100	90.80% (28 days)	100% (28 days)

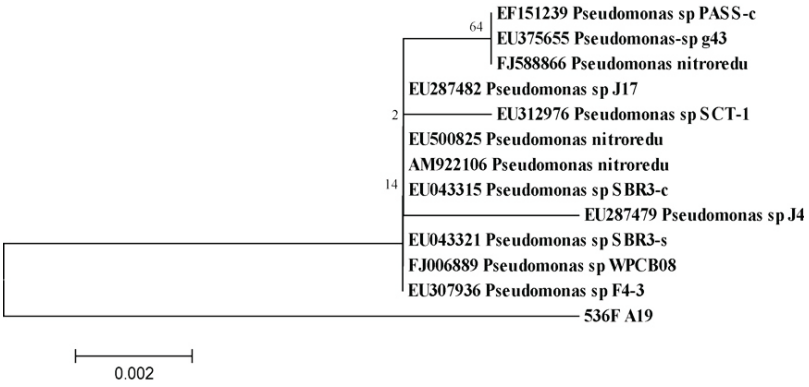

Fig. 5.22: Phylogenetic tree showing the genetic relatedness of bacteria isolated from phytoremediated rhizospheric soil.

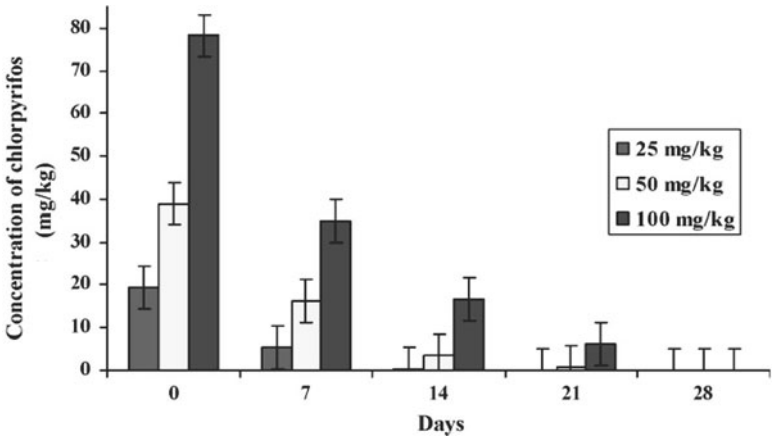

Fig. 5.23: Dissipation of chlorpyrifos in inoculated soil (bioaugmentation expt.).

The total number of heterotrophic bacteria and fungi present in the inoculated and non-inoculated rhizosphere soil were also inoculated during the bioaugmentation experiments. The microbial counts in inoculated and non-inoculated rhizospheric soils were found to be greater than the bulk or inoculated

but non-planted soil. The studies towards degradation of pesticides have been suggested to protect plants against the contamination by the degrading bacteria (I. Kuiper et al., 2004). Our findings indicated that rhizosphere bioremediation of chlorpyrifos by ryegrass and bioaugmentation by the isolate *P. nitroreducens* PS-2 have enhanced bioremediation of pesticide in the mycorrhizal soil. These techniques can highly be recommended for remediation of soil contaminated with similar group of pesticides.

Rhizosphere is considered as an ecological remediation unit to treat the contaminated soils. Micro-organisms like bacteria, fungi and actinomycetes form a symbiotic association along the root zone of the plant in the rhizosphere. The microbial enzymes and plants release exudates such as alcohols and small concentration of high molecular weight compounds (enzymes and proteins) in the Rhizosphere are beneficial in enhancing bioremediation.

5.7 Bioremediation of Dye and Dye-Stuff Effluent Using Nanotechnology-Based Sequence Bioreactor-Approach

The synthesis of dye and pigments used in textile and other industries generates the hazardous waste. The dye and dye-stuff industries have been characterized among 29 groups of hazardous industries under the Hazardous Waste Management and Handling Rules, 1989. The Environmental Protection Act, 1987 has prescribed under this rule specific provisions for the processes, operations and treatment for dye and dye-stuff waste. The waste generated during the process and operation of these dyes commonly are found to contain the inorganic and organic contaminants leading to the hazard to ecosystem and biodiversity causing impact on the environment. The waste generated from the products and processes uses the neutralization, flocculation, coagulation, settling, carbon-absorption, detoxification of organics by oxidation and biological treatment. In-spite of the present treatment technology, the organics and heavy metals (hydrocarbons, alcohols, amines, organic acids, aldehydes, ketones and heavy metals) are commonly found in the soil water environment. Therefore the recent advances made in the bioremediation technology using the bio-nanotechnological approaches are useful for the effective treatment of dye-stuff effluent for environmental protection.

In the present research study, nanotechnology based sequenced bioreactor has been designed and developed by Fulekar (2009) for the bioremediation of hazardous waste. (Fig. 5.24) with special reference to dye stuff effluent.

In the first reactor the physico-chemical and microbial characterized effluent will be taken wherein TiO_2- nano-based semi-conductor will be used under the influence of UV radiation for the oxidation/reduction and chemical degradation of dye-stuff effluent. Nanobiosensor will also be used to evaluate and/or observe the rate of chemical degradation of the hazardous compound present in dye stuff effluent. After this, the treated dye stuff effluent will be transferred to the

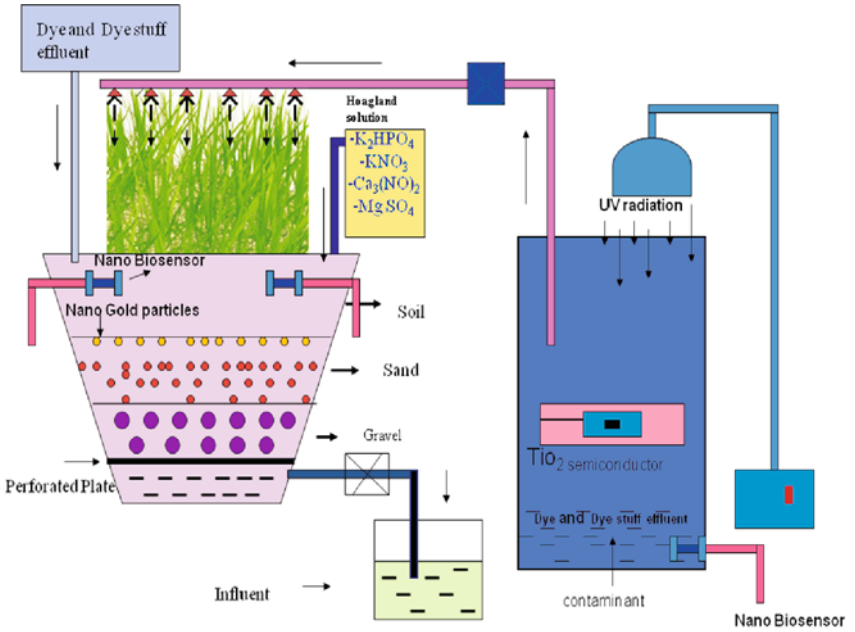

Fig. 5.24: Nanotechnology-based sequence bioreactor.

second connected bioreactor through the peristaltic pump wherein mycorrhizal soil amended with gold nanoparticles/sand/gravel will be arranged one below the other on perforated plates at the bottom inside the bioreactor for trickling the contaminants. The grasses with fibrous roots will be grown on the top of the soil for uptake of the heavy metals in mycorrhizosphere. The amended gold nanoparticles in mycorrhizosphere will enhance the uptake of heavy metals and get along with the tissue of the plants during phytoremediation. Gold nanoparticles can be recovered after harvesting the plants. Simultaneously, rhizosphere bioremediation of organic contaminants will take place through the trickling (mycorrhizal soil/sand/gravels) in the second bioreactor which would be measured by the biosensors. Rhizosphere is considered as an ecological remediation unit to treat the contaminated soils. Micro-organism like bacteria, fungi, and actinomycetes form a symbiotic association along the root zone of the plant in the rhizosphere. The plant releases exudates such as short chain organic acids, phenolics, sugars, alcohols and small concentration of high molecular weight compound (enzymes and proteins) in the rhizosphere. The plant enzymes like laccase, dehydrogenase, nitrilase, nitroreductase and peroxidase are beneficial to stimulate the degradation of organic chemicals by inducing enzymes systems of existing bacterial populations and also stimulating the growth of the new species that are able to degrade waste contaminants. The microbial enzymes in association with plants released exudates have made it possible to provide favourable conditions to degrade hazardous compounds.

Rhizosphere bioremediation has been proved effective treatment technology for biodegradation of organic compounds into environmental friendly compounds.

Further, the infiltrate collected at the bottom in the third reactor through bioremediation trickling via mycorrhizal soil/sand/gravel (second precursor) will be treated with cultured and developed mycorrhizosphere microbial consortium as immobilized biomass till the completion of bioremediation of the residual organic and inorganic contaminants. Heavy metal bioaccumulation, precipitation, biotransformation and uptake by plant in mycorrhizosphere would be assessed by bionanosensor and Atomic Absorption Spectrophotometer. Chemical degradation and bioremediation of organic contaminants and intermediate contaminants formed through biodegradable pathways would be evaluated by biosensors as well as using GC/HPLC/GC-MS/NMR techniques.

This nano-biotechnological approach would establish the new innovation in bioremediation for the removal of colour, remediation of heavy metals and organic contaminants to the acceptable level as per the standards prescribed under the Environmental Protection Acts.

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6

Biodegradation Technology for Pesticide Toxicity Elimination

E.A. El-Sheikh and M-B.A. Ashour

6.1 Introduction

Pesticides usages are intensive all over the world to control insect pests even a commitment to the rapid adoption of integrated pest management techniques. Pesticides are ranked as the most widely distributed chemical contaminants of the environment in the twentieth century. According to data compiled by the United States Environmental Protection Agency (EPA) and the World Health Organization (WHO), over 1000 compounds are used as pesticides, representing many different chemical classes: carbamates, thiocarbamates, organophoshates, dipyridyls, triazines, phenoxyacetates, coumarins, nitrophenols, pyrazoles, pyrethroids, and organic compounds containing chlorine, phosphorus, tin, mercury, arsenic, copper, etc. Millions of tons of pesticides are produced and used annually in close association with agriculture. Many articles, reviews and books are devoted to pesticides (Pesticide chemistry and bioscience, 1999 and Pesticides literature review, 2004).

Pesticide contamination is often attributed to point contaminations due to pest control with chemicals, spillages, tank washing activities, etc. To mitigate direct losses, the application of bioremediation systems on the farmyard has been proposed. Several set-ups of on-farm bioremediation systems exist, but they all can be considered as a biofilter system containing a biological active matrix that retains the pesticides and enhances their biodegradation. Due to the widespread and long-term application of pesticides in agriculture, soils, ground waters and reservoirs in many areas are now heavily contaminated. The toxicity of pesticides makes them hazardous when incorporated into the food chain.

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6.2 Pesticides Toxicity at a Glance

Dichlorodiphenyltrichloroethane (DDT) is an extremely active insecticide. This compound was first synthesized in 1874, and since 1930, when its insecticidal properties were established, was widely used against the malaria transmitter, the *Anopheles* mosquito (Konradsen et al., 2004).

The practically unlimited application of DDT led to its worldwide distribution. The highest solubility in fat favoured its incorporation into food chains. As a result, in the terminal steps of food chains the concentration of DDT is typically increased almost a million times, e.g., starting from rainwater and ending in human milk (Fellenberg, 1990).

DDT is a typical contact poison, rapidly penetrating through the skin. DDT induces apoptosis in human mononuclear cells in vitro and is associated with increased apoptosis in exposed children (Perez-Maldonado et al., 2004). It induces DNA damage in blood cells (Yanez et al., 2004), and adversely affects the normal duty cycle of nerve cell membranes. As it depresses the response of the Na^+-pump, normal restoration of the resting potential does not occur after excitation of the nerve. Large amounts of DDT induce paralysis of extremities. Maternal milk containing the insecticide can seriously damage the health of a child or disturb latent reproductive capacity by penetrating into the gonads.

Under usual conditions, DDT slowly and partially decomposes. Under aerobic conditions decomposition products are derivatives of dichloroethylene, which are less toxic than DDT; under aerobic conditions the dichloroethane derivatives are formed, which are easily transformed into derivatives of acetic acid (Fellenberg, 1990).

Since the introduction of DDT, insect pests have been controlled almost exclusively with chemical insecticides. Because of their fast acting, cheap to produce, relatively easy to deliver, and highly potent, chemical insecticides have been viewed with extreme optimism; problems associated with these compounds did not begin to become apparent to most scientists until almost two decades after their introduction.

In response to the environmental threat that these compounds pose, DDT and many other chlorinated insecticides were banned from agricultural use in many countries in the 1970s, and alternative classes of chemical compounds were developed. These compounds, some of which still have poor mammal insect specificity ratios, include carbamates, organophosphates, synthetic pyrethroids, neonicotinoids, synthetic growth regulators and metabolic disrupters.

Organophosphorus insecticides (OPPs) have been extensively used in agricultural practice for more than 40 years. OPPs are strong inhibitors of acetylcholinesterase, an enzyme essential for normal nerve impulse transmission. This affects the transmission of signals to nerve endings via the acetylcholine receptor. The decrease of enzymatic activity leads to an accumulation of symptoms of diseases such as sialorrhea, pulmonary edema, colics, diarrhoea, nausea, weakening of sight, rise of blood pressure, muscular

spasms and convulsions, speech disturbance, and paralysis of the respiratory tract. Most organophosphorus pesticides have similar general structure, containing three phosphoester linkages, and hydrolysis of one of the phosphoester bonds dramatically reduces the toxicity of the pesticides by eliminating their acetylcholinesterase inactivating properties (Horne et al., 2002). A similar clinical picture is observed in the case of phosphates and carbamates administered at intentionally increased doses. Organochlorine insecticides (e.g., chlordane, dieldrin, lindane, DDT) typically penetrate into the human organism through the digestive tract or the skin (Guttes et al., 1998]. When the membranes of nerve cells are damaged by pesticide action, their permeability for osmotic transport of Na^+-flow is maintained. Hence, their rest potential after excitation either does not return to its initial level, or is decreased. These organochlorine compounds severely change the excitability of nerve cells. At low concentrations axons are damaged; higher concentrations also cause the damage of sensory neurons. Chlordane and dieldrin are, moreover, clearly carcinogenic.

The so-called pyrethroid pesticides also have toxic characteristics and are synthetic analogues of a widely distributed insecticide pyrethrin, a natural compound found in chrysanthemums. Pyrethrins intended for insecticide use are modified to increase their stability. Synthetic pyrethrins have toxic effects on the nervous system (Spencer et al., 2001).

6.3 Pesticides Transportation in Ecosystem

As a result of agricultural practices, pesticides have been detected in many aquifers and surface waters. In structured soils, macropore flow often causes rapid nonuniform leaching via preferential flow paths, where a fraction of the contaminant percolates into ground water before it can degrade or be adsorbed by the soil (e.g., Stagnitti et al., 1994). With regard to pesticides, moderately sorbed compounds with relatively short half-lives are particularly affected (Larsson and Jarvis, 2000). Travel times for pesticides preferentially leached below the root zone are comparable to those for conservative solutes, with losses of typically less than 1% of the applied dose, but reaching up to 5% of the applied mass (Flury, 1996; Kladivko et al., 2001; FOCUS, 2001). These apparently small numbers can be put into perspective by considering the EU drinking water standard, which states that concentrations of a single pesticide may not exceed 0.1 μg L^{-1}. For a dose of 0.2 kg ha^{-1} and an annual recharge of 200 mm, this implies a maximum allowed leaching loss of only 0.1% of the applied amount (Jarvis, 2007). Hence, macropore flow should be considered in risk assessment of ground water contamination with pesticides (FOCUS, 2000).

Pesticide leaching through the vadose zone to ground water is a complex process controlled by a range of soil and environmental conditions. Accordingly, pesticide fate models account for a variety of processes including soil water

flow, solute transport, heat transport, pesticide sorption, transformation and degradation, volatilization, crop uptake, and surface runoff. A particular modelling challenge is to predict pesticide transport at very low leaching levels important for pesticide registration. These low leaching percentages may be associated with PF, where only a small fraction of the chemical percolates downward at a fast pace, while the remaining bulk of the substance leaches more slowly than would be expected from the chromatographic theory (Boesten, 2000). On the other hand, it has been argued that for very low concentrations, approaching the level of quantification, the criteria for accuracy need not be as rigorous, particularly when the analysis takes into account the uncertainty of data and model outcome (Carbone et al., 2002). An evaluation of model predictive accuracy is often made using the factor-of-fate approach, where the agreement between model estimates versus measured values is considered satisfactory within two-, five-, and 10-fold differences (Parrish and Smith, 1990). This approach allows the nature of the measured data to serve as an input to set the bounds that define the precision of the model (Carbone et al., 2002).

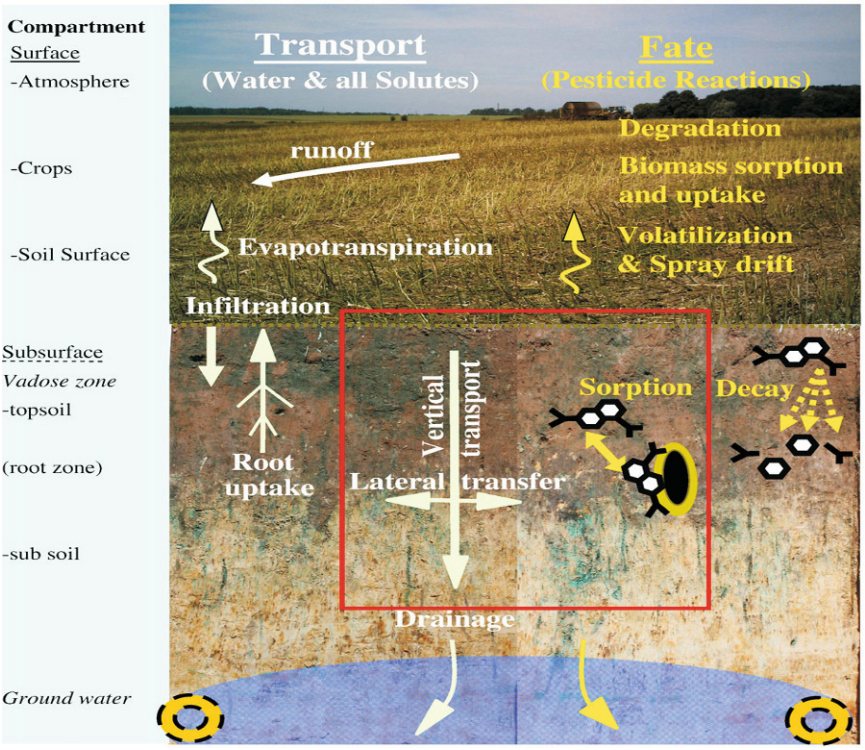

Fig. 6.1: Principal processes governing pesticide transport and fate in agricultural structured soil systems (transferred from Köhne et al., 2009). The central frame is explained in Fig. 6.2.

The principal processes governing pesticide transport and fate in agricultural structured soil systems are illustrated in Fig. 6.1. Soil matrix and macropore characteristics invoking different transport patterns are highlighted in Fig. 6.2.

Pesticide loss from aqueous systems has been well characterized and involves a combination of degradation and transport procedures. Degradation can include photolysis, chemical transformations and biological transformation (Roberts, 1998; Stangroom et al., 2000), of which microbial processes usually dominate (Vink and Van der Zee, 1997). Similarly, pesticide transport can be physicochemical or biological, but the parent compound remains unchanged; it is simply transferred from one matrix to another. Importantly, the rate of transport and breakdown of a particular pesticide depends heavily on its physicochemical properties (Stangroom et al., 2000; Crossan, 2002).

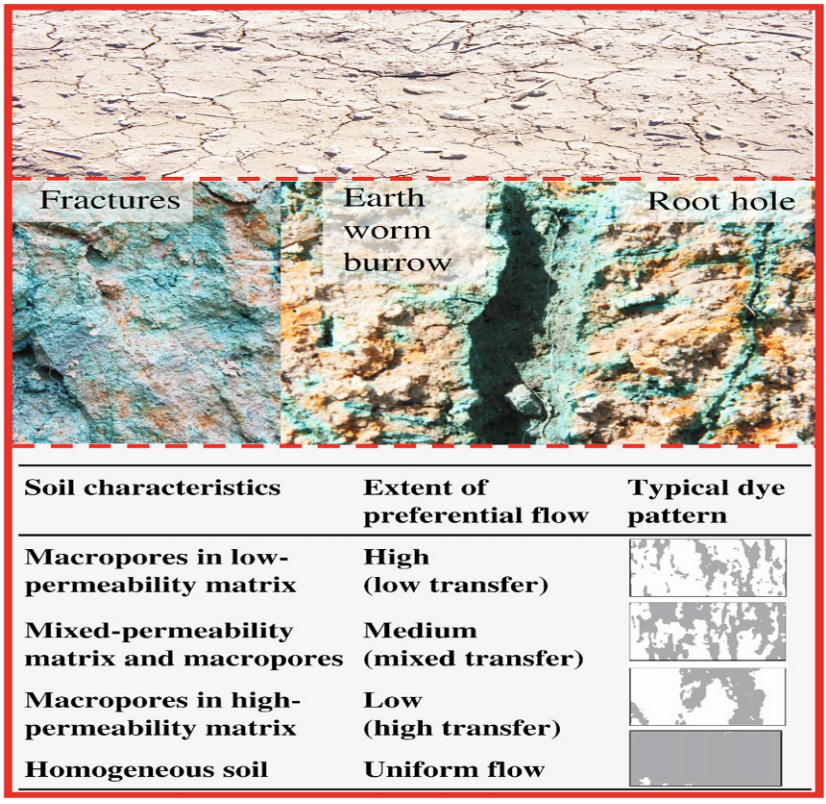

Fig. 6.2: Fractures and microtopography are triggers for preferential infiltration (top), Diverse structure/matrix interfaces stained by dye tracer visualize different preferential transport paths; these interfaces may affect lateral diffusion, sorption and degradation (middle). Soil matrix and macropore characteristics and resulting transport patterns; actual patterns also depend on the characteristics of rainfall and of overlaying soil horizons (bottom) (transferred from Köhne et al., 2009).

6.4 Pesticides Removal Efficiency Mechanisms

6.4.1 Using a Design Tool for Pesticide Removal

Practices were applied to decrease or eliminate pesticides from all environmental contents ranging from using tools to microorganisms and recently by using modified plants. For example, Apilot-scale, ponded wetland (Fig. 6.3) consisting of an open pond and a vegetated pond in series was constructed on a cotton farm in northern New South Wales, Australia (Rose et al., 2006), and assessed for its potential to remove pesticides from irrigation tail-water. Ten incubation periods ranging from 7 to 13 days each were conducted over two cotton growing seasons to monitor removal of residues of four pesticides applied to the crop. Residue reductions ranging 22–53% and 32–90% were observed in the first and second seasons respectively. Average half-lives during the first season were calculated as 21.3 days for diuron, 25.4 days for fluometuron and 26.4 days for aldicarb over the entire wetland. During the second season of monitoring, pesticide half-lives were significantly reduced, with fluometuron exhibiting a half-life of 13.8 days, aldicarb 6.2 days and endosulfan 7.5 days in the open pond. Further significant reductions were observed in the vegetated pond and also following an algal bloom in the open pond, as a result of which aldicarb and endosulfan were no longer quantifiable. Partitioning onto sediment was found to be a considerable sink for the insecticide endosulfan. These results demonstrate that macrophytes and algae can reduce the persistence of pesticides in on-farm water and provide some data for modelling (Rose et al., 2006).

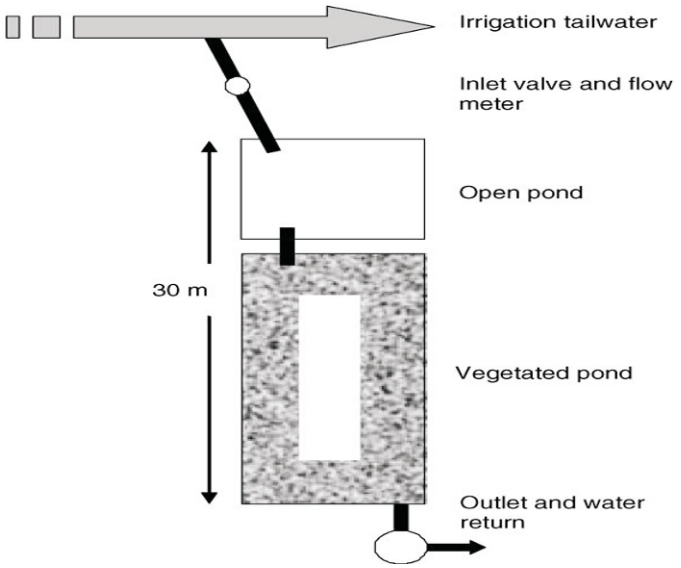

Fig. 6.3: Schematic diagram of the pilot-scale ponded wetland (transferred from Rose et al., 2006).

This model of elimination pesticides has given a preliminary insight into pesticide transport and removal efficiency in constructed wet lands on cotton farms. It is recommended that such wetlands are comprised of both open water and vegetated zones, to increase the potential for complementary chemical, photolytic, microbial and plant-mediated pesticide breakdown. Additional work is needed to examine the potential for algae in pesticide remediation of irrigation tailwaters on farm, and the determination of modelling parameters for wetland sizing and design.

6.4.2 Biodegradation Practices of Pesticides

Chemicals in the soil, water and air are affected by various chemical/physical and environmental factors. These factors are interrelated and influence the ultimate fate of the chemical as well as the degradative processes themselves. Environmental factors (rain, temperature, ultraviolet irradiation and humidity) affect the soil microbial population and resultant biological activities. Chemical/physical factors of the chemical compound include solubility, vapour pressure and structure. These factors govern the susceptibility to transformation reactions catalyzed by the microbial community. Vegetation can also affect the fate, as absorption and translocation can remove the chemical from soil or water. Once inside the plant, the chemical is then subjected to metabolism, sequestration into the vacuole or incorporation into a plant constituent such as lignin. In some cases, the absorbed chemical can be released via exudation from the root system, thus making it available for microbial or chemical processes. Presence of the chemical in the soil or water may be direct as in pesticide application for pest control, or may be indirect as in spillage or inadvertent contamination.

6.4.2.1 *Bioremediation Mechanism*

Bioremediation is a biological process by which environmental pollutants are eliminated or converted to less toxic (or even useful) substances. Natural bioremediation is often largely catalyzed by the indigenous microbial or plant populations in soil or aquatic ecosystems. These bioremediative processes have doubtlessly been active for millions of years. Farmers have been recycling animal and plant wastes back to the soil via composting for thousands of years. Yet, it is only in recent decades that detailed studies have begun to probe the underlying molecular mechanisms of the composting process as well as other approaches to bioremediation.

6.4.2.1.1 Microbial Degradation

When microorganisms are used for remediation of xenobiotics, both inoculation of microorganisms and nutrient application are essential for their maintenance at adequate levels over long periods (Eapen et al., 2007). Besides, the microbes which show highly efficient biodegradation capabilities under laboratory conditions may not perform equally well at actual contaminated sites (Goldstein et al., 1985; Macek et al., 2008). The potential of genetic engineering to

accelerate the bioremediation of xenobiotics has been recognized since the early 1980s, with initial attempts being focussed on microorganism (Rugh et al., 1998; Rosser et al., 2001; Sung et al., 2003; Rugh, 2004; Doty, 2008; Singleton, 2007). However, there are two main problems with the introduction of transgenic microorganisms: the bureaucratic barriers blocking their release into the environment and the poor survival rate of those engineered strains that have been introduced into the contaminated soil.

Bioremediation of chemicals in the soil depends on the activities of microbes in the soil, or in association with the root system. Chemical/physical properties of the chemical itself can influence the availability of the chemical to the microbe, or the susceptibility to degradative processes. Molecular alterations catalyzed by microbial processes include oxidation, reduction, hydrolysis, de-esterification, dehalogenation, dealkylation, conjugation and others. These processes usually result in a non-toxic chemical in the case of a pesticide, or in decreasing contamination levels of hazardous materials. Dissipation of pesticides in the soil involves several processes: volatilization, photodecomposition, leaching, adsorption, and microbial degradation.

Microbial degradation is considered to be a major factor determining the fate of diazinon and other organophosphorus insecticides in the environment. Many authors indicated that the bacterial strains belonging to the different taxonomic groups have a great degradation potential of the organophosphorus insecticides and other pesticides (Comeau et al., 1993; Ghassempour et al., 2002; Yasouri, 2006; Sørensen et al., 2008). Studies on microbial degradation are useful in the development of bioremediation strategies for the detoxification of these insecticides by microorganisms. Bioremediation, which involves the use of microbes to detoxify and degrade pollutants, has received increased attention as an effective biotechnological approach to clean up polluted environments. In general, the approaches to bioremediation are environmental modification, such as through nutrient application (biostimulation), and aeration (biosparging), or the addition of an appropriate degrader by seeding (bioaugmentation) (Vidali, 2001; Singh et al., 2006).

Degradation strategies exhibited by microorganisms include co-metabolism: the biotransformation of a molecule coincidental to the normal metabolic functions of the microbe; catabolism: the utilization of the molecule as a nutritive or energy source; and extracellular enzymes (phosphatases and amidases): secreted into the soil, which can act on the molecule as a substrate. Three basic types of reactions can occur: degradation, conjugation, and rearrangements, and all of which can be microbially mediated. Complete degradation of a chemical in the soil to carbon dioxide and water involves many different types of reactions; however, usually the first one or two transformations frequently result in loss of biological activity.

6.4.2.1.2 The Use of Earthworm in Pesticide Bioremediation

Due to their biological, chemical and physical actions, earthworms can be directly employed within bioremediation strategies to promote biodegradation of organic contaminants (Hickman and Reid, 2008). Their application within

waste management involves the digestion of a wide range of organic wastes (Edwards and Bohlen, 1996) into vermicast/compost. Figure 6.4 summarizes the discussed positive earthworm actions upon the soil environment, which might theoretically offset some of the previously discussed bioremediation limitations. In addition to the effects of earthworms upon microbial biotic effects, a number of studies have highlighted direct earthworm biotic effects in the form of feeding behaviours upon contaminant fate. For example, whilst Ma et al. (1995) noted that the effect of leaf litter food made only minimal difference in polycyclic aromatic hydrocarbons (PAH) loss extents, they did note that bioaccumulation of these PAHs was greatly enhanced via food limitation. Thus it is a possibility that earthworms increase their oral intake of soil particles when driven by hunger stress. When investigating total petroleum hydrocarbons (TPH) losses in with-earthworm systems that either had or had not received food, Schaefer et al. (2005) noted that residual TPH in the with-food systems was greater; however, these were not significant values.

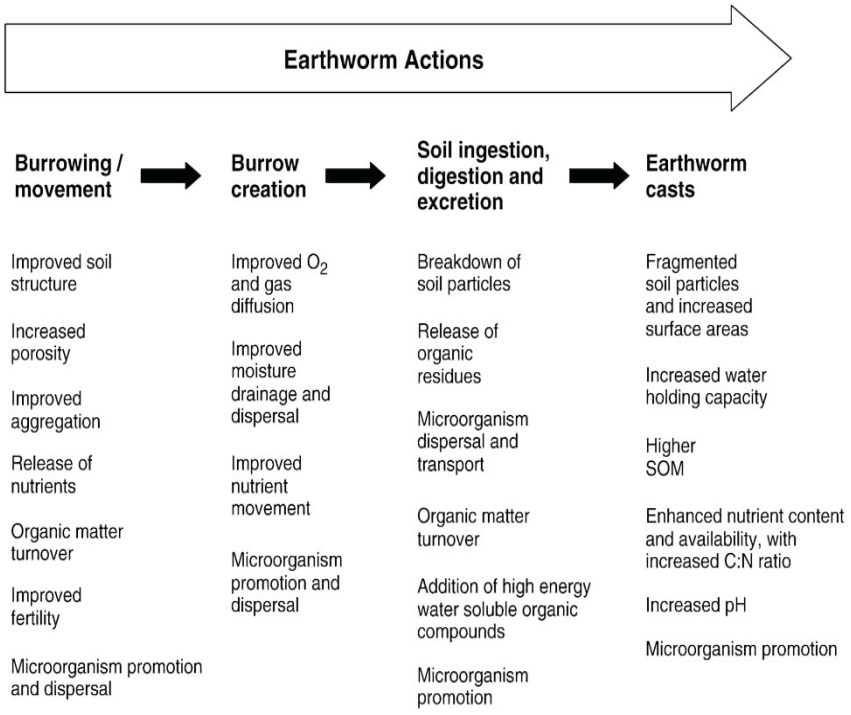

Fig. 6.4: Earthworm biological, chemical and physical effects within the soil environment (transferred from Hickman and Reid, 2008).

These results would suggest that if earthworms were applied to suboptimal contaminated soils lacking in organic matter, a greater degree of biological interaction might ensue resulting greater extents of loss. In further support of this, Haimi et al. (1992) stated that the lower the organic matter content, the

higher the bioaccumulation, which obviously implies increased soil association, a result confirmed by Peters et al. (2007) who noted significantly greater uptake of p,p-DDE from soil than compost by earthworms (*E. fetida* and *L. rubellus*). This means that earthworms must be applied only to contaminated soils that do not exert overly toxic effects. In relation to this, there are inherent differences between earthworm niche types which dictate responses and behaviour to soil type, contaminant type, food availability and a number of other environmental parameters (Edwards and Bohlen, 1996; Lavelle, 1998; Curry and Schmidt, 2007). Therefore, care would be needed to select the correct earthworm species for the correct intended conditions. Earthworm tolerances and toxicity to contaminated soils have been discussed elsewhere (Sheppard et al., 1998; Spurgeon et al., 2004).

It is envisaged that approaches for earthworm-assisted bioremediation might include:

1. Direct application of earthworms to contaminated soils (e.g. Schaefer et al., 2005)
2. Co-application of earthworms to contaminated soils with another organic media, such as compost (e.g. Ceccanti et al., 2006)
3. Application of contaminated media to earthworms as part of a feeding regime (e.g. Getliff et al., 2002)
4. Indirect use of earthworms could occur via application of vermidigested material. Such substrates could be hypothesized to be high in promoted degraders and thus high in catabolic potential (e.g. Alvarez-Bernal et al., 2006).

A number of studies have investigated the use of earthworms within bioremediation to enhance losses of pesticides (Table 6.1). These contaminants such as insecticides (Verma et al., 2006) and herbicides (Farenhorst et al., 2001; Binet et al., 2006) support the wide applicability of earthworm use. It can be observed from Table 6.1 that a variety of earthworm species were investigated, as were the effects of earthworms upon the behaviour, fate and loss of pesticides.

Studies were conducted not only to investigate earthworm effects upon agrochemicals, but also to understand the governing mechanisms in the field environment upon agrochemical fate and persistence. However, these mechanisms are consistent with those pertinent to applying earthworms to bioremediation of such compounds. Whilst research (Eijsackers et al., 2001) has identified that earthworm-assisted bioremediation is likely to increase hydrocarbon availability, and has been seen to remobilize dichlorodiphenyltrichloroethane (DDT) and hexachlorocyclohexane (HCH) bound residues (Verma and Pillai, 1991), such findings were in conflict with those of Bolan and Baskaran (1996) who investigated the effect of earthworm (Lumbricus rubellus and Allolobophora calignosa) casts upon the sorption and movement of ^{14}Catrazine, ^{14}C-2,4-dichlorophenoxy acetic acid (2,4-D) and ^{14}C-metsulforon methyl. They

stated that the casts sorbed higher amounts of herbicides than the source soil due to the higher levels of organic carbon and fine size fractions, present due to earthworm grinding actions and selective feeding. Clearly both theories are credible, and perhaps highlight not only the differences in compound behaviour, experimental set-up or earthworm species, but also the wide variability between the effects of earthworm mechanics upon compound fate, and subsequent earthworm casts upon compound fate.

Table 6.1: Overview of investigated pesticides and effects of earthworm species in the degradation

Compound	*Earthworm species*	*Effect, or aspect of use*	*Reference*
Herbicides			
Atrazine, 2,4-D and Metsulforon methyl	*L. rubellus* and *A. calignosa*	Casts increased sorption of compounds	Bolan and Baskaran (1996)
Atrazine	*L. terrestris* and *A. calignosa*	Compound sorption to gut contents and casts. Earthworms determined lower mineralization	Binet et al. (2006)
Atrazine	*L. terrestris*	Activity mixed and distributed compound, increased persistence, reduced mineralisation, accelerated binding of residues	Farenhorst et al. (2000a)
Atrazine	*L. terrestris*	Activity mixed and distributed compound, increased persistence, reduced mineralisation, accelerated binding of residues	Farenhorst et al. (2000b)
Atrazine and Metachlor	*L. terrestris*	Increased sorption of compound to burrow linings	Farenhorst et al. (2001)
Atrazine	*L. terrestris*	Increased mineralisation and bioavailability	Meharg (1996)
Atrazine	*A. giardi*	Burrows and casts increased compound sorption	Alekseeva et al. (2006)

(*Contd.*)

2,4-D, Carbofuran and Metribuzin	Species unreported	Earthworm macropores increased sorption but increased microbial mineralisation	Mallawatantri et al. (1996)
Isoproturon, Dicamba and Atrazine	*A. longa*	Released previously bound residues, limited formation of bound residues, increased mineralisation	Gevao et al. (2001)
Atrazine	*L. terrestris* and *A. caliginosa*	Reduced mineralisation and reduced microbial numbers, increased sorption of compound	Kersante et al. (2006)
Atrazine and Metamitron	*Vermicompost*	No effect upon degradation	Koocheki et al. (2005)
Insecticides			
Endosulfan	*M. posthuma*	Gut microflora promoted as specific degraders	Verma et al. (2006)
Hexachloro-hexane (HCH)	*Pheretima posthuma*	Gut microflora promoted as specific degraders	Ramteke and Hans (1992)
HCH and DDT	*P. posthuma*	Released previously bound residues	Verma and Pillai (1991)

(Adapted from Hickman and Reid, 2008)

Increased agrochemical sorption due to earthworm activity and/or presence was also noted by Farenhorst et al. (2000a,b) when earthworm (*Lumbricus terrestris*) activity, although effectively translocating, distributing and mixing ^{14}C-atrazine, had resulted in its persistence via sorption. Such sorption effects have been observed for ^{14}C-atrazine and ^{14}C-metachlor to organic rich burrow linings (Farenhorst et al., 2001), for atrazine to earthworm gut contents and humic and colloidal rich casts (Binet et al., 2006) and for atrazine to cast organic carbon content, when earthworm (*Aporrectodea giardi*) presence was investigated in combination with an atrazine degrading inoculum (*Pseudomonas* sp. strain ADP (DSM 11735) (Alekseeva et al., 2006). However, in contrast to these findings, Mallawatantri et al. (1996) determined that the soil organic carbon amount related to the earthworm macropores (species unknown) was directly correlated to microbial numbers and thus observed mineralization of 2,4-D, carbo-furan and metribuzin; organic carbon content not only appears to determine sorption of agrochemicals, but also microbial mineralization.

In relation to this, Binet et al. (2006) further suggested that earthworm (*L. terrestris* and *Aporrectodea calignosa*) activity would promote atrazine mineralization by altering the size and diversity of microbial communities. They then reported that earthworm presence, albeit at low application density (three earthworms per 1.8 kg treatment) over 86 days, determined a lower mineralization extent (11.7%) in comparison to the without-earthworm treatments (15.3%). This is in keeping with Farenhorst et al. (2000a) who also reported reduced atrazine mineralization in the presence of earthworms (*L. terrestris*), whilst also noting the acceleration of the formation of non-extractable residues in with-earthworm treatments (Farenhorst et al., 2000a,b). However, these results are in conflict with those of Meharg (1996) who determined that ^{14}C-atrazine mineralisation was regulated by substrate availability on exchange sites, and in this instance, earthworm (*L. terrestris*) presence, at high application density (one earthworm per 40 g) affected the binding of atrazine to soil exchange sites over a four-week period, with subsequent mineralisation of ^{14}C-atrazine by soil microorganisms being double that of the control. Meharg (1996) concluded that this was due to increased bioavailability on exchange sites attributed to the presence of worms, thus further reinforcing the findings of Eijsackers et al. (2001). The mechanisms for this were hypothesised to be earthworm mucilage secretions and changes in soil structure and soil microflora.

In a contrasting study, Gevao et al. (2001) applied earthworms (*Aporrectodea longa*) at a rate of five individuals per 2 kg to soils contaminated with non-extractable pesticide (^{14}C-isoproturon, ^{14}C-dicamba and ^{14}C-atrazine) residues for 28 days to evaluate subsequent degradation, release and uptake. They determined that due to earthworm physical activity, a greater degree of previously bound pesticide residue in comparison to the without-earthworm treatments was released. When the study was applied to freshly added pesticides, it was noted that the formation of non-extractable residues of ^{14}C-isoproturon, ^{14}C-dicamba and ^{14}C-atrazine were higher by factors of 2, 2 and 4, respectively, in the without-earthworm treatments. Thus, not only did earthworms limit the formation of the bound fraction, they also promoted the release and mineralisation of bound residues. From a bioremediation perspective, this clearly represents an overall beneficial scenario. The mechanisms were attributed to the promotion of pesticide degraders in earthworm gut being added to the soil, changes in carbon substrate availability and changes in soil structure subsequently altering compound availability.

Further to this study, Kersante et al. (2006) investigated the interactions between earthworms (*L. terrestris* and *A. calignosa*) and atrazine degraders (*Pseudomonas* sp. ADP) in soil micro-sites (earthworm gut contents, casts and burrow linings). They determined that atrazine mineralization was reduced in earthworm soil micro-sites and that earthworms significantly altered the soil microbial structures by reducing the size of the atrazine degrader communities. They also suggested that low atrazine mineralization, or loss, could be partly explained by low effective mineralization rates in the biostructures due to

sorption to the high organic carbon content, as previously noted by Farenhorst et al. (2001), Alekseeva et al. (2006) and Binet et al. (2006).

The effectiveness of earthworm gut microflora to effect soil contaminant losses, or degradation, has previously been discussed. For example, Verma et al. (2006) noted the growth potential of earthworm (Mataphire posthuma) gut microorganisms to the pesticide Endosulfan, whilst Ramteke and Hans (1992) isolated microorganisms from the gut of Pheretima posthuma treated with HCH noting significant subsequent HCH degradation. However, unlike Verma et al. (2006), the authors noted that HCH degraders (in guts) gradually increased over a five-week period, replacing other gut microflora, indicating the potential for specialized gut growth. However, as discussed by a number of authors (Edwards and Bohlen, 1996; Brown and Doube, 2004; Kersante et al., 2006; Curry and Schmidt, 2007) it remains questionable whether (a) microorganisms are indigenous to earthworm guts, (b) earthworm gut microflora comes from the surrounding soil and plant remains, or (c) whether specialized feeding determines distinctive gut flora.

A number of studies relating to the application of earthworms, or the effects of earthworms, upon soil associated agrochemicals have been discussed. Whilst contradictory results have been observed, especially with respect to compound sorption and release, it is apparent that there are a number of potentially positive physical effects upon agrochemical fate.

6.5 Phytoremediation of Pesticides

Phytoremediation, the use of plants to clean up polluted soil and water resources, has received much attention in the last few years. Although plants have the inherent ability to detoxify xenobiotics, they generally lack the catabolic pathway for the complete degradation of these compounds compared to microorganisms (Abhilash et al., 2009). A direct method for enhancing the efficacy of phytoremediation is to over-express in plants the genes involved in metabolism, uptake, or transport of specific pollutants. Furthermore, the expression of suitable genes in root system enhances the rhizodegradation of highly recalcitrant compounds like PAHs, PCBs etc. Hence, the idea to amplify plant biodegradation of xenobiotics by genetic manipulation was developed, following a strategy similar to that used to develop transgenic crops. Genes from human, microbes, plants, and animals are being used successfully for this venture. The introduction of these genes can be readily achieved for many plant species using *Agrobacterium tumefaciens*-mediated plant transformation or direct DNA methods of gene transfer (Abhilash et al., 2009).

Phytoremediation for removal of xenobiotics can be an alternate/supplementary method, since plants are robust in growth, are a renewable resource and can be used for in situ remediation (Cunningham and Berti, 1993; Cunningham et al., 1995, 1996; Cunningham and Ow, 1996; Suresh and

Ravishankar, 2004; Parameswaran et al., 2007). It has almost certain public acceptance. Further, plants may survive higher concentrations of hazardous wastes than many micro-organisms used for bioremediation. Phytoremediation increases the amount of organic carbon in the soil, which can stimulate microbial activity and augment the rhizospheric degradation of the pollutants. Phytoremediation also yields other benefits including carbon sequestration, soil stabilization, and the possibility of biofuel or fibre production. The development of phytoremediation technologies for the plant-based clean-up of contaminated soils is therefore of significant interest (Hooker and Skeen, 1999; Dietz and Schnoor, 2001; Eapen and D'Souza, 2005).

Although much research has been done to demonstrate the success of phytoremediation, resulting in its use on many contaminated sites (Aprill and Sims, 1990; Gunther et al., 1996; Binet et al., 2000; Liste and Alexander, 2000; Mattina et al., 2000; White, 2000, 2001, 2002, 2003; Fismes et al., 2002; Li et al., 2002; Maila and Cloete, 2002; Yoon et al., 2002; Singh and Jain, 2003; Sung et al., 2003; Sunderberg et al., 2003; Trapp et al., 2003; Gao and Zhu, 2004; Ma et al., 2004; Mattina et al., 2004; Suresh et al., 2005; Parrish et al., 2006; Mills et al., 2006; Aslund et al., 2007), the method still lacks wide application. Further, detoxification of organic pollutants by plants is often slow, leading to the accumulation of toxic compounds in plants that could be later released into the environment (Aken, 2008). There are also concerns over the potential for introduction of contaminants into the food chain. The question of how to dispose off plants that accumulate organic pollutants is also a serious concern. A direct method for enhancing the effectiveness of phytoremediation is to overexpress in transgenic plants the genes involved in metabolism, uptake, or transport of specific pollutants (Shiota et al., 1994; Rugh, 2004; Cherian and Oliveira, 2005; Kramer, 2005; Eapen et al., 2007; Macek et al., 2008; Aken, 2008; Doty, 2008). If the plants are able to degrade the xenobiotics to non-toxic metabolites or completely mineralized into carbon dioxide, nitrate, chlorine etc., there is no apprehension over hazardous waste management strategies for disposing off harvested plants. The purpose of this review is to provide recent advances in development of transgenic plants overexpressing catabolic genes including bacterial and human cytochrome P450 for the enhanced degradation and mineralization of xenobiotic pollutants.

6.5.1 Transgenic Plants for Enhanced Biodegradation and Phytoremediation of Organic Xenobiotics

Environmental pollution with organic xenobiotics (pesticides, pharmaceuticals, petroleum compounds, PAHs, PCBs etc.) is a global problem, and the development of inventive remediation technologies for the decontamination of impacted sites are therefore of paramount importance. Physical, chemical and biological methods can all be used for the remediation of contaminated sites (Lee and Huffman, 1989; Felsot and Dzantor, 1995; Johnson et al., 1997;

Hatakeda et al., 1999; Wirtz et al., 2000; Kummling et al., 2001; Perrin-Ganier et al., 2001; Matsunaga and Yashuhara, 2003; Yang et al., 2007); however, phytoremediation has long been recognized as a cost effective method for the decontamination of soil and water resources (Salt et al., 1998; Macek et al., 2000; Eapen and D'Souza, 2005). Further, a variety of pollutant attenuation mechanisms possessed by plants makes their use in remediating contaminated land and water more feasible than physical and chemical remediation (Glick, 2003; Huang et al., 2004, 2005; Greenberg, 2006; Gerhardt et al., 2009). As a result of their sedentary nature, plants have evolved diverse abilities for dealing with toxic compounds in their environment. Plants act as solar-driven pumping and filtering systems as they take up contaminants (mainly water soluble) through their roots and transport/translocate them through various plant tissues where they can be metabolized, sequestered, or volatilized (Greenberg et al., 2006; Abhilash, 2007; Doty et al., 2007).

With respect to their direct roles in remediation processes, plants use several different strategies for dealing with environmental chemicals: phytoextraction, phytodegradation, phytovolatilization, and rhizodegradation (Fig. 6.5) (Schnoor, 1997). Phytoextraction involves the removal and subsequent storage of contaminants by the plant and is often applied to the exclusion and storage of metals that may undergo speciation in plants, but cannot be metabolized. However, certain organic chemicals may also be treated in this manner due to inherent resistance to degradation. Conversely, phytodegradation describes processes in which plants metabolize the contaminants they take up. Components of this mechanism are often utilized by plants exposed to herbicides and thus have been researched extensively. The metabolic processes involved in phytodegradation have strong similarities to those used by animals for modification and degradation of drugs and other toxins. This has given rise to a conceptual model for phytodegradation known as the "green liver" model (Sanderman, 1994). A further attenuation mechanism, referred to as phytovolatilization, involves the release of contaminants to the atmosphere following their uptake from the soil or water. This mechanism has been observed for both organic and heavy metal contaminants, including trichloroethylene (TCE), which has been observed in the off-gas from plant leaves in the laboratory and field (Compton et al., 1998), and in the production of volatile, elemental mercury by genetically-engineered *Arabidopsis thaliana* grown in the presence of ionic mercury (Rugh et al., 1996; Bizily et al., 1999). An indirect mechanism, rhizodegradation, refers to the transformation of contaminants by resident microbes in the plant rhizosphere (i.e., the microbe-rich zone in intimate contact with the root vascular system). As mentioned above, the presence of plants on contaminated sites can drastically affect soil redox conditions and organic content (often through the secretion of organic acids from roots), as well as soil moisture. Rhizodegradation is the dominant mechanism in the removal of total petroleum hydrocarbons from soil by deep-rooted trees (Carman et al., 1998), as well as annual species (Schwab and Banks, 1994).

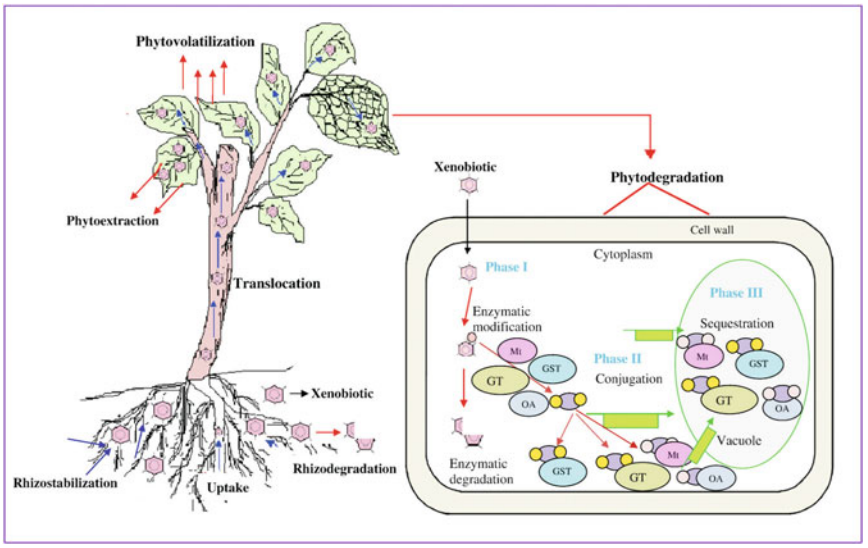

Fig. 6.5: Typical attenuation mechanism possessed by plants against xenobiotics. The xenobiotics can be stabilized or degraded in the rhizosphere, adsorbed or accumulated into the roots and transported to the aerial parts, volatilized or degraded inside the plant tissue. Plant detoxification generally involves conversion or enzymatic modification (phase I) followed by conjugation (phase II) followed by active sequestration (phase III). (GST = glutathione S-transferases; GT = glucosyltransferases; Mt = Malonyltransferases; OA = organic acids (Transferred from Abhilash et al., 2009).

6.5.2 Environmental Remediation

The concept that plants can degrade xenobiotics emerged in 1940s, when plants were shown to metabolize pesticides (Sanderman, 1994). Since then, the development of genomics, proteomics, and metabolomics has contributed much to enhance or manipulate the plant metabolism of many xenobiotic pollutants (Raskin, 1996; Eapen et al., 2007; Aken, 2008). Although phytoremediation was first applied for the removal of inorganic pollutants from soil, this technology has gradually proven to be efficient for the treatment of organic pollutants including chlorinated solvents, polyaromatic hydrocarbons and explosives (Pilon-Smits, 2005; Salt et al., 1998). The first generation of commercially available transgenic plants were produced to reduce the loss of crop yield caused by insect damage at the same time as reducing the amount of pesticides required (e.g. plants expressing Bt toxin). However, transgenic plants for phytoremediation were first developed for remediating heavy metal contaminated soil sites; for example, *Nicotiana tabaccum* expressing a yeast metallothionein gene for higher tolerance to cadmium, or *Arabidopsis thaliana* overexpressing a mercuric ion reductase gene for higher tolerance to mercury (Misra and Gedama, 1989; Rugh et al., 1996).

The first attempt to develop engineered plants for phytoremediation of organic pollutants targeted explosives and halogenated organic compounds in tobacco plants (French et al., 1999; Doty et al., 2000). The efficiency of transgenic plants to degrade chlorinated solvents, explosives, phenolics etc. have been extensively acknowledged in the literature (McCutcheon and Schnoor, 2003; Mackova et al., 2006; Meagher, 2000; Eapen et al., 2007; Doty, 2008; Macek et al., 2008). These plants have been developed to contain either transgenes responsible for the metabolization of xenobiotics or transgenes that result in the increased resistance of pollutants.

CYP450 for Enhanced Pesticides Metabolism

Cytochrome P450s enzymes comprise a superfamily of heme proteins crucial for the oxidative, peroxidative, and reductive metabolism of a diverse group of compounds, including endobiotics, such as steroids, bile acids, fatty acids, prostaglandins, and leukotrienes, and xenobiotics, including most of the therapeutic drugs and environmental pollutants (Klingenberg, 1958; Nelson et al., 1996; Kreuz et al., 1996). The first report on the existence of a CYP enzyme or a microsomal carbon monoxide-binding pigment was published in 1958 by Klingenberg et al. This enzyme gave a unique 450-nm optical absorption peak, and when its hemoprotein nature was recognized, it was given the name cytochrome P450 (Omura and Sato, 1962, 1964; Omura, 1999). In almost all living organisms, these enzymes are present in more than one form, thus forming one of the largest families of enzymes. The enzyme system is located in microsomes and consists of several cytochrome P450 isoforms and a nonspecific NADPH-cytochrome P450 oxidoreductase. The notable diversity of CYP enzymes has given rise to a systematic classification of individual forms into families and subfamilies. The protein sequences within a given gene family are at least 40% identical (e.g. CYP2A6 and CYP2B6), and the sequences within a given subfamily are N55% identical (e.g. CYP2A6 and CYP2A7) (Nelson et al., 1996). However, the number of families and enzymes varies among different organisms.

Although cytochrome P450 (P450 or CYP) monooxygenases in higher plants play an important role in the oxidative metabolism of endogenous and exogenous liphophilic compounds (Inui et al., 2000; Eapen et al., 2007; Doty, 2008), molecular information on P450 species metabolizing xenobiotics in plants is quite limited. On the other hand, there are a number of P450 species metabolizing xenobiotics in the microsomes of human liver (Inui et al., 2000). A study of 11 human P450s in the CYP1, 2, and 3 families using a recombinant yeast expressing system showed that they can metabolize 27 herbicides and four insecticides (Inui et al., 2001). Further, another study conducted by same research group found that human CYP1A1 metabolized 16 herbicides, including triazines, ureas, and carbamates, and CYP2B6 metabolized more than 10 herbicides, including chloroacetanilides, oxyacetamides, and 2,6-dinitroanilines, three insecticides, and two industrial chemicals (Inui et al., 2001).

During the last two decades, numerous experiments were conducted on the overexpression of human and mammalian (e.g. rat, mouse, rabbit) CYP450 isoenzymes (CYP1, CYP1, CYP3) in higher plants such as *Nicotiana tabaccum*, *Solanum tuberosum*, *Oryza sativa* or *Arabidopsis thaliana*. The introduction of these genes can be readily achieved for many plant species using Agrobacterium tumefaciens-mediated plant transformation or direct DNA gene transfer (Doty, 2008). Microsomes containing selected human P450s are commercially available which are produced by means of bacterial (*Escherichia coli*) or baculovirus expression system (Schmidt et al., 2006a,b). The integration and expression of this transgene can be confirmed by southern, northern and western blot analysis (Kawahigashi et al., 2007). The primary objective of these genetic manipulations were the production of either herbicide resistant plant (e.g. tolerance towards atrazine, simazine) or plants capable for enhanced metabolization of xenobiotics (herbicides or volatile halogenated hydrocarbons) and their subsequent removal from contaminated soil and ground water. Many of these plants were overexpressed with a single (e.g. CYP1A1 or CYP2E1) or several P450 genes (e.g. CYP1A1, Cyp2B6, and CyP2C19). Partially, plants were additionally modified with NADPH-cytochrome P450 reductase, or P450 and reductase were expressed as fusion enzyme (Shiots et al., 2000; Schmidt et al., 2006a,b). Due to the broad substrate specificity of human and mammalian P450s, the transgenics showed remarkable improvement of metabolic degradation towards single or multiple xenobiotics (Table 10.2).

Herbicides are economically important because they prevent losses in crop yield due to weed infestation (Lockhart et al., 1990; Kawahigashi et al., 2008). However, the overuse and repeated use of same herbicide can lead to the development of herbicide-resistant weeds. According to the Weed Science Society of America, over 310 biotypes of herbicide-resistant weeds have been reported in agricultural fields and gardens worldwide. As a result of these herbicide tolerance, larger amount of herbicides are needed to kill these weeds, so that residues contaminate the soil and nearby water bodies (Kawahigashi et al., 2006a,b; 2007). Plants used for decontamination of these contaminated system should be resistant to herbicides. The two primary strategies in agricultural crops against herbicide tolerance are: (i) modification of target sites and (ii) development of enhanced detoxification (Putwain, 2005). Among the various enzymatic group, cytochrome P450 and glutathione S-transferase play major roles in the enhanced degradation of herbicides (Ohkawa et al., 1999). Molecular information on plant P450 related to organic pollutant metabolism is limited; however, many P450-dependent oxidations in plant microsomes have been reported (Kawahigashi et al., 2007), including oxidation of chlorotoluron in maize (Fonne-Pfister and Kreuz, 1990) and wheat (Mougin et al., 1990); linuron in wheat (Frear, 1995) and maize (Moreland et al., 1993); atrazine in tulip (*Tulipa generiana* L.); and isoproturon in yam bean (Belfrod et al., 2004). Although numerous cytochrome genes are reported in plants, only some herbicide metabolizing P450 genes have been cloned and characterized, such

Table 6.2: Transgenic plants for enhanced phytoremediation of pesticides

Target plant	*Gene (s)*	*Enzymes*	*Source*	*Transgene effects*	*Reference*
O. sativa	CYP1A1, CYP2B6 and CYP2C19	Cytochrome P450 monooxygenase	Human	Phytoremediation of atrazine and metolachlor	Kawahigashi et al., 2006a
Solanum tuberosum, *O. sativa*	CYP1A1, CYP2B6, and CYP2C19	Cytochrome P450 monooxygenase	Human	Resistance to sulfonylurea and other herbicides	Inui and Ohkawa, 2005
N. tabaccum	CYP105A1	Cytochrome P450 monooxygenase	*Streptomycesgri-seolus*	Resistance to sulfonylurea	O'keefe et al., 1994.
O. sativa	CYP2C9	Cytochrome P450 monooxygenase	Human	Tolerance to sulfonylurea	Hirose et al., 2005.
O. sativa	CYP2B22, CYP2C49	Cytochrome P450 monooxygenase	*Sus scrofa*	Tolerance to several herbicides	Kawahigashi et al., 2005
N. tabaccum, *A. thaliana*	CYP71A10	Cytochrome P450 monooxygenase	*Glycine max*	Tolerance to phenyl urea herbicide	Siminszky et al., 1999
N. tabaccum	CYP76B1	Cytochrome P450 monooxygenase	*Helianthus tuberosus*	Tolerance to herbicide	Didierjean et al., 2002.
Alfalfa *N. tabaccum*	atzA	Atrazine chlorohydrolase	Bacteria	Enhanced metabolic activity against atrazine	Wang et al., 2005
O. sativa	Protox	Protoporphyrinogen IX oxidase	*Bacillus subtilis*	Tolerance to diphenyl ether herbicide oxyflufen	Jung et al., 2008
N. tabaccum	ophc2	Organophosphorus hydrolase (OPH)	*Pseudomonaspse-udoalcaligenes*	Enhanced degradation of organophosphorus (methyl parathion).	Wang et al., 2008
Populus trichocarpa	γ-ECS	γ-Glutamycysteine synthetase	Poplar	Overexpression of γ-ECS resulted in increased tolerance to chloroacetanilide herbicides.	Gullner et al., 2001
N. tabaccum	CYP450E1	Cytochrome P450 monooxygenase	Human	Enhanced degradation of anthracene and chloropyriphos	Dixit et al., 2008
A. thaliana	LACI	Root specific laccase	Cotton	Secretes laccase to the rhizosphere and have shown enhanced resistance to phenolic alleleochemicals and enhanced tolerance to 2, 4, 6,-trichlorophenol.	Wang et al., 2004

(Adapted from Abhilash et al., 2009)

as CYP73A1 and CYP76B1 from Jerusalem artichoke (*Helianthus tuberosus*) (Pierrel et al., 1994; Robineau et al., 1998), CYP71A11 from tobacco (*Nicotiana tabaccum*) (Yamada et al., 2000), and CYP71A10 from soy bean (Glycine max) (Siminszky et al., 1999). Fischer et al. (2001) reported 16 cytochrome P450 species responsible for the herbicide detoxification and cross-tolerance (De Prado et al., 2005) in Lolium rigidum.

6.5.3 Glutathione S-transferase (GST) for Enhanced Pesticide Degradation/Conjugation

In addition to P450 oxidation, glutathione conjugation is an important mechanism for xenobiotic detoxification. Glutathione S-transferases (GSTs) (EC. 2.5.1.18) are a family of multifunctional enzymes involved in the cellular detoxification and excretion of many physiological and endogenous substances (Wilce and Parker, 1994), which are found in animals, plants and microorganisms (Santos et al., 2002). In addition, studies on GSTs are further characterizing their role in xenobiotic metabolism. Under normal conditions, glutathione is predominantly present in its reduced form (GSH), with only a small proportion present in its fully oxidized state (GSSG) (Dixon et al., 1998a,b). GSTs catalyze the nucleophilic addition of the thiol of reduced glutathione (γ-glutamyl-cysteinyl-glycine) to electrophonic centres in organic compounds. The glutathione conjugates formed are more hydrophilic, thus facilitating their exclusion. Thus GSTs catalyzed transformation is one of the early steps along the mercapturic acid pathway in which hydrophobic xenobiotics are detoxified and eliminated from the organisms (Habig et al., 1974). Subsequently, an ATP dependent efflux pump that mediates the export of glutathione conjugates from cells (Hayes and Wolf, 1990; Ishikawa, 1992) (Fig. 6.6).

According to Dixon et al. (1998a), all plant GSTs have native relative masses of around 50 kDa and are composed of two similarly signed (~25 kDa) sub-units. Furthermore, each sub-unit contains a kinetically independent active site with distinct binding domains for glutathione and co-substrates (Marrs, 1996). The sub-units may be identical, giving rise to homodimers, or distinct but related, resulting in heterodimers, with each distinct sub-unit encoded by a different gene (Dixon et al., 1998a). Since plants contain complex multigene families of GSTs, the various sub-units may be able to produce multiple homo- and hetro-dimeric GST isoenzymes (Dixon et al., 1998a, b). The presence of a large number of isoenzymes with a differential, overlapping substrate selectivity, affords the organism the possibility to detoxify a wide range of reactive xenobiotics, by catalyzing their conjugation with GSH, or by ‘trapping’ them through non-covalent or covalent binding. The extent to which detoxification and/or activation will occur depends on the number and amount of specific isoenzymes present in a tissue (Vos and Bladeren, 1990). Therefore, the overexpression of these genes in suitable plant species is essential to enhance their catabolic potential.

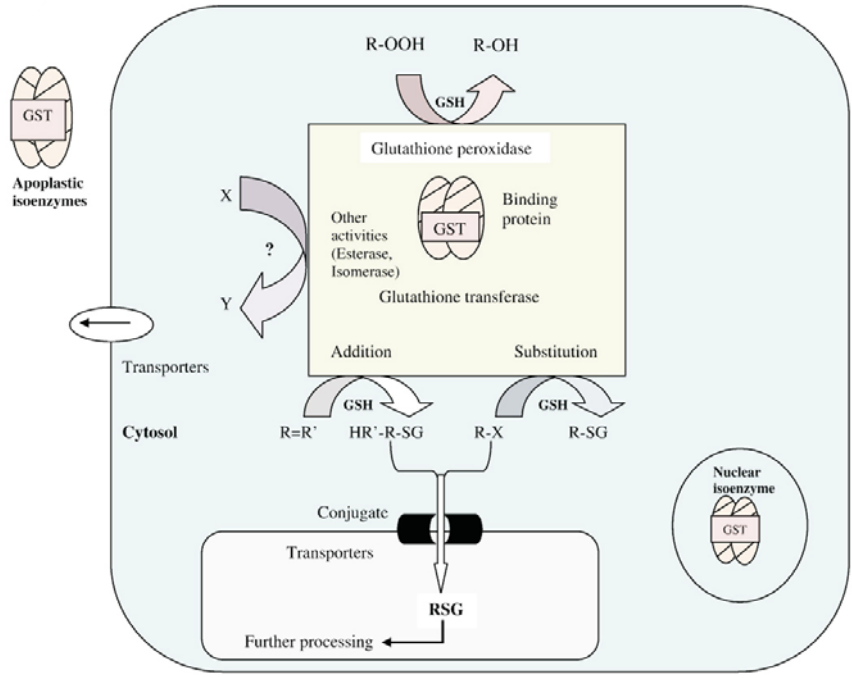

Fig. 6.6: Schematic representation of the role of GSTs in xenobiotic detoxification and endogenous metabolism. It is a proven fact that true detoxification reactions in Phase II performed by GSTs, rendering the compound under consideration less toxic because of conjugation (usually in a substitution reaction but occasionally as an addition reaction), and then a set of further reactions that include cleavage, rearrangement, secondary conjugation etc. Majority of the plant GSTs are supposed to be cytosolic; however, there is evidence for the existence of apoplastic and nuclear isoenzymes (Dixon et al., 1998a, b). The conjugated molecules (R-SG) are then transported to the vacuoles for further processing. The alternative activities of GSTs include glutathione peroxidase, esterase, isomerase and binding activities, which may play additional roles in endogenous metabolism (modified from Dixon et al., 1998a, b).

Poplar plants were transformed to overexpress the bacterial gene encoding γ-glutamyl-cysteine-synthetase (γ-ECS), which is the rate limiting regulatory enzyme in the biosynthesis of GSH (Noctor and Foyer, 1998). The transformed plants showed enhanced levels of GSH and its precursor γ-ECH (Noctor et al., 1996). The increased production of GSH contributes to the antioxidative protection of plant cells against oxidative stress caused by various environmental factors (Noctor and Foyer, 1998). Gullner et al. (2001) reported that increased levels of glutathione have been shown to increase the resistance to chloroacetanilide herbicides in transgenic poplar plants expressing γ-glutamylcysteine synthetase. Karavangeli et al. (2005) developed transgenic tobacco plants overexpressing maize glutathione S-transferase I for enhanced phytoremediation of chloroacetanilide herbicide. The isoenzyme GST I from maize exhibits

significant catabolic activity for the chloroacetanilide herbicide alachlor and appears to be involved in its detoxifying process. The transgenic plants showed substantially higher tolerance to alachlor compared to non-transgenic plants in terms of growth and development (Karavangeli et al., 2005).

Recently, Schroder et al. (2008) reported the glutathione dependent detoxification (conjugate detoxification) of organic xenobiotics (acetyl salicylic acid, lamotrigin, paracetamol, 1-chloro-2, 4-dinitrobenzene (CDNB), fenoxaprop, and propachlor) in Phragmites australis [(Cav.) Trin. Ex. Steud.]. A study of Brenter et al. (2008) showed that expression of glutathione Stranferases in poplar trees (Populus trichocarpa) resulted in a significant increase of gene expression to GST, peaking at levels of 25 and 10 fold the expression level of non-exposed plants after 24 h of each of the GST genes, respectively (Brenter et al., 2008). Brassica juncea over-expressing γ-glutamyl-cysteine-synthetase and glutathione synthetase also have shown enhanced tolerance to atrazine, CDNB, metolachlor and phenanthrene (Flocco et al., 2004). Recently, Dixit et al. (2008) introduced human P4502E1 and GST from fungus Trcihoderma virens in *N. tabacum*. The transgenic plant has shown enhanced degradation of anthracene and chloropyriphos. Thus, it is expected that the transgenic expression of both human P450 and glutathione conjugation enzymes in plants will provide enhanced detoxification and therefore improved remediation of organic xenobiotics.

6.5.4 Rhizoremediation of Organic Xenobiotics

One of the most promising approaches to enhancing the phytoremediation technology is the insertion of xenobiotics degrading genes into the root system of suitable plant species for the enhanced rhizospheric secretion and the subsequent degradation of pollutants (Glick, 2003; Gerhardt et al., 2009; Kawahigashi, 2009). The advantage of this method is that the plants do not need to take up the pollutants in order to detoxify them; instead, the secreted enzymes can degrade the pollutants in rhizospheric zone (Kawahigashi, 2009). The rhizosphere is the soil in the immediate vicinity of a root that is affected by root processes. It comes into being when a root tip enters a volume of soil and disappears some time after the root has died and decomposed (Darrah et al., 2006). Many studies demonstrate significantly enhanced dissipation and/or mineralization of persistent organic pollutants at the root–soil interface (Anderson et al., 1993; Anderson and Coats, 1995; Kuiper et al., 2004; Chaudhry et al., 2005; Abhilash and Singh, 2009b). This rhizosphere effect is generally attributed to an increase in microbial density, diversity and/or metabolic activity due to the release of plant root exudates, mucigel and root lysates (enzymes, amino acids, carbohydrates, low-molecular-mass carboxylic acids, flavonones and phenolics) (Curl and Truelove,1986; Kidd et al., 2008). Further, plants can also increase the physical and chemical properties of the contaminated soil, and increase contact between the root-associated microorganisms and the soil contaminants (Fig. 6.7).

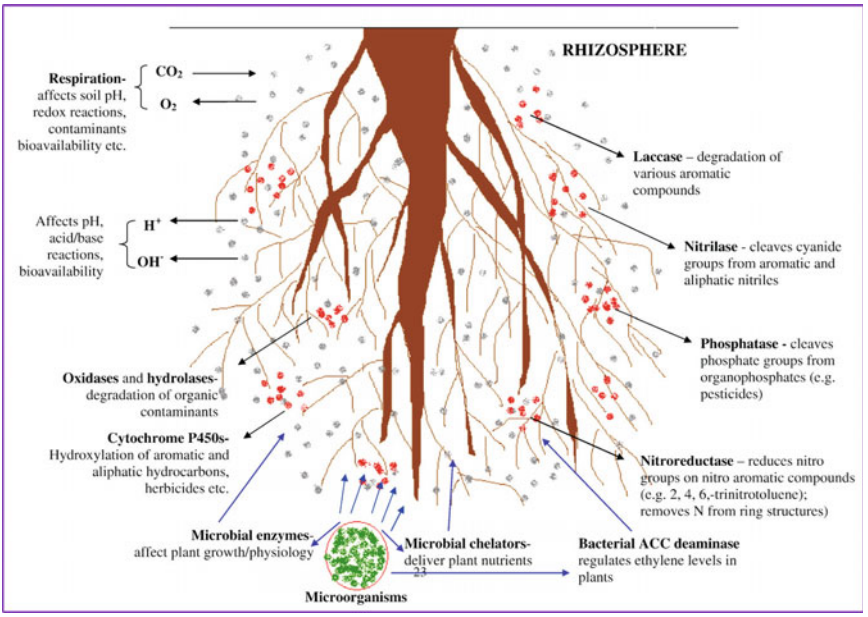

Fig. 6.7: Schematic representation of the enzymatic and microbial activities responsible for the enhanced remediation in rhizospheric zone (Transferred from Abhilash et al., 2009).

Transgenic plants that secrete detoxifying enzymes can be useful for the rhizoremediation of wide range of hydrophobic chemicals. Wang et al. (2004) developed transgenic Arabidopsis plants secrete a root-specific laccase (LAC1) from cotton plants to the rhizosphere and have shown enhanced resistance to phenolic allelochemicals and 2, 4, 6-trichlorophenol. Similarly, Sonoki et al. (2005) transformed tobacco plants with an extracellular fungal laccase from Coriouls versicolar for the rhizoremediation of bisphenol A and PCP. Furthermore, the overexpression of a bacterial biphenyl dioxygenase (BPDO) from Burkholderia xenovorans LB400 resulted in the enhanced oxygenation of 4-chlorobiphenyls (Mohammadi et al., 2007). BPDO catalyzes a stereospecific oxygenation of biphenyl, producing cis-2,3-dihydro2,3-dihydroxybiphenyl-2,3-dehydrogenase. The 2,3-dihydroxybiphenyl is then cleaved by the 2, 3-dihydroxybiphenyl-1,2-dioxygenase (2, 3DHBD) and the resulting 2-hydroxy-6-oxo-6-phenyl-hexa-2,4-dienoic acid (HOPDA) is hydrolysed producing benzoic and pentatonic acids (Sylvestre et al., 2009). The encoding genes for PCB degradation in Burkholderia xenovorans LB400 are bphA (BphAE subunit), bphE (BphAE β subunit), bphF (BphF) and bphG (BphG) (Sylvestre et al., 2009). Gene bphC from Pandoraea pnomenusa B-356 was successfully cloned in tobacco plants for the enhanced remediation of PCB (Francova et al., 2003; Novakova et al., 2009). Transgenic Arabidopsis plants expressing the aromatic-cleaving extradiol dioxygenase (DBfB) resulted in the enhanced degradation of 2, 3-dihydroxybiphenyl (2, 3DHB) (Uchida et al., 2005).

Similarly, transgenic tobacco plants expressing haloalkane dehydrogenase (DhaA) accelerated the detoxification of 1-chlorobutane in rhizospheric zone (Uchida et al., 2005).

Apart from the presence of catabolic genes, the success of phytoremediation depends upon the morphological and intrinsic properties of the plant species itself. Therefore, the choice of plants is likely to impact on the success of the rhizoremediation technology (Sylvestre et al., 2009). A plant species with large aboveground biomass is crucial for the phytoextraction, whereas the plant species with extensive root system or belowground biomass is important for the rhizoremediation. However, the growth of the plants in contaminated sites is normally hindered by the pollutants. Accelerated ethylene production in response to stress induced by pollutants is known to inhibit the root growth and is considered a major obstacle to phytoremediation (Kawahigashi, 2009). Previous studies proved that bacterial 1-aminocyclopropane-1-carboxylate (ACC) deaminase regulates ethylene levels in plants by metabolizing its precursor ACC into α-ketobutyric acid and ammonia (Bernard, 2005; Arshad et al., 2007). Interestingly, this ACC deaminase has been detected in some plant growth-promoting bacteria (PGPR), and thus regulates the biosynthesis of ethylene in inoculated plant roots (Glick et al., 1998; Glick, 2005). Transgenic plants that express ACC deaminase genes can reduce ethylene levels, resulting in a more extensive root system (Arshad et al., 2007). It is expected that the resultant increase in root growth provided by ACC deaminase might enhance the rhizoremediation potential. Furthermore, incorporation of multiple genes related to the different phases of xenobiotics degradation, together with ACC deaminase, may improve the remediation potential of transgenic plants (Kawahigashi, 2009).

6.6 Modeling for Simulation of Pesticide Degradation

The efficiency of a bioremediation system is estimated from effluent composition showing the result of removal by sorption, degradation and volatilization. The degradation activity of the system can be assessed in degradation assays on matrix samples. These degradation data can be entered in transport models to estimate the bioremediation efficiency (Sniegowski et al., 2009). To model the pesticide degradation kinetics in a biofilter, models that either take microbiological growth into account or models that do not can be applied. The former is of relevance in case the biofilter system contains microbial populations which mineralize the compound with formation of biomass, CO_2 and other harmless minerals, which is the preferential outcome in treatment of pesticide-contaminated water. Sniegowski et al. (2009) propagated the use of pesticide-primed soils as inoculum for on-farm bioremediation systems. Pesticide-primed soils originate from long-term treated agricultural fields and contain often adapted microbial populations possessing pathways to mineralize the pesticide.

Currently, they are examining the potential of this bioaugmentation strategy in laboratory biofilter microcosms (BM) using linuron (3-(3,4-dichlorophenyl)-1-methoxy-1-methyl urea) and a linuron-treated soil harbouring a population which mineralizes linuron and uses it for growth as models.

Sniegowski et al. (2009) compare pesticide degradation models which simulate the response of biofilters for treatment of pesticide-contaminated waste water to time-irregular pesticide supply in which the pesticide is used for growth and mineralized. Biofilter microcosms containing a mixture of straw, peat and soil and harbouring micropopulations which use the herbicide linuron for growth, were irrigated with linuron for 28 weeks with a stop in its supply between week 12th and 17th. Matrix samples were regularly taken to assay linuron mineralization. A first-order approximation of the Monod model was used to simulate the observed mineralization data, while an inverse modelling framework combining a sensitivity analysis (Morris Sensitivity Analysis) with an inverse modelling approach (Shuffled Complex Evolution Metropolis) adopted to parameterize the model. Lag times in linuron mineralization decreased during the initial weeks of linuron irrigation but increased after supply of linuron ceased. The model well-simulated the lag time dynamics which were related to the dynamics of the predicted linuron-degrading population size in the microcosms. It was predicted that the population size decreased at a rate of 0.031 d^{-1} after pesticide supply ceased to reach its initial population size after 25 weeks. They find that modelling pesticide degradation in biofilters should incorporate biomass dynamics in case the pesticide is used as C-source. First-order approaches without incorporating biomass dynamics could lead to underestimation of the risk of pesticide leaching.

6.7 Conclusion

At the global level, the use of pesticides has proved to assist solving of many problems facing human health and food production. However, such usage has occasionally been accompanied with hazards to man and the environment. At national, regional, and international levels, the problem of pesticide harm should be handled scientifically within collaborative action plans; to take as much benefits as possible from using these toxicants without significant hazard to human beings. To promote initiation of the concerned plans, the situation of pesticides in each country should be thoroughly investigated and clarified.

Microbes are degrading pesticides in environment and use them for their normal metabolic processes as carbon or phosphorus source or consume the pesticides along with other source of food or energy. This bioprocess of microbes can be utilized for the development of pesticide decontamination and restoration of health of the environment. The use of pesticide-degrading microbial systems for removal of pesticide compounds from the contaminated sites requires an understanding of ecological requirements of degrading strains involved in degradation processes. There is a need for further research on the biochemical and genetic aspects of pesticide degradation by microbes.

Hydrolytic enzymes, responsible for degradation of pesticides to non-toxic products in the environment, provide informed decisions on which genes to engineer. It has been suggested that increased understanding of the enzymatic process involved in plant tolerance and detoxification of pesticides will provide new directions for manipulating plant with superior remediation potential. Further, some of the engineered plants are unsuitable for field application because of its small biomass and growth rates. Although more focussing on transgenic plants has been made, their potential as engineered phytoremediation plants was not examined extensively in field trials. The ecological impact and underlying economics of phytoremediation with transgenics should be carefully evaluated and weighed against known disadvantages of conventional remediation techniques or risks of having the recalcitrant heavy metal or metalloid species in our environment. Investigations needed for the detailed biochemical and physiological analysis of the whole process of phytoremediation—a group of innovative technological approaches (detailed characterization of all types of oxidases catalyzing degradation of organic contaminants, and of transferases and other enzymes participating in the detoxification process etc.) should be continued; the creation of new, modified, genetically stable, environmentally safe, highly effective vegetation (growing under different climatic conditions) trees, grasses, legumes, terrestrial or aquatic plants; the selection of microorganisms (bacteria, fungi, actinomycetes) with special attention to symbiotic action and scaling up of phytoremediation processes.

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7

Genomics Approach to Bioremediation

V.V. Lakshmi

7.1 Introduction

A range of pollutants are being added to the environment by human activities. Majority of these contaminants are chemically synthesized compounds also termed as xenobiotics. Some of these persist in the environment for longer periods due to presence of structural elements or constituents that do not occur naturally in nature and therefore are resistant to attack by degradative enzymes. Pollutants like nitro aromatics, polycyclic aromatics and biphenyls are degraded relatively within a short period of time or transformed into non-toxic end products owing to microbial degradation. However, highly nitrated and halogenated compounds as well as pesticides and explosives are chemically inert with longer half-lives under native conditions making them recalcitrant. Due to their poor water solubility they tend to enter the food web and are subsequently biomagnified. In addition, many of these pollutants are often metabolized in the mammalian cells resulting in intermediates that are potentially mutagenic or carcinogenic agents (Henschler et al., 2001; Galloway and Handy, 2003). This has led to imposition of prohibition on use of several of these compounds. Apart from this, large quantity of radioactive waste is also generated as a byproduct from nuclear plants generating atomic energy. This bio-hazardous waste is disposed in multiple waste dumping sites worldwide. Currently there is no efficient technology to store this waste safely. Radio nuclides need to be detoxified or effectively immobilized to prevent leaking from the contaminated sites. Ecological disasters resulting from accidental spills of the accumulated toxic waste, not only affects terrestrial ecosystem and marine life but also influences the economy of the area (Samanta et al., 2002; Brim et al., 2003; Regueiro et al., 2007).

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Physical and chemical methods like thermal desorption, soil washing, incineration, land filling etc. have been employed in treatment of several contaminated sites. However these treatments do not effectively restore flora or fauna of the area (Spain, 2000). The rate of xenobiotic bioremediation is significantly influenced by external factors/unfavourable conditions including adverse temperature, nutrients deficiency, availability of appropriate electron acceptor, toxicity of the compound or the intermediates produced resulting due to slow conversion in nature (Dua et al., 2002; Carvahlo et al., 2005). Techniques like biotransformation, bioaccumulation, biosorption, biostimulation and bioaugmentation have been tried in situ with limited success. In bioaugmentation native biotype is supplemented by strains or population of known biodegrading organisms to accelerate cleanup process. However, implementation of such techniques is not always successful in view of difficulties to control and scale up the process from laboratory to on-site scale. This is highly dependent on an in-depth understanding of the physico-chemical characteristics of the contaminated environment as well as characterization of the microbial communities in terms of their biocatalyst capabilities, functional potential and interactions in environment (Atlas and Unterman, 1999; Alexander, 2001).

To address the challenges of clean up of contaminated sites several emerging technologies are utilized in environmental biotechnology. Bioremediation of xenobiotics and radio nuclides has emerged as a promising approach to remove toxic pollutants from the environment. It is a multi faceted process in which both biotic and abiotic factors mediate the removal process. Bioremediations take advantage of the catabolic versatility of microorganisms to convert complex waste (Abed et al., 2002). A remarkable range of detoxification abilities involving complex pathways are exhibited by the microbial communities. Radionuclides including uranium, technetium and chromium have been subjected to enzymatic detoxification by metabolic cooperation of microbes. Microbial mediated restoration of contaminated sites is an ecologically acceptable, environmentally friendly, safe and effective approach (Abraham et al., 2002; Brim et al., 2003; Kumar et al., 2007).

7.2 Genomic and Metagenomic Approaches

Microorganisms represent two out of three domains of life and have vast diversity. Traditionally identification and characterization of microbial communities in contaminated sites was limited to culturable organisms. In post-genomic era, genome-wide analysis provided a deeper insight into the metabolic diversity in microorganisms. The first step in this daunting task of exploration was to sequence the genomes of several adaptable bacteria identified to be involved in bioremediation that possess a variety of catabolic genes. However, it is increasingly being realized that only a fraction of microorganisms involved in biodegradation are culturable. The present culture techniques used are biased towards organisms that are best adapted to the laboratory conditions. This

preferential growth distorts the actual microbial community composition of the environment. Hence investigations employing traditional cultivation approaches alone have become unacceptable as only tiny portion of environmental bacteria that are cultivable in laboratory are targeted (Torsvick, 2002; Daniel, 2005; Malik et al., 2008).

The biggest boom in microbial ecology started in 1995 with application of molecular tools to study the biodiversity and understand dynamics of microbial community in finer details. Culture-independent methods (CIM) emerged crucial in understanding the genetic diversity, population structure and ecological roles of the majority of habitats. The molecular methods rely on extracting and characterizing biomolecules such as nucleic acids, proteins, fatty acids and other compounds directly obtained from the environment, that are specific for the taxa. CIM has revealed startling richness of the uncultured microbial world leading to renewed trend of exploring the unknown microbial territories similar to that observed in 16^{th}-17^{th} century, but with employing molecular tools. The initial attempt in this direction was to sequence clones from 5S rRNA cDNA library. However, most convincing scenario of vastness of microbial diversity emerged from various studies employing 16S rRNA, which were highly successful in reconstructing phylogenies and compared microbial distributions among different samples. To achieve this nucleotide sequence along with fingerprinting were widely employed in addition to quantifying the relative abundance of each taxonomic group using membrane hybridization or fluorescent in situ hybridization (FISH) (Lane et al., 1985; Pace et al., 1985; Woese, 1987; Handelsman, 2004; Sanz and Kochling, 2007).

Genome representation of about four to seven thousand has been estimated per gm of soil using molecular tools. However, even utilizing the current r-RNA based phylogenetic approaches also only 50-200 microbial forms/gm are monitored, thus still leaving a major fraction unexplored. This scenario is mainly accounted for by the issues related to DNA extraction efficiency, primer specificity sensitivity and lower limits of detection for the available technologies, in addition to high initial amount of biomass load. These limitations destroy or distort the community structure and diversity present within both micro as well as macro habitats (Polz and Cavanaugh, 1998; Rappe and Giovannoni, 2003). The present Gene Bank's entries of 16S rRNA genes from uncultured prokaryotes have significantly out numbered those from cultured microorganisms, the former being more than double. This uncultured fraction includes diverse organisms that have no affiliation or are only distantly related to the phyla of cultured ones. Thus bulk of microbiota still remains a vast untapped resource for application (Lovely, 2003; Handelsman, 2004, 2005).

Similarly, in environment also, the characterized organisms—known to play role in biodegradation by culture techniques—do not accurately represent the microbial community composition in nature either qualitatively or quantitatively. This has been clearly depicted on comparison of gene pools capable of degrading several xenobiotics. The uncultivable organisms in these

cases are predicted to account for a significant fraction of total diversity which is not reflected in culture based methods. In the past two decades integration of newer CIM techniques and improvisation of existing ones have contributed significantly to detection of several novel catabolic genes from contaminated sites especially from non-cultivable organisms (Widada et al., 2002, Van Hamme et al., 2003).

Limitations to bioremediation in natural conditions and low specificity of the conventional methods have paved the way for development of alternate novel methods in the form of Genetically Modified Organisms (GMOs) or 'designer biocatalysts'. These incorporate modified enzymes that mediate novel metabolic routes, or have increased efficiency of catabolic activity by widening substrate range thereby improving existing pathway for bioremediation. With technical advancements in several fronts it has now become feasible to construct GMOs by reshuffling structural genes encoding desired properties from various species (Furukawa, 2003; Boubakri, 2005). The selected gene modules are then tailored and put under control of a strong promoter to construct novel/improved biocatalyst strains that can handle xenobiotics in laboratory as well as in in situ conditions. Further, accessibility to rapidly growing metabolic pathway databases and increased understanding of the structure-function relationships of the enzyme components is assisting in rational optimization of biocatalysts (Pieper and Reineke, 2000; Krause et al., 2006).

Several successful attempts for designing potential biocatalysts have been made in the last two decades including those for treating site contaminated with polychlorinated biphenyls (PCBs) and chloro-aromatic compounds. Just to consider a few examples: Substrate specificity of biphenyl dioxygenase enzyme from *Pseudomonas* sp. was altered by combining homologs obtained from *Pseudomonas* sp. LB400 and *Pseudomonas alcaligenes* KF707 species. The resulting variant of the active enzyme could hydroxylate both double ortho- and double para-substituted PCBs efficiently. In another attempt, site-directed mutagenesis, gene shuffling, assisted by computerized modelling of 3D protein structures, assisted the successful tailoring of haloalkane dehalogenase. The resulting enzyme with specific amino acids replaced at the active site had several-fold increased enzyme activity thus enhancing efficiency for bioremediation of pollutants (Bohac et al., 2002; Chaloupkova et al., 2003; Okuata, 2004; Cases and Lorenzo, 2005).

With the prospective use of GMOs in situ, interesting debates have been initiated on the containment of these constructs and potential ecological risks involved with leakages. Problems associated with horizontal gene transfer, tracking modified genes/strains and schedule cell death of the biocatalyst after completing the desired task were addressed to minimize ecological risks. To achieve this, 'killer-anti-killer' gene pairs associated with bacterial plasmids/chromosomes or bacteriophages are being exploited. Molin et al. (1987) proposed the first containment system based on expression of two different genes—the toxin or poison and a respective antitoxin or antidote

placed under the regulation of catabolic regulatory genes. The killer toxin was stable for hours whereas its anti-killing counterpart was very labile. In this process cells escaping the contaminated environment are killed by the induction of a controlled 'suicide' system thereby restricting their survival and spread (Molin, 1993). A further refinement with constitutive expression of cognate anti-killer gene controlled by environmental signals was designed to decrease the probability of escape from programmed cell death. In recent years, a dual lethal system was introduced wherein lethal genes like colicinE3, RNase and EcoRI endonuclease that simultaneously attack different cellular targets to control cell death. This ensured drastic reduction in the chance of escape as simultaneous mutations in several enzymes was more improbable. Thus 'Bacterial containment systems' were designed to prevent their escape into the environment thereby ensuring a 'fail-safe' system for introduction GMOs into the environment for bioremediation (Zielenkiewicz and Ceglowski, 2001; Torres et al., 2003).

7.2.1 Metagenomic Shift

Describing the phylogenetic diversity of uncultured microorganisms is only the first step in understanding biodiversity. The greater challenge lies in assigning ecological roles to the organisms as uncultured microbiota are increasingly being realized to play pivotal roles in natural environmental processes. Thus it was vital to determine function of gene at protein or metabolism levels than mere sequencing. The shift in emphasis to genome-wide analysis of the currently available genome data rather than individual genes has revealed that about 30-40% of the fully sequenced organisms have no known functions assigned at biochemical level, many of which are unrelated to the known genes. Thus functional genomics has emerged as a new discipline employing major innovative technologies utilizing traditional as well as novel approaches for genome-wide searches supported by bioinformatics tools (Langer et al., 2006; Valenzuela et al., 2006; Falk and Hugenholtz, 2007; Ferrer et al., 2009).

Metagenomics goes a step forward and aims to transcend the limitations of the individual organism to the "meta level" by targeting community profile. Microbial community from environmental sample is mined directly by extracting data from both known and novel microbial forms. The term "metagenomics" was coined by Handelsman et al. (1998), while describing acid mine biofilms. Thus, whereas genomics determines the complete genetic complement of an organism only, by contrast metagenomics involves sampling the genome sequences of a community of organisms inhabiting a common environment. The definition, in application, excludes studies using PCR to amplify gene cassettes or access genes of interest only as they do not provide genomic information beyond the genes being amplified. In principle, any environment is amenable to metagenomic analysis provided that nucleic acids can be extracted from sample material. Metagenomics has become the centre of focus approach in last five years and has been applied to several niches/

environments, including oceans, soils, thermal vents, hot spring samples and human mouth and gastrointestinal tract (Sebat et al., 2003; Langer et al., 2006; Warnecke et al., 2007; Hugenholtz and Tyson, 2008). The approach is also termed as community genomics, ecogenomics, or environmental genomics. The process requires no prior separation of organisms from its habitat or maintaining microorganisms as either pure or mixed culture in laboratory for sequencing thus by-passing major limitations of classical approaches in microbiology. Strategies of metagenomics also circumvent several limitations due to direct cloning of large DNA as well as proper representation of finite number of clones to screen. Metagenomic studies involve massive sequencing to capture entire communities which have become possible with advances in sequencing technology like pyrosequencing. Thus accessing the potential of the collective microbial genomes in a given environment simultaneously using functional sequence-based analysis employing high-throughput methods is now feasible (Cowan, 2005; Tringe and Rubin, 2005; Dinsdale, 2008).

Metagenomics has been initiated at all three scales. Small scale target a slice of community for a selective function of interest or by employing specific techniques and initiated mainly by single-investigator or laboratory. Middle scale metagenomic projects employ multidisciplinary approach to thoroughly investigate a community of interest. The scope of these projects is diverse and is undertaken by group of collaborating investigators each targeting one facet. Large-scale project on the other hand encompass global initiatives on big scale to understand selective microbial communities in depth with detailed characterization. Metagenomics applied to community genomics of environment had the greatest impact in the last few years (Cowan, 2005; Daniel, 2005; Dinsdale, 2008; Ferrer, 2009). Pipeline of the metagenomic project design can be broadly categorized into following stages.

7.2.2 Pre-sequencing Considerations

Community composition has a great influence on mode and results of metagenomic analysis. Prior knowledge of dominant population is helpful in designing subsequent strategies. The issue of assessing how much sequence data is to be obtained for a community also becomes very crucial as there is no fixed end point, unlike in complete genomes. Since species differ in gene coding densities of their genome and do not have uniform abundance in a community, it is simple to address the coverage of individual populations. To obtain greater than single sequencing coverage, the size of a moderate metagenomic library would need to be many times the size of the metagenome which can reach upto 100 Mbp with a typical Sanger sequencing. Generally a 6× to 8× coverage of microbial isolates is a common target to obtain a draft genome suitable for metagenomic project (Dinsdale, 2008; Kunin et al., 2008; Ferrer, 2009). Ultimately, an objective of the study guides the sequence allocation. Thus, although a coverage of 20× or higher may be ideal to determine SNP frequency profile for the dominant population, to identify over-represented

gene functions in a community as a whole; however, a much less sequence data would be adequate. Recent studies have shown that even extremely low coverage of <0.01× dominant population of stratified hypersaline mat community were still sufficient to detect genetic gradients (Goldberg et al., 2006, Kunin et al., 2008).

7.2.3 Sampling Strategies

Extraction and purification of high-quality DNA still remain main bottlenecks in metagenomic studies as there is no "one-size-fits-all" extraction method for all samples. Low-biomass of environmental sample yields insufficient DNA for library construction or pyro-sequence analysis, where microgram quantities of genomic DNA are required (Kunin et al., 2008). During sampling it is essential to collect additional sample material and all possible collateral non-sequence data associated with an environmental sample or "meta data". Type of metadata can vary considerably depending on the sample type. Some of the common ones for environmental studies are geographical data such as global positioning system coordinates, depth/height of sample collection, data on pH, temperature, salinity of the site etc. (Venter et al., 2004; Kunin et al., 2008; Urich et al., 2008). This complementary information is crucial for analyses as re-sampling might not be always directly comparable. Meta data enhances the ability to interpret sequence data significantly, particularly for a comparative analysis of temporal or spatial organization. Co-localization of organisms by combining FISH with digital image analysis can provide spatial information in structured ecosystems. This can provide vital support to derive metabolic interactions between community members as inferred from metagenomic data (Lebaron et al., 1997; Sanz and Kochling, 2007). Tyramide signal amplification FISH (TSA-FISH using fluorescence labelled tyramides are being employed to increase the sensitivity by enhancing the hybridization signal. Non-radioactive probes like digoxigenin uracil triphosphate ([DIG]-UTP) label are among the method of choice currently (Schonhuber et al., 1997; Pernthaler et al., 2004).

7.2.4 Data Generation

Prior to metagenomic data generation, a Pre-Metagenome Community Composition Profiling (PCCP) is usually undertaken as a prescreening. This aids subsequent decision on sequence allocation and processing based on community composition of the environmental sample. This is usually followed by shotgun library preparation wherein at least three different average sizes of 3, 8, and 40 kbp cloned DNA in fosmids are used. Global metagenomic projects undertaken by Joint Genome Institute (JGI) have evolved a 4:1 ratio between the smaller and larger fragment sizes to produce high-quality draft assemblies (Warnecke, 2007; Kunin et al., 2008).

Selection of sequencing technology is critical for quality as well as cost effectiveness of the project. Recently pyrosequencing has been added to the

universally used Sanger's approach. Though Sanger (dye terminator) sequencing still remains the primary source of metagenomic sequence data, pyrosequencing strategies have been increasingly applied to viral and bacterial communities. Advantages of pyrosequencing over Sanger approach is a much lower per base cost and no necessity for prior cloning whereby cloning bias are avoided. The major disadvantage of pyrosequencing is its short average read lengths. This ranges from a maximum of 100 bps on the GS20 to 200 bps on the GS FLX platforms (Nulton, 2006; Wommack et al., 2008). Any further enhancement in this capability would be an asset for metagenomics. Due to this short read lengths of sequence, the approaches mainly based on similarity searches against a reference database instead of gene calling or assembly which are not feasible in majority of instances. Combining different sequencing technologies together is being explored for producing high-quality draft assemblies. At least two stages of quality control are considered to be mandatory to get reliable data information (Edwards et al., 2006a, b; Goldberg, 2006).

7.2.5 Sequence Processing

This step encompasses a critical step in pre-processing of sequence reads prior to assembly, gene-prediction, and annotation. Screening by base calling of raw data coming off the sequencing machines is carried to remove cloning vector sequences followed by quality trimming to remove low-quality bases. Errors in these steps have enormous impact on downstream consequences in metagenomics. In contrast to genomics, metagenomic projects tend to retain only high quality reads and contigs as the process has low tolerance for contaminating reads, and are not easily distinguishable thereby skewing downstream analysis (Chou and Holmes, 2001; Kunin et al., 2008). Other issues that need attention to minimize mis-assembly include non-uniform read depth repetitive regions, etc. Overlooking these aspect can greatly hamper both the quality and reliability of metagenome output. Assembly process combines sequence reads into contiguous stretches of DNA based on sequence similarity, called contigs (Raes et al., 2007).

Genome closure or finishing is not possible for most of metagenomes though it finds common place in isolated microbial genome projects. However, finishing may be considered as a viable option only for data sets pertaining to dominant populations within a metagenome (Hallam et al., 2006). Complete or near-complete draft-level coverage genomes have been assembled from metagenome populations present in biofilms in acid mine drainage, in activated sludge and in marine environment (Kunin et al., 2008a, b, c). Metagenomic sequences of highly complex microbial communities are generally expected to result in little or no assembly of reads, which excludes the use of micro-heterogeneity for analyses. Still high coding density of bacterial and archaeal genomes and their average gene size have yielded acceptable reads that capture most of the coding sequence. Thus metagenomic data is found to encompass the genetic micro-heterogeneity present in that population.

7.2.6 Gene Prediction and Annotation

Genes and gene fragments from a given metagenomic data set are mapped into gene families, thereby getting an estimate of their relative representation. To enhance the assignment of function to sequenced data, gene-centric trends can be adopted in metagenomics which was first described by Tringe et al. (2005). The method focusses on assigning roles to proteins encoded in the sequenced genes. Two different possible approaches are adopted in this mode of analysis namely unselective or targeted metagenomics. In unselective mode there is no prior selection based on either growth or PCR amplification. The data generated is a result of gene 'fishing' expedition. The approach though has advantage of providing highly valuable information of the metabolic activity of community not known earlier; it suffers from the disadvantage of possible low hit rate (Raes et al., 2007; Kunin et al., 2008a, b). In contrast, in the second approach of targeted metagenomics, the isolated pool of DNA is deliberately 'biased' to enhance the hit rates. Numerous strategies are adopted like pretreatment of the microbial community with target pollutant to increase the metabolic activity of the prospective genes, pre-fractionation of the DNA on the basis of G+C%, amplification of target regions by PCR before metagenomic analysis, selection of particular cells by cell-sorting procedures using high throughput modes to collected enriched population of organisms etc. This is followed by functional screenings of the libraries adopting techniques like dual culture assay, substrate induced gene expression system (SIGEX), and florescent activated cell sorting (FACS) enabling identification of potential active products. This approach was successful in identification of several hydrocarbon-induced genes followed by capture of relevant genes, utilising expression of green florescent protein (Yun and Ryu, 2005; Raes et al., 2007).

There are two principal modes of gene prediction: evidence-based gene calling and "ab initio" approach. The evidence-based method depends on using homology searches to identify ORFs and has been adopted in tools like BLAST CRITICA, Orpheus etc. (Badger et al., 1998; Badger and Olsen, 1999). The other prominent approach of "ab initio" gene calling relies on intrinsic features of the DNA sequence to discriminate between coding and non-coding regions, allowing the identification of genes even in absence of any homologs in the available databases. Gene training sets are used to improve the quality of the obtained data and enhance prediction of genes. Training sets could be derived by setting parameters derived from known genes of the same or related organisms like in case of fgenesB programme, while several others rely on generating self-trained data sets from the target sequence e.g. GLIMMER, MetaGene etc. whereas combination of these tools is employed in mORFind (Delcher et al., 1999; Noguchi et al., 2006). The gene prediction is generally followed by functional annotation utilizing available meta data similar to the genomic annotation. However, the fragmentary nature and lack of neighbourhood context makes it more challenging in metagenomics. In

genomics, gene predictions and annotations are finally checked manually to increase reliability as a part of quality control but such manual curations are not presently feasible in metagenomes which is recommended only at large contigs levels. Thus metagenomic has been successful in study of single genes, pathways, organisms and communities (Tyson, 2004; Tringe et al., 2005; Mavromatisey et al., 2007).

7.2.7 Data Analysis

The collection of reads, contigs, and genes, resulting from metagenome pipeline, have to be associated with the organisms from which they were derived in terms of taxonomic groups. This is highly desirable for meaningful interpretation of the contributing species of the ecosystem and this process is called binning or classification. Present available binning tools rarely have the resolution to discriminate between strains of the same species, so strain co-assembly is not practical. The standard processing steps in Post Sequencing Community Composition (PSCC) are re-evaluation of the community composition estimates directly from the metagenomic data itself. This is important for interpreting data and avoiding skewing (Kunin et al., 2008a, b, c). This is followed by analyzing dominant populations by mapping of conserved phylogenetic marker genes to assess both organism identity as well as their relative abundance. If more than one dominant population are sequenced, the probable metabolic interplay among the populations is considered (Whitaker and Banfield, 2006; Raes et al., 2007).

7.2.8 Tool Box of Genomics/Metagenomics

Several tools are applied for the visualization and identification of polymorphism in composite population. Prominent tools used in genomics and metagenomics address microbial communities profile from diverse facets (Gilbride et al., 2006; Gabor et al., 2007). Nucleic acid based techniques like polymerase chain reaction (PCR) and its numerous variants are applied for analysis of catabolic genes involved in the biodegradation of organic contaminants as well as for microbial community profiling (Stenuit et al., 2006, 2008). Inherent limitations of extraction and purification of nucleic acids both in quality and quantity from environmental samples significantly effect analysis by PCR amplification. The presence of inhibitory substances which are co-extracted like humic acids, organic matter and clay particles significantly interferes in amplification step. Multiplex PCR technique as a single reaction has been used for the detection of mono, or dioxygenase enzyme attacking aromatic naphthalene, toluene, xylene, phenol etc. thereby saving time and resources (Wilson et al., 1999; Baldwin et al., 2003; Kirk et al., 2004; Gilbride et al., 2006).

Real-time PCR (RT-PCR) enables simultaneous detection and quantification of the amplified product while the reaction is still in progress as fluorescent markers are employed. With quantification limit typically of 1–2 genome

copies, RT-PCR is 100- to 10,000-fold more sensitive than microarray-based methods (Inglis and Kalischuk, 2004; Eyers et al., 2004b). Moreover, it does not require any time-consuming post-PCR steps for the quantification of amplicons as they are monitored in real-time and intensity of fluorescence is directly proportional to product concentration. Hence, real time PCR which allows quantification at the end of each cycle is appropriate for high-throughput mode requirements of metagenomics. Further its high analytical sensitivity for identification of specific genes in complex DNA mixture also makes it highly suitable for analysis of environmental samples (Scow and Hicks, 2005; Ritalahti et al., 2006). However the obtained results do not always link gene expression with a specific measurable microbial activity or population due to the short half-life of RNA. Being a very sensitive PCR technique, contaminants are observed to significantly influence the outcome resulting in high false signals also (Spiegelman et al., 2005). Several studies have employed RT-PCR in environmental studies to monitor activities of gene for biodegrading enzymes, a few of which include like aromatic oxygenase, reductive dehalogenase (RDase) carbazole 1,9 a-dioxygenase (carAa), benzylsuccinate synthase (bssA), atrazine catabolic (atz), arsenate reductase (arsC) etc. (Widada et al., 2002a; Baldwin et al., 2003; Lerat et al., 2005; Saleh-Lakha et al., 2005). Community profiling in hydrocarbon-contaminated Antarctic soil was also successfully achieved by observing real time changes. Speed, sensitivity, accuracy and advantage of possible robotic automation makes RT-PCR to occupy a prominent position among genomic and metagenomic tools (Watanabe and Hamamura, 2003; Powell et al., 2006).

Genetic fingerprinting techniques provide specific pattern or profile of a given microbial community. These are based on the separation of amplicons after PCR amplification of phylogenetic markers such as rRNA (16S and 23S rRNA) along with RecA (DNA repair protein), EF-Tu, EF-G HSP70 (heat shock protein), and RpoB (RNA polymerase subunit) or functional genes using universal or specific primers. The application potentials and limitations of various fingerprinting techniques are reviewed (Fromin et al., 2002; Nocker et al., 2007). Among the several genetic community profiling methods available amplified ribosomal DNA restriction analysis (ARDRA), and terminal-restriction length polymorphism (T-RFLP), ribosomal intergenic spacer analysis (RISA) along with denaturing gradient gel electrophoresis (DGGE)/temperature gradient gel electrophoresis (TGGE) are the most popular tools used for environmental samples (Marsh et al., 2000; Eyers et al., 2004a; Forney et al., 2004; Fedi et al., 2005; Bai et al., 2006). Unlike traditional gel separation methods, DGGE/TGGE separate amplified rDNA fragments of the same length but have different base pair compositions. The number of bands produced during DGGE or TGGE is observed to be proportional to the number of dominant species in the sample. The main limitations of DGGE/TGGE is an incomplete phylogenetic specificity provided by the analyzed 500 bp

fragments of 16S rRNA sequences, overestimation of a community multiple bands of the single species due to presence of several copies of rRNA gene, or underestimation due to different rRNA bands having same mobility. In spite of some of these limitations, the method allows monitoring of the spatial/temporal changes in prominent microbial community structure at moderate phylogenetic resolution. This is especially prominent in the samples where microbial diversity is largely unknown (Watanabe et al., 2002; Singh, 2006; Malik, 2008).

ARDRA is a simple, rapid and cost effective method to explore the bacterial community structure or screen clones while analyzing contaminated sites. Though it is good measure for detecting structural changes of microbial community it does not provide an effective picture of phylogenetic diversity in complex population (Weidner et al., 1996; Bai et al., 2006). T-RFLP is a modification of ARDRA that utilizes fluorescent labeled PCR primers allowing quantification of terminal fragments of the digestion. The advantage of this approach is simplification of the banding pattern still retaining reliability. This is highlighted in monitoring of several complex environmental communities as each band represented a single operational taxonomic unit or ribotype (Tajida et al., 1999; Konstantinidis et al., 2003; Reardon et al., 2004; Macbeth et al., 2004; Fahy et al., 2005). Despite the high resolution and sensitivity, T-RFLP being highly dependent on PCR amplification, the biases related to PCR also influence this analysis. This can lead to erroneous conclusions about the abundance of a particular strain or species in a complex microbial community (Kirk et al., 2004; Osborne et al., 2006). Combination of ARDRA with T-RFLP and DGGE has helped to better characterize microbial communities from contaminated sources (Watts et al., 2001; Haack et al., 2004). Application of several fingerprinting techniques in combination with real-time PCR for in situ analysis of xenobiotic degrading bacterial communities and quantification of their catabolic genes showed good correlation between microbial abundance and corresponding pollutant concentration at the site (Widada et al., 2002; Watanabe and Hamamura, 2003; Scow and Hicks, 2005).

RISA, another PCR-based technique targeting intergenic spacer region between the 16S and 23S rRNA operons, has been found to exhibit unique variability in both sequence and length. This is exploited as taxonomic identification marker to distinguish strains or closely related organisms (Eriksson et al., 2003; Spiegelman et al., 2005). Though rapid and simple, in view of shorter sequences analyzed in this method also, complex community profile levels of lower taxonomic resolution are generated (Spiegelman et al., 2005). A quantitative fingerprinting method combining real-time PCR and T-RFLP was utilized for simultaneous determination of microbial abundance and diversity within a complex wastewater community. RT-PCR and RFLP can be integrated with SIP to obtain quantitative data for concomitant identification and quantification of active microorganisms involved in naphthalene degradation in soil microcosms (Yu et al., 2005).

Some of these fingerprinting tools like RISA, T-RFLP, length heterogeneity analysis by PCR (LH-PCR), single strand conformation polymorphism (SSCP) and DGGE have potential for high-throughput design. In their high-throughput versions fluorescent labeled fragments are separated and analyzed on automated DNA sequencers. In DGGE high-throughput version, DNA fragments can be separated within minutes with Denaturing High Performance Liquid Chromatography (D-HPLC), employing fast and repeatable reverse-phase ion-pair chromatography (Barlaan et al., 2005; Spiegelman et al., 2005). The fragments are then collected at the end of the column and subjected to further automated sequencing.

The term environmental gene tags are used akin to expressed sequence tags because of the fragmentary nature of the resulting data of metagenomics. Serial analysis of ribosomal sequence tags/ribosomal DNA (SARST or SARD, respectively) and single-point genome signature tags (SP-GSTs) are two prominent tagging methods that have been evolved for high-throughput profiling of complex microbial communities in addition to the above fingerprinting techniques (Neufeld et al., 2004; Ashby et al., 2007; Kysela et al., 2005; Yu et al., 2006; van der Lelie et al., 2006). These techniques provide fingerprint of microbial communities in the form of concatemers of short information rich PCR-amplified tag sequences like V1, V5, V6 and other hypervariable regions of bacterial 16S rRNA genes. These are further cloned and sequenced to characterize microbial community composition. In this way, multiple ribosomal sequence tags (RSTs) from many different organisms can be obtained simultaneously from a single sequencing reaction. RSTs, as expected, provide only a lower phylogenetic resolution than longer 16S rDNA but are useful to explore the ecological role of low abundance/novel bacteria both in qualitative and quantitative terms (Kayesala et al., 2005; Yu et al., 2006; Ashby et al., 2007). SP-GSTs, though similar to the SARST method in principle, offer a broader probability to study distribution of specific functional genes while examining microbial community composition. The study provided support to the experimental mechanisms proposed for genomic evolution bacteria in nature by lateral transfer of gene fragments and shuffling in the microbial genomes (Boubakri et al., 2006). Metagenomic DNA shuffling approach aims to assess the potential genomic evolution in environmental metagenomes in terms of creation of novel genes under recent environmental pressure like contamination with xenobiotics. Further these approaches hold great promise when combined with the habitat-specific microarrays.

7.2.9 DNA Microarrays

Microarray technology is a powerful taxonomic and functional tool that is widely used at genome and transcriptome analysis including mixed microbial communities. The property of a single stranded DNA or RNA target molecule to hybridize to a complementary probe molecule that is attached to a solid support of slide/chip forms the basic principle of microarrays (Zhou, 2003; Ehrenreich,

2006). Microchips differ according to the immobilization technology used to attach the probes, the length and nature of the probes as well as synthesis and labeling of the targets. The choice between the different technologies is based on parameters such as cost, probe density, specificity, sensitivity and quantification as reviewed (Nees and Woodworth, 2001; Zhou, 2003). Probes can either be directly synthesized in situ using photolithographic masks (e.g., "GeneChip" arrays), electrochemical reactions, or spotted onto the solid support after ex situ synthesis. As fluorescent dyes are enzymatically or chemically incorporated in the sample the readout is based on the detection of fluorescence signals. Thus microarray presents the advantage of miniaturization by spotting thousands of probes on a single slide for simultaneous gene function analysis in real time. In comparison to traditional nucleic acid hybridization techniques, microarrays provide rapid and highly sensitive detection even up to single mismatch (Eyers et al*.,* 2004b). In addition, environmental samples can be incubated in the presence of a radioactively labeled substrate prior to hybridization, in order to identify microorganisms involved in the metabolism of a specific substrate as in "isotope arrays" (Adamczyk et al., 2003; Wagner et al., 2006).

Three major classes of environmental microarray formats have been developed for microbial community analysis, namely,

- Functional gene arrays (FGA) where probes target identifying or measuring genes encoding key enzymes involved in specific metabolic process (Wu et al., 2001; Rhee et al*.,* 2004),
- Community genome arrays (CGA) which are constructed from whole genomic DNA of multiple cultured strains or species (Zhou, 2003; Wu et al., 2004, 2006) and
- Phylogenetic oligonucleotide arrays (POA) contain probes targeting taxonomic genes (El Fantroussi et al., 2003; Chandler et al*.,* 2006).

Due to high throughput capacity of microarrays and the availability of extensive rRNA sequence databases, POA have emerged as a very convenient means for simultaneous identification of many microorganisms from a sample. Microarray approach has been successfully used to discriminate shifts in a soil microbial community associated with hexachlorocyclohexane (HCH), TNT, U(VI) bioremediation and characterization of the phylogenetic and catabolic diversity of the contaminated environments (Brodie et al., 2006, Chandler et al., 2006; Garmendiaet et al., 2008). Among other results, a strong correlation was observed between total concentration of xenobiotic and probe signals corresponding to unknown γ-Proteobacteria, and also between probes targeting *Sphingomonas* sp and HCH concentration. Ribosomal Sequence Tag (RST) arrays is another "dynamic" way to monitor changes in the phylogenetic composition or changes in catabolic gene expression levels during biodegradation processes (Neufeld et al., 2006).

Array technology faces great challenges in environmental applications in terms of specificity, sensitivity and quantification that vary in intensity with different array formats. Several approaches are proposed to tackle these

limitations (Zhou and Thompson, 2002; Cook and Sayler, 2003; El Fantroussi et al., 2003; Gentry et al., 2006). As the design of probes for POA and FGA formats is mainly based on sequences retrieved from databases alone, these microarrays are unable to give access to unknown phylogenetic affiliations and functional activities, which is a major drawback for environmental applications (Gentry et al., 2006). In order to circumvent this limitation, clone libraries from environmental bacterium whose complete genome sequence are still unknown were spotted on a microarray making it Whole Genome or open reading frame arrays. Further clone library of a specific microorganism printed on a microarray could be hybridized with mRNA isolated prior to and after exposure of the microorganism to a contaminant treatment. Differences in hybridization patterns obtained are utilized to identify clones harbouring expressed (or repressed) genes in response to the treatment. To achieve comprehensive picture of complex communities and detection with quantification of rare bacterial species without sensitivity biases, a new generation of large scale "universal" phylogenetic arrays with all known genes and pathways involved in biodegradation are designed (Desantiz et al., 2007). These are rapidly replacing relatively small-scale, earlier generation phylogenetic microarrays (Palmer et al., 2006; Ashby et al., 2007). The use of dissociation curves holds promise for accurate quantification of environmental samples (Wick et al., 2006). The use of microarrays for studying the dynamics of microbial communities in situ is also facilitated by the recent development of a comprehensive FGA termed Geochip. This microarray provides direct linkages between biogeochemical processes and functional activities of microbial communities of different environments. Geochip 3.0 has several additional phylogenetic markers such as gyrB in addition to functional analysis (He et al., 2007).

Another prominent step forward in DNA arrays is to use probes produced directly from environmental DNA without any cultivations step, i.e. combining metagenomics with microarrays. Such a metagenomic array (MGA) was proposed by Sebat et al. (2003) for the first time to characterize microbial community of a groundwater microcosm. The MGA format is still in its early stages of development, but holds great promise for high-throughput screening of natural environments; with no prior sequence knowledge requirement for microbial communities' analysis (Gentry et al., 2006). Further whole community RNA amplification (WCRA) coupling with CGA can allow monitoring functional activities in microbial communities from contaminated environments with diverse organic solvents, hydrocarbons, uranium etc. (Wu et al., 2006; Gao et al., 2007).

Stable isotope probing (SIP) is another technique of choice for linking microbial phylogenetic identity with ecological function by incorporating stable isotope labelled substrates as cellular biomarkers (Radajewski et al., 2000). The main features of SIP enable direct access to the nucleic acid of metabolically active members in microbial communities, without any prior knowledge of their identity or presence in the sample. This approach was

employed for isolation of anaerobic benzene-degrading bacteria from gasoline-contaminated groundwater arsenic reducing bacteria and uncultured bacteria from PAH contaminated soil (Wackett, 2004a; Manefield et al., 2005; Yu and Chuy, 2005; Kasai et al., 2006; Lear et al., 2007). An alternative enrichment method using bromodeoxyuridine (BrdU) takes advantage of the fact that the DNA of microorganisms that metabolize BrdU can be immunoprecipitated. DNA-SIP techniques have been used for isolation of large DNA fragments from uncultured microbial communities for metagenomic analysis (Dumont et al., 2006). However insufficient substrate incorporation during incubation during DNA/RNA-SIP investigations can be a major source of enrichment bias that represented in the natural substrate metabolism situation in the environment. There is also a potential for enhanced cross-feeding of substrate and these issues need to be monitored using appropriate controls (Neufeld et al., 2007).

To overcome the generic biases with PCR dependent approaches, new PCR-independent amplification techniques like whole genome amplification are emerging as solutions. These techniques allow access to genomic information from very low abundance microbial sources that would otherwise remain inaccessible. Multiple Displacement Amplification (MDA) using phi29 DNA polymerase and random exonuclease resistant primers without thermal cycling exhibited surprisingly uniform amplification across the genomic targets compared to PCR based whole genome amplification (Dean et al. 2002; Abulencia et al., 2006; Binga et al., 2008). The method generated larger sized products with lower error frequency holding promise for single cell genome sequencing for metagenomic studies. DNA hybridization of microbial community amplified using the ø29 DNA polymerase to CGA was successful in analysing structure of the environmental community (Wu et al., 2006). T7 polymerase-based linear amplification approach using fusion primers has been proposed for mRNA based metatranscriptomics analyses (Gao et al., 2007).

Though function-driven screening strategy have allowed discovery of several new enzymes, antibiotics with biotechnological and therapeutic applications for years now, it involves low-throughput screens based on the visual detection that are difficult to be adopted for metagenomic scale (Gentry et al., 2006; Kunin et al., 2008). However, automated colony picking, pipetting robotics, use of microtiter plates, and availability of sensitive activity assays targetting biomolecules for screening, informatics-assisted data management aid in automation of the metagenome screens. This is particularly an advantage, as "hits" in metagenome screening are typically very low being less than two out of 10,000 clones. Diverse strategies are employed to enrich/select community genomes with desired traits through promoter activity rather than phenotypic expression before metagenomic library construction is tried (Lorenz and Eck, 2005).

Intracellular fluorescent biosensors or whole-cell-based biosensors are applied widely for environmental sensing and reporting of specific or classes of contaminants (Bhattacharyyaa, 2005; Handelsman, 2005; Williamson et al.,

2005). Bioreporters have two principle components: a microbial biorecognition element and a transduction element that converts the response into an output signal (Rogers, 2006). Genetically modified microbes, in which a promoterless gene e.g. lacZ, lux, gfp encoding for an easily measurable protein like β-galactosidase, luciferase fluorescent proteins respectively are fused with a natural regulatory circuit that regulates biodegradation pathway of a xenobiotic compound. The promoter activity being detected visually or compatible with fluorescence-activated cell sorting allow a high throughput level screening of the clones and possible integration on a chip-based device (Robertson and Steer, 2004; Harms et al., 2006). For genomics applications to reach its optimum potential in environmental studies it would be advantageous if the automated technology is made cheaper, compact and field deployable for in situ analysis (Gentry et al., 2006). With the recent advances in micro-fabrication and micro-fluidics technologies, it has become possible to make the concept of 'laboratory-on-a-chip' (LOC) a reality. The device being a miniaturized system assembling have all the components of sample preparation i.e., concentration, separation and purification together with hybridization/detection/analysis steps on a single portable platform offering simple and versatile applications to undertake in situ studies (Liu and Zhu, 2005).

7.3 Advantages and Limitation of Metagenomics Approach

Strength of metagenomics lies in its potential for serendipitous discovery. Thus, it is presently applied to explore biosynthetic diversity of microorganisms from varied environments by identifying genes involved in whole biosynthetic pathways or bioremediation of important xenobiotic compounds. Discovery of a light-driven proton pump from bacterioplankton mediated by proteorhodopsin protein, was first identified in environmental DNA samples using the metagenomic approach. A more recent discovery is regarding ammonia oxidizers among archaea was thought that bacteria were solely responsible for aerobic ammonia oxidation, although their numbers often could not account for observed rates of ammonia oxidation in many habitats. Identification of an ammonia monooxygenase gene next to an archaeal marker gene encoding small subunit ribosomal RNA using bioinformatic tools implicated archaea as one of probable main ammonia oxidisers in several marine and terrestrial ecosystems which was established experimentally later (Baldwin, 2003; Daniel, 2005; Schneikeret et al., 2006). Metagenomic assemblage of microorganisms has potential to answer several fundamental questions in microbial ecology as well as to assess the diversity and functionality of microorganims in situ quantitatively, in an objective way. In this bio-prospecting trend, several unique and previously unrecognized microbial forms are identified at the contaminated sites without the need of cultivation. Co-localization studies by combining FISH and digital image analysis has provided comparative analysis of temporal or

spatial information in structured ecosystems to support metabolic interactions between community members as inferred from metagenomic data (Wagner et al., 2006; Sanz and Kochling, 2007; Malik, 2008).

In spite of huge impact of metagenomics in different fields, there are several challenges faced with this kind of genome analysis. Three notable limitations of the basic metagenomic approach include low resolution, inability to classify short metagenomic fragments and uphill task of functional verification (Cowan et al., 2005; Hendelsman, 2004, 2005; Kunin, 2008a, b). The major challenge is determining the minimum size of metagenome library. Constructing metagenomic libraries from environmental sample, though conceptually simple, are technically very challenging. Extraction and purification of high-quality DNA for different environmental samples is still main bottleneck in metagenomics. In fact there appears to be no "one-size-fits-all" extraction method. Assuming environment sample has 100 species, it is estimated that there will be 0.5 Gbp of unique DNA. The present estimates of microbial diversity suggest a range of between 3,000 and 11,000 genomes per gram of soil. To get more than single sequence coverage, metagenomic library would need to be many times the size of the metagenome. The resolution of microbial communities by shotgun sequencing is rather low, with sufficient sequence coverage only of dominant populations to levels of permitting sequence assembly. Considering no other biases, a population representing 0.1% of a community would account for only 100 kilobase pairs (kbp) of a 100 Mbp metagenome constructed for a 4 Mbp sized genome, resulting in very little coverage of ~0.025× coverage. A recent study on the microbial diversity in the deep sea in terms of species-abundance distribution strongly suggested that rare community members comprising the bulk of the diversity in many environmental samples are completely missed by current levels of shotgun sequencing (Langer et al., 2006; Goldberg et al., 2006; Gabor et al., 2006; Johnson & Slatkin, 2008).

Compared to currently adopted PCR based WGA methods, the newly explored isothermal amplification with phi29 DNA polymerase is giving high yields of longer fragments, with least biased amplification. Due to the outstanding processivity of the enzyme, virtually significant amounts of DNA from previously inaccessible habitats or organisms are being obtained. The robust activity of phi29 DNA polymerase even in the presence of typical enzyme inhibitors suggests another important application of MDA in moderate to high abundance species in highly polluted DNA samples. As only nanogram amounts of template DNA are required to yield micrograms of DNA, in this method samples can be massively diluted, thereby reducing contaminants to sub-threshold concentrations (Dean et al., 2002; Abulencia et al., 2006; Binga et al., 2008).

The large scale sequencing cost involved for metagenomes from complex environments can still be prohibitive in spite of several advances in novel sequencing techniques like pyrosequencing. To address this challenge microbial community can be divided into simpler subsets that facilitate contig

identification and greater genomic coverage of populations. The divide and conquer approach, though difficult to achieve in cultivation approach, can be more easily addressed via metagenomic studies. The resulting data provides valuable guidance for subdividing microbial communities into enriched populations. The other important challenge is in identifying the source species of metagenomic fragments. Current methods to classify fragments do not perform well on sequences of less than 8 kbp. The bulk of the sequence data obtained in most metagenomic studies being smaller remains bottleneck for characterization. And thirdly, similar to all DNA sequence data, metagenomics data can provide information on metabolic potential for only the genes with recognizable homology with characterized proteins (Goldgerg et al., 2006; Johnson and Slatkin, 2008; Cardenas & Tiedje, 2008; Shendure, 2008). Lack of reference genomes is widening the gap between characterized and hypothetical proteins at an alarming rate in metagenomes data. Lack of availability of ideal phylogenetic anchors that can be globally used is still a prominent limitation. A good phylogenetic anchor needs to be equally represented in all species and serve as an indicator for the diversity both in qualitative as well quantitative terms. This ideal requirement is not met in phylogenetic anchors presently available. 16S rRNA genes analysis, though being the most widely used phylogenetic marker, does not meet this ideal requirement. The number of *rrn* operon copies vary from 1 to 15 in microorganism which mainly correlates with the growth rate. This leads to poor representation of slow-growing bacteria in the PCR generated 16S rRNA metagenome libraries as compared to rapidly growing counterparts. This can result in ignoring or overlooking low abundance species thereby distorting the in situ representation. Lack of reference genomes for interspecies heterogeneity/polymorphism, high level sequence conservation between phylogenetically unrelated genomes, confounded by low precision assembly of available softwares result in false or chimeric assemblies not represented in nature (Kysela et al., 2005; Langer et al., 2006; Johnson and Slatkin, 2008; Kunin et al., 2008a, b, c).

Further presently with sequencing becoming more and more reachable, complete genome sequences of a number of strains/individual species of some prominent organisms/community are growing in number and diversity. Hence an array of challenges are emerging to address comparison of these multiple bacterial genomes. Integrating the information from multiple sources using comparative genome analysis for various species of a bacterial genome is attempted and is collectively described as pan-genome. These are composed of a "core genome" containing genes present in all characterized strains, and a "dispensable genome" containing genes present in two or more strains and genes unique to single strains also. The pan-genome repertoire of genome sequences of a set of related bacterial species thus turns out to be many orders larger in magnitude than any single genome. The estimated minimum number of strains/species of given organism to be characterized, ultra-high-throughput required thereof and current models designed to manage public collections

of genomes in novel ways and their applicability are reviewed (Nocker et al., 2007; Malik et al., 2008; Tetteli et al., 2008).

Global oceanic projects and other metagenomic projects have added more than 8000 Mbps of genomic data essentially doubling the current amount of prokaryotic sequence data along with voluminous metadata in the last few years (DeLong, 2005; Overbeek, 2005; Dubchak et al., 2006; Seshadri et al., 2007). Metadata pertains to the metabolic information microbial population diversity, temporal spatial and physico-chemical data associated with sampling sites. For standardizing metadata, presently there are no established standards. Genomic standards consortium is involved in evolving acceptable minimal set of metadata for genomic/metagenomic projects in consultation with research communities. Further it would be significant to correlate metadata provided with deciphered ecological and environmental conditions to elucidate the factors that favour one population structure over the other (Goldberg et al., 2006; Hallam et al., 2006; Nulton, 2006; Stepanauskas and Sieracki, 2007).

The diverse data generated by metagenome projects at different scales present a tremendous challenge from the data management and data mining point of view also. The mounting data is increasing at a tremendously higher rate than the increases in the laboratory computational power. More than simple storage of sequence data, the other demanding issue is 'all-versus-all' sequence comparisons required to interpret metagenomes that pushes up the computational requirements exponentially. This is forcing to adopt radical breakthrough in computing approaches like quantum computes to carry out the comparisons of metagenomic data. A pioneering effort to manage rich metadata information that include geographical location, sampling procedures and other interesting additional information regarding environment was exemplified by CAMERA project, which stands for Community Cyber infrastructure for Advanced Marine Microbial Ecology Research and Analysis. This mega project initiated in January 2006 with estimated outlay of $24.5-million for seven years is funded by the Gordon and Betty Moore Foundation. The focus of this metagenome initiative is to serve the needs of the microbial ecology research community by creating a rich, distinctive data repository dataset of global prokaryotic marine diversity of both species and genes (Overbeek, 2005; Dubchak et al., 2006; Seshadri et al., 2007; Stepanauskas and Sieracki, 2007).

7.4 Integration of -omic Approaches for Effective Bioremediation

As observed, several xenobiotic pollutants are unusually recalcitrant as either microbes are unable to completely mineralize them or their sister metabolites accumulate in the environment creating health hazards. Exploring or engineering new catabolic pathways and studying the regulatory control of primary and secondary metabolites produced can provide solution to effective bioremediation. Sequencing the genomes is only a preliminary step in the

daunting task of exploring the physiological functions of complex and highly adaptable organisms. Several of these genes generate a number of distinguishable functional entities at protein level based on differential splicing. In view of the importance of these post-transcriptional modifications (PTMs) in nature, study of global protein profile during ex situ and in situ bioremediation is crucial. Further metabolomics targeting holistic analysis of the entire set of metabolites produced by cellular proteins in response to various environmental stimuli can provide critical clues to pathway analysis. Several metabolomics studies reveal precise correlation between true metabolic fluxes analysed with proteins and mRNA abundance (Casellas et al., 1997; Kromer et al., 2004). As this approach examines all the metabolites present in a biological system there is little bias associated with the choice of metabolites to be studied. However, metabolites of a site-specific organism being part of an in vivo metabolite flux regulate entire metabolic pathways. Hence, metabolism-based wide fluxes (fluxomes) in turn allows pinpointing scenarios of physiological regulation. Thus far, proteomics data have been deemed necessary to complement the metabolomics approach and techniques. However, exploring proteomics and metabolomics is gaining central positions among post-genomic techniques.

Currently there is no single molecular technique that can be adequate to describe entire microbial diversity and the associated catabolic genes. Each approach has its own limitation in respect of bias for investigation of the diversity. While proteomics target the global expression of proteins, metabolomics characterize and quantify the end products namely the metabolites that are produced under a given set of conditions. Hence proteomic and metabolomic approaches have enormous potential and are powerful tools in describing biological and ecological functions of an environment. These approaches and application potentials have been reviewed comprehensively (Hou et al., 2003; Stenuit et al., 2005, 2008; Dinsdale et al., 2008). Functional proteomics is defined as the use of proteomics to monitor and analyze the spatial and temporal properties of the molecular networks and fluxes involved in living cells. Conversely, functional proteomics is also the use of proteomics methods to identify the molecular species that participate in such networks via functional stimulation, perturbation, or isolation of these networks. Similarly the term metaproteome was used by Rodriguez-valera in 2004 to characterize entire protein complement of environmental micro biota at a given point of time. Until recently, proteomics had been mostly restricted to microbial isolates in culture due to technical difficulties in the preparation of protein extracts. Benndorf et al. (2007) developed a protein extraction protocol for a wide range of environmental samples, that can be coupled to the tools of protein analysis on-line linked to MS/MS via electrospray ionization source (LC-ESI–MS/MS) for automation.

Metagenomics constructions of contaminated sites appear prerequisite for adapting bioremediation in situ. Further biomined genes and enzymes have

to be integrated into the complex regulatory and metabolic network of the host organism in order to be properly expressed. Bioremediation processes thus is increasingly recognized as a frame in a complex web of metabolic and regulatory interactions which are difficult to approach with the traditional molecular approaches. A combination of metabolite identification, genome and proteome analysis is vital to elucidate the degradation pathway. Metagenomics is increasingly being viewed as a baseline technology representing the cornerstone of what can be achieved along with a range of complementary -omics technologies. Integration of emerging -omics technologies such as transcriptomics, proteomics, metabolomics can go long way in providing answers to fundamental questions in microbial ecology (Paul et al., 2005; Malik et al., 2008; Stenuit et al., 2008; Zhao and Poh, 2008). Application of these ideas and methods for network analysis is offering new insights into the biodegradation process. This outlook of 'systems biology' provides a comprehensive overview to understand the ecology and evolution of microbial ecosystems, upon which hypotheses and experimental strategies are being built up. A simultaneous study combining functional proteomics and metabolomics, i.e., proteometabolomics can create a system-wide approach to study site specific microorganisms during active mineralization and it would be possible to develop models that can predict microbial activities under various bioremediation strategies. This dynamic information will allow one to follow degradative pathways and track their intermediates and responses during mineralization. This information can be particularly valuable for studying complex in situ mineralization which evolves dynamically, with indirect effects on heterogeneous cell populations (Carbajosa et al., 2006; Cardenaas and Tiedje, 2008).

7.5 Systems Approaches Using High-throughput Technologies

Over the years qualitative and quantitative techniques have proved to be invaluable tools to identify new catabolic operons and to describe environmental microbial communities. However, their application is often time consuming and therefore limited to a small number of samples, thereby making comprehensive characterization of an ecosystem difficult. To tackle these issues, high-throughput approaches are progressively applied in environmental microbiology. They offer the advantage of miniaturization, automation and massive parallelization of time consuming steps, allowing the simultaneous "real-time" analysis. Expanding sequencing infrastructure to numerous samples at a reasonable price was possible adopting the novel pyrosequencing or "454" technology with rapid drop in genome-sequencing costs. Thus there is a dramatic increase in sequenced bacterial genomes and metagenomes degrading xenobiotics. The approach has emerged as a rather low cost alternative for studying the metabolic potential of uncultivated microbes (Golyshin et al., 2003; Kube et al., 2005b; Deutschbauer et al., 2006, Schneiker et al., 2006).

This rapid accumulating data of degradation process has made it mandatory to formalize structured databases for global analysis of biodegradation as a whole and their metabolic networks. This global approach is highly informative providing details regarding their structure, behaviour, complex biological interactions and evolution in a scale-free manner with increased intrinsic robustness. In addition it is accelerating the development of several valuable tools that can predict degradability of compounds or assist in design of artificial pathways.

Metabolic networks are often designed in 'scale-free' or 'small world' and hierarchical manner. This is an intermediate state between completely randomly connected networks and very regular one. In spite of some controversies, it provides a useful conceptual framework for the analysis of global biological process (Barabasi and Bonabeau, 2003; Arita, 2005; Khanin and Wit, 2006). The 'scale-free' nature of metabolic network implies a high heterogeneity in the number of connections of their nodes. New nodes can be added to the system by adding connections to already existing nodes. Hierarchical nature of metabolic networks allows clustering of enzymes into interconnected modules or groups that work together to perform a discrete function. These in turn are connected among themselves by specific nodes that acquire critical importance in maintaining flow in the entire system. A novel metabolic reactions' selection by evolution is more likely if the product is used in several other metabolic reactions. The analysis of a number of biodegradation network topologies have revealed a funnel-like structure in which several pathways converge to a set of common intermediates, which constitute the hub of the network. This property is specific to the global biodegradation networks unlike the hierarchical modularity of the general metabolism. Similar to other catabolic networks, larger and more hydrophobic compounds tend to accumulate far from the central metabolism. Also, enzymatic activities are not homogenously distributed in the network. The biodegradation network grows mainly from inside to outside, adding new enzymes towards periphery of the network, thereby allowing expansion of compounds to be biodegraded. Several theories have been proposed to explain the evolution of metabolic pathways (Schmidt et al., 2003). Prominent of these are retro-evolution, patchwork model and duplication of pathways. Duplication mechanism is favoured in several bacteria where genes coding for enzymes mediating same pathway are often clustered on the genome thereby facilitating simultaneous duplication. The patchwork and duplication models are presumed to play an important role in the formation of some biodegradation pathways (Jensen, 1976; Huynen and Snel, 2000; Rison and Thornton, 2002).

Thus systems approach attempts to answer interesting questions of how degradative pathways are evolved and the probable selection mechanisms operating at the ecosystem level. It is also helpful to predict the response of functional properties to internal and external variations (Feist et al., 2007; Feist and Palsson, 2008; Trigo, 2009). Typically systems biology approaches consider the 'complete system' to be a single cell. This formulation is clearly

insufficient in some case of biodegradation where the 'system' is a complex environment including multiple biotic and abiotic components. However, global biodegradation networks consider it as supra-organism metabolism, thereby transcending the limitation to some extent as properties appear similar to single-organism. The wealth of knowledge on cellular metabolism has accumulated into several rich databases such as KEGG, Metacyc Biocatalysis/Biodegradation Database (UM-BBD), Bionemo etc. (Karp et al., 2002; Ellis, 2002; Kanehisa et al., 2006; Ellis et al., 2008).

Rapid advances in microfabrications for nanolitre DNA processing, submicrolitre PCR DNA amplification and protein microarray and microfluidic technologies for 'sequencing factory-on-a-chip' have enabled the development of fully-integrated, miniaturized systems termed 'laboratory-on-a-chip' (LOC) devices. The technology provides automated, high-throughput and possible on-site solutions right from sample preparation (e.g. sample collection, concentration, extraction and purification), to final detection using immunological or biochemical reaction signal (optical or electrochemical) for assay. The whole system is assembled as a portable device with effective packaging for on-site use. Laboratory-on-a-chip (LOC) devices, also known as 'micro total analysis system (mTAS)' or 'biological microelectronic mechanical system (BioMEMS)' are emerging as important platform in several disciplines (Reyes et al., 2002). LOC for PCR is another rapid and sensitive means to develop a miniature analytical thermal cycling instrument on a chip. The mode integrates cell trapping and lysis steps along with PCR amplification, real-time detection through fluorescence resonance energy transfer mechanism utilizing TaqMan and molecular beacon probes followed by analysis in an array format. Another recent approach is to couple a microfabricated PCR chip to perform a commonly used community fingerprinting method in bioremediation including T-RFLP on capillary electrophoresis (CE) chip. The amplification and identification of several distinct gene markers by this method was highly encouraging (Liu and Zhu, 2005; Ottesen, 2006; Stenuit et al., 2008).

Optimization of automated sample preparation along with integrated packaging are major bottleneck for full fledged development of a universal system for rapid detection of microorganism in situ. Improved designs are extensively being explored for creation of interfaces to integrate the growing module components. The current trend is focussing on the 'all-in-one' concept by miniaturization and integrating buffer storage, pumps and valves into a single device. Significant efforts to reduce the volume and weight of the whole system by minimizing sample loss and reagent consumption are envisaged. Further 'plug-and-play' concept is introduced by integrating plastic chips for different functional modules of analysis and an electrochemical detection chip onto a printed circuit board (PCB) along with control circuits for regulation. Micro-mixers, microvalves and micropumps embedded resistive heaters are used to achieve homogeneous solutions and direct fluid flow in and out of different chambers (Liu and Zhu, 2005). Though challenges in automated sample

preparation, system portability and integration remain to be fully resolved, even in the present stage of developments LOC devices are rapidly explored in environmental metagenomics and proteomics along with nanofabrication and nanoparticle detection (Liu et al., 2004). The imminent impact of LOC in these studies was significant simply because the sample concentration, reagent and reaction volume and detection time could be minimized by 100–1000 folds compared to current methods. LOC provides several of the essential features demanded by ideal detection systems like speed, sensitive, specificity, automation along with portable features. Hence this approach brings in situ analysis a step closer to 'true' microbial diversity existing in nature (Liu and Zhu, 2005; Abgrall et al., 2007).

7.6 Conclusion

Potentially now bioremediation can be targeted to eliminate any compound that microorganisms can capture or absorb. Combination of high-throughput technologies like genomics, metagenomics, and proteomics applied in novel and imaginative way along rich metadata tagging could allow us to address interesting aspects of bioremediation. Together, these approaches hold great promise for the study of microbial ecology and evolution. Synergy in currently emerging -omic approaches will hasten emergence of alternative tools to address challenging frontiers of the complex of biodegradation pathways. This will deepen the understanding of microbial community structure and diversity and correlate them with vital niche-specific functions within a micro space.

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8

Recombinant DNA Technology for Bioremediation of Pollutants

Pradeep Kumar and Gayatri Baul

8.1 Introduction

Pollutants are artificially generated by-products of modern human world and most of them are known to have little to severe ecotoxicological impact on nature. The growing awareness of environmental pollution and its direct or indirect impact on ecosystem warrants development of cost effective, efficient and environmentally safe methods. The excessive use of chemicals in every walk of life due to unabated industrialization of modern society and growing market of cheap but harmful products due to globalization are disturbing the homeostatic balance of nature and its environment. Being the most intelligent creature of the universe, it is the prime duty of human beings to keep nature free of pollutants and provide environmentally safe ambience not only to the society but also to those innocent creatures who cannot express their discomfort.

8.2 Need of Biological Methods

A number of methods are available to deal with pollution; however, many of them are of limited value due to high cost and minimal effectiveness. Natural methods are effective and safe but are very slow and becoming ineffective because of the rapid rate of pollutant generation by modern man through industrialization and generation of non-biodegradable artificial synthetic materials, also termed as recalcitrant. Physical methods are labour intensive and costly while chemical methods are considered hazardous. In view of such growing concern biological methods are becoming preferred method of choice.

Biological methods are very frequently referred to as bioremediation and are very similar to natural methods in combating pollutants from environment.

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In more precise manner they are extension of natural methods that are made effective by augmentation using various effectors such as microbes, their enzymes, biosurfactants, bioemulsifiers, adsorbents, plants and other agro products. The most effective biological methods are based on biodegradation of pollutants through degradative enzymes such as hydrolases and/or transferases etc. The methods that include biodegradation are termed bioremediation and are considered ecofriendly, as they degrade pollutants either completely into carbon dioxide, water and biomass or into other harmless intermediates.

8.3 Bioremediation Advances

Bioremediation is a process of environmental biotechnology with promise of promoting a clean environment. It makes use of natural metabolic properties of microorganisms in transforming contaminants to harmless substances in the environment. The pollutants that are new to the biosphere can also be called xenobiotics. Introduction of such chemicals into the environment is known to impact the self cleaning ability of recipient ecosystem that results in the accumulation of pollutants to unmanageable and harmful levels.

Bioremediation explores gene diversity and metabolic versatility of microorganisms. The genetic architecture of these organisms makes them valuable in biodegradation, biotransformation, biosorption and bioaccumulation. The necessary blue print of genes encoding for biodegradative enzymes is present in chromosomal and extrachromosomal DNA of such microbes. Recombinant DNA techniques facilitate to evolve the ability of an organism to metabolize a xenobiotic by detection of such degradative genes and transforming them into an appropriate host via suitable vector under the tight control of appropriate promoters. It depends on susceptibility to alteration and exchange of genetic information. The recombinant DNA technology explores PCR, anti-sense RNA technique, site directed mutagenesis, electroporation, and particle bombardment techniques in developing effective, safe and economical techniques.

Mother earth is able to handle pollution by natural bioremediation through plants called phytoremediation and microbes termed microbial bioremediation. Traditionally desalination of agricultural land using plants has been practiced in many countries. Augmented bioremediation technology was first proposed by Robinson who explored it further during 1960's using microbes on petroleum hydrocarbons. Bioremediation can be undertaken at site and termed as in-situ or away from the polluted site termed as ex-situ. The later method involves physical removal of pollutant from polluted site to the site of treatment. The conventional bioremediation methods are land farming, bioventing, biocomposting, bioreactor, bioaugmentation, rhizofiltration and biological stimulation. As these methods are described in detail elsewhere in this book, we will restrict this chapter only to exploration of recombinant DNA technology in bioremediation of pollutants.

8.4 Emergence of Recombinant DNA Technology

Emergence of recombinant DNA technology, in 1972, paved the way of biotechnology, making it a cutting edge technology leading to appearance of new industry in the modern world. The delivered products of biotech industry include human insulin, interferon, human growth hormone and hepatitis vaccine (Steinberg and Raso, 1998). These deliverables are proven milestones of recombinant DNA technology in the field of medical sciences. The recombinant DNA technology is also facilitating development of promising methods to combat pollution. Many reports describe genetically modified organisms (GMO) as the prime product of recombinant DNA technology and it is becoming a promising option in environmental biotechnology as well (Glick and Pasternak, 1988; Cowan, 2000). As bioremediation being ecofriendly, it is becoming a method of choice and in this chapter we will discuss value addition by effective tools of recombinant DNA technology in pollution control.

The bioremediation, in its natural format in many cases, is affected by slow rate, impact of physico-chemical conditions and competing community structure at site. These limitations can effectively be tackled in modern bioremediation by use of defined community of degradative microorganisms with ingeniously engineered traits and by creating hybrid organisms or GMO's. Further, use of biological mediators to enhance biological availability of targeted substrate molecules to facilitate biodegradation at a rapid rate without generation of harmful products. The recombinant DNA technique is also proving to be a valuable tool in generating such mediators ex-situ (in fermentor or treatment vessels) and can also be used in-situ (at the site of pollution).

As value addition to conventional bioremediation, recombinant DNA technology displays great potential making future of this approach very promising (Lovley, 2003). The common pollutants that are cause of concern and need to be addressed include high density petroleum hydrocarbons, heavy metals (lead, mercury, cadmium, arsenic etc.), pesticides, chlorinated hydrocarbons, insecticides, polymers (polyethylene, polypropylene, polycarbonates, polyurethane etc.), detergents and explosives (TNT, GTN and RDX) etc.

Bioremediation being environmentally safe is considered a method of choice for many pollutants like polychlorinated biphenyls (PCB), polycyclic aromatic hydrocarbons (PCAH), many other harmful chemicals, hydrocarbons, petroleum fuels and heavy metals. The biotechnology armed with recombinant DNA technology is now fine tuning the bioremediation technology by improving pollutant degrading microbes through strain improvement and genetic modification of specific regulatory and metabolic genes that are crucial in biodegradation. The milestone patent of Anand Chakrabarty for petroleum oil pollution bioremediation was first step towards application of recombinant DNA technology (Chakrabarty, 1985).

It is appropriate to briefly understand fundamentals of this technology in subsequent paragraphs. For details readers are referred to Molecular Cloning

Manual by Sambrook et al. (1989). The GMO's are the most prominent and potential tool of recombinant DNA technology in bioremediation of pollutants. Although, they are the main deliverables but their use in open environment needs statutory clearance in many countries. They are used as per regulatory guidelines of that country.

8.5 Fundamentals of Recombinant DNA Technology

This technology enables to generate copies of DNA that can be combined specifically at a desired position on a genomic or plasmid DNA. The resultant construct is said to be a hybrid of recombinant and genomic DNA. Mostly such a hybrid DNA represents copy of one or more genes that can be expressed in a new host organism that is referred to as recombinant clone. Such clones are able to express the properties of foreign genes that are acquired through this technique. The same has been demonstrated by many investigators in bacteria, algae, fungi, nematodes (*Cenorahabditis elegans*) and mammalian cells. The said technology is today's most powerful tool of biotechnology that is being explored in various fields including environmental pollution control, through bioremediation. The most prominent deliverable of this technology is genetically modified or engineered organism (GMO or GEM). Although in several countries GMO's cannot be released but can be explored in enclosed vessels (fermentor) to generate various recombinant products having applications in medicine, agriculture and environment.

Generation of such recombinant hybrid involves isolation of target gene from complex mixture of biological molecules of living cell. The isolated DNA is then digested with enzymes called restriction endonucleases or obtained by PCR amplification of specific target sequence. The former method is termed restriction digestion that are used as molecular scissors to cut desired sequence at specific site that is appropriate for joining to vector DNA in gene cloning. The vector DNA is also described as a plasmid or extra chromosomal self replicating DNA molecule. In simple words, recombinant DNA technology signifies generation of DNA from two different origins, their recombination and transformation or transfection in a host to generate new or hybrid characters. Each cell receiving this hybrid DNA can multiply under defined conditions generating thousands of copies by replication. Further advances in this technology facilitated modification of gene prior to cloning to express an altered protein, to attach a reporter gene, to add a specific promoter for controlled expression, to have increased protein expression, to modify characteristics of expressed protein, to alter the time and site of a protein expression etc. The techniques that are used to introduce such properties are PCR, site directed mutagenesis, promoter fusion, reporter gene fusion, signal sequence integration and restriction enzyme digestion etc.

The enzymes play a crucial role in this technology. These enzymes are originally isolated from bacteria (e.g. *Eco*R1 from *Escherichia coli* and *Bam*H1

from *Bacillus amyloliquefaciens*) and are capable of cleaving a nucleic acid molecule. The natural presence of such enzymes in these bacteria confers them resistance to bacterial viruses (Bacteriophages). Such bacterium protects its own DNA by methylating those specific sequence motifs. In recombinant DNA technology they are explored for various functions.

Several types of enzymes confer specific function to them like, Type I enzymes cut DNA on both strands but at a non-specific location at varying distances from the particular sequence that is recognized by the enzyme giving random/imprecise cuts. Such DNA fragments are not very useful. Whereas, Type II enzyme cut both strands of DNA within the particular sequence recognized by the restriction enzyme. Type II restriction endonucleases are the most important enzymes in recombinant DNA technology as they are used to cut a DNA molecule from specific location. It reads the same in the $5' \rightarrow 3'$ direction on both strands (Palindromic Sequence 5′GGATCC3′, 3′CCTAGG5′). Some of the type II restriction enzymes generate cut in middle or across both strand generating "blunt ends" on digested DNA (*Hae*III), while others generate "sticky ends" (staggered cuts, e.g. *BamH*1, *Eco*R1, *Hind*III etc., Table 8.1). H-bonding is possible with complementary tails. DNA ligase is another important enzyme that is used to link two DNA fragments covalently together by forming phosphodiester bonds of the phosphate-sugar backbone in the DNA. Prior to ligation it is appropriate to select the vector DNA or plasmid carefully.

The choice of a vector depends on the design and objective of the experiment. Most vectors contain a prokaryotic origin of replication (Ori/replicon e.g. *Col*E1 from *E.coli*) allowing maintenance in bacterial cells. Some vectors contain an additional eukaryotic origin of replication allowing autonomous, episomal replication in eukaryotic cells. Multiple unique cloning sites are often included for versatility and easier library construction.

Table 8.1: Recognition site of a few common type II restriction endonuclease enzymes

Restriction enzyme	*Recognition sequence*	*Digestion product*
• Sticky end generating		
*Eco*R1	5′GAATTC 3′CTTAAG	5′…GAATTC…3′ 3′…CTTAAG…5′
*Bam*H1	5′GGATCC 3′CCTAGG	5′…GGATCC…3′ 3′…CCTAGG…5′
• Blunt end generating		
HaeIII	5′…GGCC… 3′…CCGG…	5′…GGCC…3′ 3′…CCGG…5′
*Alu*1	5′…AGCT… 3′…TCGA…	5′…AGCT…3′ 3′…TCGA…5′

These vectors also contain antibiotic resistance genes (Ampicilline/Tetracycline/Spectinamycin/Chloramphanicol) and/or other selectable markers that enable identification and selection of cells in the medium containing antibiotics. As the antibiotic resistance is an artificially engineered character in the vector it can be used for the selection of clones.

Some vectors contain inducible or tissue-specific promoters permitting controlled expression of introduced genes in transfected cells or transgenic organism. Modern vectors contain multi-functional elements designed to permit a combination of cloning, DNA sequencing, in-vitro mutagenesis, transcription and episomal replication.

Main Types of Vectors

The various types of vector DNA used in molecular cloning experiments are based on modified plasmid, bacteriophage, cosmid, bacterial artificial chromosome (BAC), yeast artificial chromosome (YAC), yeast 2 micron plasmid, retrovirus and baculovirus vectors etc. The choice of vector depends on nature of target DNA, type of host cell to accommodate rDNA (Prokaryotic or Eukaryotic) and objective of the cloning. A plasmid vector is a covalently closed, circular, double stranded DNA molecule that occurs naturally and replicate extra-chromosomally in bacteria. Most vector plasmids contain a minimum of three main elements in the sequence namely origin of replication, selectable marker gene (Ampicilline/Tetracycline/Spectinamycin) and a multiple cloning site for the insertion of foreign DNA. Many confer drug resistance to bacterial strains. A most common laboratory generated plasmid vector pBR322 contains two selectable markers (*Amp* and *Tet* resistance genes). Several unique restriction sites are scattered throughout plasmid. Some lie within antibiotic resistance genes and are used as means of screening of inserts. *Col*E1 *ORI* is the replicon in this vector. Another important plasmid vector is pUC18, derived from pBR322. Being a small vector it can accommodate larger DNA fragments than pBR322 (5-10 kbp). It is a high copy number plasmid i.e. 500 per cell, 5-10 times more than pBR322. Multiple cloning site (MCS) is clustered in the same location.

Interruptible gene encoding for enzyme beta galactosidase (*lac*Z) is commonly used in many vectors as a selectable marker gene. The MCS in the middle of the *lac*Z sequence is conventionally used as marker and insertion. Disrupted gene (*lac*Z) being non-functional due to the presence of foreign gene, the resultant phenotype appears as a white colony (disrupted *lac*Z) on media containing XGAL chromogenic substrate indicating successful cloning (intact lacZ gene gives blue colonies). The presence of antibiotic resistance gene (Beta lactamase) allows growth of both transformed and non-transformed colonies on media containing antibiotic (Ampicilline). The resultant clones can be further explored in subsequent steps as may be required for specific application such as PCR amplification, DNA library construction, sequencing, sub-cloning, mutagenesis and transfection.

The recombinant DNA technology is now being explored to further boost the rate of biodegradation several fold by judicious use of GMOs and mediators produced through this technology. Further, along with other molecular biology techniques such as RAPD, DGGE and FISH it provides valuable tools to monitor bioremediation by determining community structure before and after and factors affecting survival of bacteria involved in the process. A brief description of these techniques is given below.

RAPD: It stands for Random Amplification of Polymorphic DNA. This technique is based on random amplification of parts of polymorphic target DNA by PCR using short primers. The randomly amplified DNA fragments are then compared in agarose gel electrophoresis. It is useful in detecting species-specific patterns and is feasible with relatively small quantity of sample DNA.

DGGE: It stands for Denaturing gradient gel electrophoresis. This technique is a molecular fingerprinting technique which separates small DNA fragments (200-700 bp) on the basis of differences in their melting behaviour and single base substitution. It is useful in comparing the melting behaviour of the polymorphic DNA fragments and to detect fragments that have mutations.

FISH: This stands for Fluorescent in situ hybridization. It is a molecular probe technique used to identify presence or absence of specific DNA sequences on chromosomes. This technique uses fluorescent probes which binds to high degree of sequence similarity parts on the chromosome.

8.6 Applications of Recombinant DNA Technology in Pollutant Control

Many reports described 1990s as the decade of environmental biotechnology when molecular and recombinant DNA technology gained its importance in environmental pollution control. The tools of molecular biology and recombinant DNA technology unleashed the microbial diversity and population dynamics of microbial ecosystem in diverse ecological, geographical and environmental conditions. Application of bioremediation aided by recombinant DNA products for environmental clean up provides a robust, cost effective and efficient technology.

Recombinant DNA technology is now able to provide effective bioremediation strategies to many environmental pollutants using one or a combination of following techniques (Table 8.2).

8.6.1 Reporter Gene based Pollutant Biosensors in Bioremediation Monitoring

The process of bioremediation conventionally being monitored indirectly by measuring the *Oxidation Reduction Potential* or redox of polluted site, together with pH, temperature, oxygen content, electron acceptor/donor concentrations, and concentration of breakdown products (e.g. carbon dioxide) and bacterial

biosensors are being used to analyze petroleum-contaminated environments. Microbial biosensors are now being tested and proven useful in monitoring pollutants in a reporter gene based system (Table 8.2). The metal resistance genes are known to be responsible for microbial metal resistance. These genes are found organized in operons and are often found in plasmids carried by the metal resistant bacteria (Ramanathan et al., 1997). The expression of the resistance genes is tightly regulated and induced by the presence of specific metals in the cellular environment (Ramanathan et al., 1997). The specificity of this tight regulation is now being exploited in such biosensors. The promoters and regulatory genes from these resistance operons are being used to construct metal-specific biosensors (promoter-reporter gene fusions). By using metal specific bacterial sensors in addition to chemical analyses it is possible to regulate pollutant concentration in a bioremediation process and also to distinguish the bioavailable metal concentration from the total metal concentration of the samples.

Table 8.2: Microbial sensors developed using recombinant DNA technology for toxic heavy metals

Metal	*Reporter gene*	*Bacterial host strain*
Antimony	*Luc*	*Staphylococcus aureus* *Bacillus subtilis* *Escherichia coli*
Arsenic	*Luc*	*Staphylococcus aureus* *Bacillus subtilis* *Escherichia coli* *Pseudomonas fluorescens*
	Lux	*Escherichia coli*
Cadmium	*Luc*	*Staphylococcus aureus* *Bacillus subtilis*
Cobalt	*Lux*	*Ralstonia eutropha*
Copper	*Lux*	*Pseudomonas* *Fluorescens*
Lead	*Luc*	*Staphylococcus aureus* *Bacillus subtilis*
Mercury	*Lux*	*Escherichia coli*
	Luc	*Escherichia coli*
	Lux	*Pseudomonas putida*
	Lac	*Escherichia coli*
	Gfp	*Escherichia coli*
	Luc	*Pseudomonas fluorescens*
Nickel	*Lux*	*Ralstonia eutropha*

8.6.2 Recombinant Monoculture for Multi-pollutant Bioremediation

The efficient recombinant microorganisms as a monoculture may have the ability to degrade more than one pollutant. The use of recombinant DNA technology to evolve or generate efficient organisms specifically tailor made for bioremediation displayed proven potential. For example bacterium *Dienococcus radiodurans* has been genetically modified to utilize and degrade toluene and ionic mercury from highly radioactive nuclear waste.

8.7 Metabolic Intermediates and Bioremediation

The genetic modification through recombinant DNA technology offers several advantages over natural isolates. The technology facilitates design and development of microorganisms capable of operating efficiently through multiple degradative pathways for partial or complete degradation of even previously non-biodegradable compounds. The development of recombinant microorganisms with the ability to degrade xenobiotics may be preferable over consortia of natural isolates. The consortia of wild or laboratory strains may degrade a xenobiotic compound or mixture through different metabolic pathways. Further, bioavailability and exchange of intermediates may reduce the degradation rate and efficiency (Erb et al., 1997). The resistant intermediates are known to be toxic to the bioremediation process (Timmis et al., 1994). Many investigators therefore recommend use of multi substrate acting monoculture of GMO's.

To avoid the formation of potentially inhibitory metabolites, Erb et al. (1997) developed a GM pseudomonad (*Pseudomonas* sp B13 SN45RE). This GMO was made to degrade both chloro- and methyl aromatic compounds by the *ortho* cleavage pathway. Another GMO (*Pseudomonas* sp B13 FR1 (pFRC20P), was made capable of utilizing chloro- and methyl aromatic compounds by a constructed *ortho* cleavage pathway to degrade a mixture of 3-chlorobenzoate (3CB) and 4-methylbenzoate (4MB; 25 μM of each) in sediment cores under the water column throughout a four-week period (Pipke et al., 1992). This development further highlights the enormous potential of GMOs in the environment to overcome problems inherent with non-GM microorganisms.

Data analysis of the biodegradation of 3CB and 4MB by *Pseudomonas* sp B13 FR1 (pFRC20P) in water and in sediment microcosms indicated that the observed and theoretical half-lives correspond well, proving that GM pseudomonad functioned optimally in these environments. The physiological characteristics of the GMOs and their performance in aquatic environment make this microorganism an excellent candidate for in situ bioremediation at sites contaminated with mixtures of chlorinated and methylated aromatics (Heuer et al., 1995).

8.8 Environmental Stress-proof Bioremediation

Genetic modification can also be used to develop microorganisms that are more tolerant to the environmental stresses likely to be encountered in a contaminated environment. Although microorganisms indigenous to such sites are likely to be adapted to the stresses to some extent, stress factors such as poor oxygen, water and nutrient availability, high concentrations of toxic pollutants and extreme pH may be expected to have some restriction on the level of in situ biodegradation. Genetic modification has the potential to confer improved tolerance or resistance to the likely stresses, and consequently improve the performance of the degradative microorganism (Timmis et al., 1994). The coupling of the catabolic genes to promoters is responsive to environmental stresses such as low pH. These modifications enhance the degradative properties of the GM microorganism even in stressful environments. Placing the catabolic genes under the control of such promoters also means that the expression of those genes is restricted to the particular stressed environments.

Recent advances in molecular biology have extended our understanding of the metabolic processes related to microbial transformation of petroleum hydrocarbons. The physiological responses of microorganisms to the presence of hydrocarbons, including cell surface alterations and adaptive mechanisms for uptake and efflux of these substrates, have been characterized. New molecular techniques have enhanced our ability to investigate the dynamics of microbial communities in petroleum contaminated ecosystems. By establishing conditions which maximize rates and extent of microbial growth, hydrocarbon access, and transformation, highly accelerated and bioreactor-based petroleum waste degradation processes have been implemented.

The development of a GMO tailored for specific pollutant always aimed at rapid rate of biodegradation through specific enzymes and is made or can be made robust to survive at contaminated site to function efficiently as demonstrated under test conditions. They are also designed in such a way that they enhance bioavailability of pollutant. In many situations non-availability of substrate to the degrading organism impairs the effectiveness of the bioremediation process. Many contaminated sites, particularly those that are continuously polluted by industrial activities, are polluted with a cocktail of both organic and inorganic compounds. A consortium of GMO's capable of degrading both organic and inorganic substrates appears promising in such situations. The in-situ success of GMO in bioremediation also depends on chemical constituents or contaminants present at polluted site. A GMO may display excellent bioremediation efficiency under laboratory conditions but may not necessarily be so efficient in the field conditions. Such effects can be observed due to inhibition of the catabolic steps of the bioremediation or as a result of toxicity of local contaminants to added GMO (Timmis et al., 1994). For example, at a site polluted with a mixture of chlorinated solvents and radionuclides, the introduced GMO to degrade chlorinated solvents must be

resistant to radiation. In other words, an efficient GMO must be engineered to degrade either both pollutants or be resistant to one while degrading the other. Such radiation resistant strains are described in the literature. A genetically modified *Deinococcus radiodurans* strain, described in literature, is found capable of degrading 125 n mole per ml of chlorobenzene at a site having radioactivity up to 60 Gy per hour (Lange et al., 1998).

8.9 Transcriptional Modifications of Metabolic Genes

General approaches to applications of recombinant DNA technology in bioremediation also include transcriptional modifications of important metabolic genes encoding for enzymes of pollutant biodegradation or modification to storable non-toxic forms. It is considered to be a molecular biology tool in which gene is modified in the coding region or in the gene sequence that is transcribed into modified mRNA. Such modifications are aimed at effective enzyme substrate binding or effective biological interaction due to increased or modified active binding site. It may also be done for desired control or regulation of a metabolic gene. Such modifications allow strain modification for bioremediation and sensing of pollutants level endpoints.

Table 8.3: Recent developments of recombinant DNA technology in the field of pollutant bioremediation

Genetic modification	*Bioremediation strategy*	*Reference*
Suicidal GEMs	Killer-anti killer genes susceptible to apoptosis	Pandey et al., 2004
Mercury biosensor	Mercury inducible *mer* promoter fusion with *luc* gene (Minimum detection 0.1fM)	Marko et al., 1995
Organophosphate and carbamate pesticide degrading GEM	Methyl parathion hydrolase encoding gene (*mpd*) and cognate regulator	Liu et al., 2006
Coal-tar waste contamination detection by FISH	mRNA transcripts related to naphthalene dioxygenase and tyramide signal amplification	Bakersmans and Madsen, 2002
On-line in-situ biosensor	Whole cell and electrochemical biosensor	Paitan et al., 2003
Safe vectors for field release	Pseudo wild type bacteria having no heterologous DNA other than gene of interest	Davison, 2002

Genetic modification allows development of robust modified organisms to specifically attack pollutants through efficient enzyme cascade and bioavailability enhancing mediators of biological origin. Further the technology is capable of providing efficient bioremediation and pollutant monitoring tools (Romantschuk et al., 2000). Several reports describe naturally occurring microorganisms equipped with the pathways required to mineralize the more recalcitrant xenobiotics compounds like pentachlorophenol (PCP) and PCBs (Wilson and Lindow, 1993). Recombinant DNA technology has the potential to improve existing catabolic pathways or to extend such pathways to include additional target compounds that may otherwise not be degraded (Timmis et al., 1994; Brazil et al., 1995). It can also be optimized to overcome the toxic or inhibitory effect of a particular pollutant or a metabolite (Mason et al., 1997).

The scope of catabolic pathways can be widened through the introduction of additional genetic sequences, or the alteration of existing genes is reported to offer the simplest application of GM techniques to bioremediation strategies (Kumar et al., 1996; Johri et al., 1999).

8.10 Improvements in Transcription of the Gene Sequences

The genes involved in the degradation of pollutants are usually arranged in operons carried on wide-host range, conjugative or mobilisable plasmids. For transcription to occur correctly, genes are required to be arranged in most appropriate sequence in operons to produce the specific enzyme. Transcription of such catabolic operons is controlled by individual operons and also at a whole cell level.

Individual operons are controlled by positively acting regulatory proteins which are activated by substrates or metabolites (termed effectors) present in the catabolic pathway. The control of transcription in this way ensures that the microorganism will only operate the catabolic pathway in the presence of specific substrates, and will not waste carbon and energy synthesizing unnecessary proteins in the absence of those substrates.

The amendment of contaminated sites, with compounds that act as effectors for particular catabolic pathways, may improve the degradation of xenobiotics by activating the respective catabolic pathway. The promoter P*lac* is activated by the addition of isopropyl-β-D-thiogalactoside (IPTG), and is reported to function well in *Escherichia coli* under laboratory conditions. The P*tac* promoter also responds to IPTG and is reported to work better than P*lac* in a variety of Gram-negative bacteria. However, IPTG is relatively expensive, and this means that the development of GMOs with promoter systems based on this effecter are unlikely to be commercially viable for field release applications (Timmis et al., 1994).

The P*m* promoter of the *meta*-cleavage pathway of the TOL plasmid functions well in a number of Gram-negative bacteria, and is induced when the XylS regulatory protein is activated by the presence of benzoate and its derivatives (Keasling and Bang, 1998; Ramos et al., 1986). Unlike IPTG, benzoate is relatively cheap and is likely to be produced in-situ during the degradation of aromatic pollutants. Because of its existing use as a food preservative and its poor persistence in the environment benzoate has been described as an environmentally friendly inducer (Timmis et al., 1994).

Other systems that are reported to be applicable to field release systems are the salicylate-induced P*sal*-NahR promoter-regulator, the T7 promoter and the XylR/P*u* expression system which controls the upper pathway of the TOL plasmid and is induced by effectors such as toluene, ethylbenzene and xylene present at many contaminated sites (Keasling and Bang, 1998; de Lorenzo et al., 1993). The T7 promoter has been incorporated for use in a number of soil bacteria (Tabor and Richardson, 1985; Davison et al., 1989). Future developments in this area are proposed to include expression systems based on responses to metals, such as CadC (cadmium regulatory protein) and ArR, a regulatory protein for the arsenic resistance system and other environmental contaminants (Keasling and Bang, 1998).

Operons, at a whole-cell level, are controlled by global regulatory circuits. These are designed to ensure that the operons operate as part of the overall nutrient and energy requirements of the microbial cell. The global regulatory circuit responsible for the coordination of the transcription of catabolic operons is known as catabolite repression and acts to repress the operons used for the catabolism of certain substrates when other preferred substrates are available (Timmis et al., 1994). Therefore, where the target compound is not a preferred substrate, degradation of the target compound may be blocked by catabolite repression.

Inactivation of the catabolite repression circuit through genetic modification may however lead to a reduction in the competitiveness of the microorganism and for the purposes of bioremediation is therefore undesirable. A preferred approach is to block the catabolite repression of the specific operon used in the degradation of the target compound. This can be achieved through the identification and elimination of the sites in the operon promoter where repression is exerted (Timmis et al., 1994).

Activation of the catabolic operons occurs when the relevant effector is present at a sufficient concentration also known as effector concentration threshold. For example, the catabolic promoters are activated at an effectors threshold concentration of 5-50 μM of the TOL plasmid (Mermod et al., 1998). The *Pm* promoter of the TOL plasmid pWWO in *Pseudomonas putida* is activated at a concentration of 1 ppm of benzoate (de Lorenzo, 1994). The *Pm, Pu* and *Psal* promoters, which are activated by alkyl- and halobenzoates, alkyl, and halotoluenes, and salicylates respectively, have a broad host range and can function in a number of genera (de Lorenzo, 1994). However, if

the aim of the bioremediation strategy is to degrade the target pollutant to a concentration lower than the effector concentration threshold, then an alternative catabolic pathway may be required. Otherwise, when the pollutant drops to a concentration below the effector concentration threshold, transcription of the catabolic operon will not occur, and no degradation will take place. If an alternative catabolic operon is not available then the microorganism may be genetically modified to place the catabolic genes under the control of a different promoter that is not activated by the target compound (Timmis et al., 1994).

Alternative promoters allow activation of the catabolic operon to degrade a non-degradable pollutant and can be effectively separated from the needs of the microorganism enabling it to utilize the pollutant as a carbon source. In addition such modifications facilitate use of the pollutant to be degraded to below the level required to support microbial growth. The approach can also be used to degrade pollutants that would provide little or no benefit to the microorganism in terms of carbon or energy. As described for trichloroethylene (TCE), where the bioremediative function of the microorganism is separated from the requirement to use the pollutant as a carbon source, the microorganism is enabled to utilize other carbon sources to maintain its energy balance (Timmis et al., 1994).

The genetic modification that involves insertion of alternative promoters also increases the range of effectors that may activate the biodegradative pathway, and hence offers greater flexibility to the process of bioremediation. Such effectors may confer greater transcription of the catabolic operon leading to improved efficiency of the catabolic process. However, to ascertain that the inserted character is expressed well in the environment, it is important to select a promoter that is known to function in the target environment, and then fit the modified genes to that promoter. This is likely to be more successful than attempting to modify a promoter that works well under laboratory conditions to operate in the field (de Lorenzo, 1994).

If suitable promoters are not available, then the modified genes can be inserted into the host microorganism using a mini transposon vector. The location of the modified genes adjacent to the terminus of the mini transposon will result in the genes being controlled by a promoter sequence indigenous to the host microorganism. Screening of the GM microorganism is subsequently required to identify the most suitable mutant strain (de Lorenzo, 1994).

8.10.1 Indigenous Promoter

The use of an indigenous promoter sequence of the organism being modified to control the inserted gene may confer a greater level of expression than the gene's own promoter sequence. For example, insertion of the *opd* gene from *Flavobacterium* sp ATCC 27551 and from *P diminuta* into other Gram-negative bacteria under the control of its own promoter sequence results in the poor expression of the gene. However, expression of the *opd* gene, which is

responsible to degrade the pesticides parathion and methyl parathion, is much better if it is placed under the control of the modified organism's own promoter (de Lorenzo, 1994).

8.10.2 Starvation-induced Promoters

On many occasions catabolic genes are not expressed due to non-availability of micronutrients or conditions of starvation. Expression of these catabolic genes under starvation in polluted environments can be improved if expression of these genes is linked to starvation-induced promoters such as *groEL* and *tra* (Little et al., 1991; Matin, 1992; Davison, 1999). Under such environmental stress, GroEL can constitute 3-4 percent of total cell protein. Starvation linked promoters activate heterologous gene sequences through a universal signal and are found suitable to environments where availability of nutrients are too low to support exponential growth of the microorganism (de Lorenzo, 1994). Promoters responsive to carbon, nitrogen, iron and phosphate starvation have been characterized in many Gram-negative bacteria (de Lorenzo, 1994). A further advantage of using starvation induced promoters is that it avoids the problems associated with the introduction of large quantities of nutrients into contaminated sites (Matin et al., 1995), used starvation promoters from *E. coli* to control synthesis of toluene monooxygenase in pseudomonads, with a resulting 60-90 percent reduction in nutrient demand for transforming a given amount of TCE compared to wild type microorganisms.

8.11 Improving Translation

Improving the translation of a genetic sequence is particularly useful in optimizing the rate-limiting step of a reaction. The rate-limiting stage is often due to the relative lack of specific protein compared to the others required for the pathway. To alleviate rate limiting step of reaction, improvements in translation are targeted to a specific protein and the necessary gene is kept under the control of a separate promoter. To ensure increased synthesis of required protein as compared to other proteins, modification of the translation initiation region (TIR) of the relevant gene is recommended by site directed mutagenesis (Timmis et al., 1994). Translational enhancers are short sequences (40-50 bp) present in some plant viruses and can also be explored to ensure that translation of the desired sequence occurs in a range of host organisms as a means to circumvent the potential problems of host specificity encountered with TIRs (de Lorenzo, 1994). The insertion of a translational enhancer from tobacco mosaic virus enabled the gene for chloramphenicol acetyl transferase to be expressed in *E. coli, S. typhimurium, Erwinia amylovora, A. tumefaciens, A. rhizogenes, Rhizobium meliloti* and *Xanthomonas campestris* (Gallie and Kado, 1989).

8.12 Improving Protein Stability and Activity

Stability of the mRNA is known to influence the expression of the desired trait. The mRNA of gene 32 of the bacteriophage T4 is extremely stable due to structural determinants at its 5' end. The fusion of the native promoter/TIR region of gene 32 to various heterologous genes has been shown to result in the production of more stable transcripts when expressed in heterologous hosts. The fusion of this transcript to the *xylE* gene (encoding catechol 2, 3 dioxygenase) has been reported to result in the expression of high levels of the reporter product in Gram-negative bacteria such as *A. tumefaciens* and *X. maltophila* (Frey et al., 1988). The introduction of DNA cassettes into the 5' untranslated region of a gene of interest is reported to improve the stability of the mRNA by introducing hairpin structures at the 5'-end of the mRNA (Keasling and Bang, 1998). Because the hairpin-containing mRNA had a half-life three times greater than that of equivalent mRNA with no hairpins, the introduction of hairpins can be effectively explored to increase the amount of mRNA and protein that is intended for production (Keasling and Bang, 1998).

Improvements in transcription and translation will result in an increased production of the enzymes and proteins required for the biodegradation of the target contaminant. However, poor stability and activity of the proteins and low substrate specificity may still restrict the efficiency of the whole biodegradative process. The stability of a protein may be improved by altering the sequence of its amino acids (Murdock et al., 1993). For example, the aromatic ring cleavage enzyme catechol-2, 3-dioxygenase is slowly inactivated by its substrates oxygen and catechol derivatives such as 4-ethylcatechol. Ramos et al. (1987) reported that by modifying the catechol-2, 3-dioxygenase by two single amino acids caused the enzyme to be less susceptible to inactivation by 4-ethylcatechol and more stable in the presence of this substrate. The development of hybrid gene cluster with altered enzymes activity and substrate specificities has been reported in the literature. These hybrids encode for subunits of different enzymes to produce a single enzyme with superior transforming capability. For example, Furukawa et al. (1994) described the degradation of TCE by a genetically modified *E. coli,* which was engineered to express a hybrid gene cluster consisting of genes from the toluene metabolic *tod* operon and the biphenyl metabolic *bph* operon. This engineered *E. coli* was much faster in degrading TCE than that of a wild non-modified *E. coli* cells that express either the original toluene dioxygenase genes (*todC1C2BA*) or the original biphenyl dioxygenase genes (*bphA1A2A3A4*). This modified *E. coli* had hybrid gene cluster that is consisted of *todC1* that encodes for the large subunit of toluene terminal dioxygenase in *P. putida* F1, and *bphA2, bphA3*, and *bphA4* in *P. pseudoalcaligenes* KF707, encoding for small subunit of biphenyl terminal dioxygenase, the ferredoxin and ferredoxin reductase respectively.

Hybrid enzymes have also been developed to produce a single enzyme system capable of degrading benzene and tetrachlorobenzene. The enzyme

TecA chlorobenzene dioxygenase is able to dehalogenate partially 1,2,4,5-tetrachlorobenzene but has no activity against benzene, whereas the TodCBA toluene dioxygenase can dioxygenate benzene but has no activity against tetrachlorobenzene. The genetic modification of *E. coli* to express a hybrid enzyme consisting of the large alpha-subunit of the TodCBA dioxygenase whose specific todC1 alpha-subunit subsequences had been replaced by equivalent sequences of the tecA1 alpha-subunit, resulted in a GMO with activity towards benzene and tetrachlorobenzene (Beil et al., 1998).

Random mutagenesis and selective screening of desired traits are described in the literature for improvements in protein activity. However, a more rational approach based on the three dimensional structure of the protein and its structure sequence relationship is also described for improvements in protein activity by these investigators (Timmis et al., 1994; Mason et al., 1997; Kellner et al., 1997). Site-directed mutagenesis of the *bphA* gene of *Pseudomonas* sp LB400 for example increased the specificity of the expressed biphenyl dioxygenase to include congeners not degraded by the non-GM strain (Erickson and Mondello, 1993). However, the information required for the more rational approach exists for relatively few degradative enzymes (Mason et al., 1997), including cytochrome P450cam, haloalkane dehalogenase, dihydroxy biphenyl dioxygenase and methane monooxygenase hydroxylase (Timmis et al., 1994).

8.13 Extending the Scope of Existing Catabolic Pathways

Extending the scope of existing catabolic pathways to degrade new compounds avoids the requirement to develop wholly new degradative pathways and may therefore offer the most immediate route to the bioremediation of previously non-degradable xenobiotics pollutants. One route for improving the scope of a catabolic pathway is to widen the types of effectors that can regulate that particular pathway. This may allow the catabolic pathway to operate in contaminated sites that do not contain the effectors used in the non-modified pathway.

Studies with the XylS regulator of the catabolic operon of the TOL plasmid found that although the pathway was activated by benzoate, some other benzoate analogues such as 4-ethylbenzoate competitively inhibited the effector-mediated activation of XylS. Mutation of the XylS regulator resulted in all benzoate analogues being able to activate the system (Ramos et al., 1986). The inclusion of new effector compounds may also confer a greater efficiency of transcription (Timmis et al., 1994).

Extension of the catabolic pathway to include more substrates could be either lateral or vertical. Lateral extension needs the incorporation of more analogues of existing substrates while vertical extension may require addition of totally new substrates into an existing pathway. Due to the modular nature of

many catabolic pathways, the substrate range can often be extended by adding a biochemically compatible module to the microorganism which enables a new substrate to be channeled into an existing pathway (Timmis et al., 1994).

For example, the addition of dehalogenase genes to microorganisms able to co-metabolize PCBs can extend existing pathways beyond the chlorobenzoate intermediate and improve the mineralization of PCBs by single microorganism (Hrywna et al.,1999). Lateral expansion of a pathway can be achieved through the utilization of isofunctional routes for the degradation of structurally related compounds, the preferential use of enzymes with relaxed substrate specificities and site-directed mutagenesis to alter the specificity of proteins for their substrates (Timmis et al., 1994).

The ease with which protein mutagenesis can be used to alter existing pathways to include previously non-metabolized compounds depends on the number of proteins that need to adapt before catabolism of the new target compound can occur. Where only a single protein needs to be altered and the required changes can be achieved by the direct selection of a mutant that may be able to grow on the target compound. However, where multiple non-permissive proteins require alteration, the necessary mutations are likely to occur at too low a frequency to produce the desired microorganism. In this case, the proteins that are non-permissive for the new target compound must be identified and either sequentially modified to achieve the required specificity, or replaced by permissive proteins from other organisms (Timmis et al., 1994).

The scope of biodegradation of aromatic compounds can be increased in a catabolic pathway by genetic modification (Timmis et al., 1994). Following aspects needs to be addressed carefully for alteration either laterally or vertically in a metabolic pathway:

- substrate/metabolite-activated transcriptional regulators,
- mono- or di-oxygenases that mediate the initial attack on the substrate,
- ring cleavage enzymes and
- enzymes that transform the substituted muconolactones to oxoadipate.

The inability of *Pseudomonas* sp B13 to 4CB or 3,5-dichlorobenzoate (3,5DCB) is reported to be due to the narrow specificity of the first enzyme in the pathway (benzoate 1,2-dioxygenase). This enzyme only allowed the pseudomonad to degrade 3CB (Reineke and Knackmuss, 1980). Insertion of the genes encoding the isofunctional enzyme toluene 1, 2-dioxygenase enabled the bacterium to degrade 3CB and 4CB. Toluene1, 2-dioxygenase has a much broader substrate specificity than benzoate 1, 2-dioxygenase, and includes all alkyl- and chlorobenzoates.

In environmental management of pollutants bioremediation has become an obvious choice due to its ecofriendly nature. It offers cost effective, efficient and robust methods for the control of environmental pollutants. The future of bioremediation armed with recombinant DNA technology to deliver tailor-made

reagents and specific approaches for every target pollutant appears promising. Further, well defined approaches with meaningful application of software driven modelling of pollutant density and kinetics data may extend well defined bioremediation strategies to combat a single or multiple pollutants in various environmental niches.

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9

Bioaccumulation and Biotransformation of Heavy Metals

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9.1 Introduction

The strategy for increasing and improving the efficiency of remediation techniques is to increase the bioaccumulation and biotransformation potential of plant and microbes for detoxification of toxic metals. With an increase in anthropological practices, more and more toxic metal ions are being added to the natural environment disrupting the ecosystem. Metals like Cd, Pb, Cr, As etc. when present in high concentrations in soil show potential toxic effects on overall growth and metabolism of plants and microbes (Yadav et al., 2009; Juwarkar et al., 2008). Bioaccumulation of such toxic metals in the plants poses a risk to human and animal health. Removal of excess of metal ions from the contaminated site is brought about by chemical as well as biological means. However, the existence of many classes and type of chemical species make the removal of the toxic metals from the environment very complicated.

The use of biological means to clean the natural environment includes phytoremediation as well as microbial remediation techniques. The technology is based on the use of naturally occurring or genetically engineered microorganisms to restore contaminated sites and protect the environment. Other than microorganisms, certain plant species that accumulate high concentration of heavy metals also have a potential towards restoration of environment. Success of microbial remediation technology depends on the application of such microbial strains which have the ability of self replenishment, continuous metabolic uptake of metals after physical adsorption and the potential for optimization through development of resistant species and cell surface

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modification (Joshi and Juwarkar, 2009; Juwarkar et al., 2007). The rate of phytoremediation is directly proportional to plant growth rate and the total amount of phytoremediation is correlated with a plant total biomass, making the process very slow (Kumar et al., 2008). This necessitates the identification of a fast growing (largest potential biomass and greatest nutrient responses) and more strongly metal-accumulating genotypes. The technique makes use of the intrinsic capacity of plants to accumulate metals and transport them to shoots, ability to form phytochelatins in roots and sequester the metal ions. Harbouring the genes that are considered as signature for the tolerance and hyperaccumulation from identified hyperaccumulator plant species into the transgenic plants provide a platform to develop the technology with the help of genetic engineering. This would result in transgenics that may have large biomass and fast growth, a quality essential for removal of metal from soil quickly and in large quantities. Despite so much of a potential, the progress in the field of developing transgenic phytoremediator plant species is rather slow. This can be attributed to the lack of our understanding of complex interactions in the soil indigenous mechanisms in the plants that allow metal translocation, accumulation and removal from a site.

The phytoremediation technology involves several techniques like phytoextraction, phytomining, phytovolatilization, phytofilteration etc. all of which employ plants for cleaning of contaminants from the soil. A plant's ability to phytoextract certain metals is a result of its dependence upon the absorption of metals such as arsenic, chromium, zinc, manganese, nickel, and copper to maintain natural function (Yadav et al., 2009; Lasat, 2002). Research shows that the hyperaccumulators often do not exclude non-essential metals in the absorption process, thus resulting in plants that can extract high contents of pollutants (1-2% of their biomass) from contaminated soil. It is believed that plants initially developed the ability to hyperaccumulate non-essential metallic compounds as a means of self protection from herbivorous predators, who would experience toxic side effect from ingestion of the hyperaccumulator's foliage. Phytoextraction has attracted attention in recent years for the low cost of implementation and environmental benefits. This approach makes use of high-biomass crops that are induced to take up large amount of metals when their mobility in soil is enhanced by chemical treatments. Several chelating agents, such as citric acid, ethylene-diamine-tetraacetic acid (EDTA), diethylene-triamine-pentaacetic acid (DTPA), ethylene-bis[oxyethylene-trinitrilo] tetra acetic acid (EGTA), nitriloacetate (NTA) and other synthetic chelators, have been studied for their ability to mobilize metals and increase accumulation of metals in different plant species (Blaylock et al., 1997).

The bioaccumulation and transformation potential of plants and microorganisms are the key factors controlling the efficiency of remediation technology. The success of remediation of heavy metals is dependent on the accumulation and transformation rate; therefore the approaches to

increase these potential have attracted the attention of scientific community. Hyperaccumulator plants have an ability to extract heavy metals from soil and aqueous media. Roots exudates play a significant role in bioaccumulation and biotranformation/detoxification of heavy metals in plant as well as in microorganisms. Microorganisms can sustain themselves in toxic metallic environment and substantially accumulate and transform toxic metals to non-toxic metals through different ways like biotransformation, biosorption and bioaccumulation. The plants and microorganisms produce metal chelators called phytochelatins, metallothioneins, organic acids and amino acids in response to metal toxicity. These chelators are able to convert the high toxic forms to less or non-toxic forms of metals. Some microorganisms produce organic ligands such as siderophore and biosurfactant, which enhance the solubility and accumulation of the metals. Identification of mutants, which is involved in tolerance and metal hyperaccumulation in plants, is required to develop ideal transgenic hyperaccumulators. The present chapter has been focussed on the recent developments regarding enhancement of the capabilities of the bioremediation technologies for heavy metals through biotransformation and bioaccumulation potential and different mechanisms involved in detoxification process of toxic metals.

9.2 Modern Approaches for Improving the Remedial Potential

9.2.1 Metals Accumulation Potential of Plant Species

Plants can accumulate metals that are essential for growth and development (such as Cu, Mn, Fe, Zn, Mo and possibly Ni) and also some that have no known biological function (Cd, Cr, Pb, Co, Ag, Se and Hg) (Baker and Brooks, 1989; Raskin, 1996). In this context, plants have been described as solar-driven pumping stations (Cunningham et al., 1995) which can actually remove these contaminants from the environment. Although some hyperaccumulators appear to be capable of accumulating elevated concentrations of several heavy metals simultaneously, there still remains considerable specificity in metal hyperaccumulation. Tolerance to heavy metal in plants may be defined as the ability to survive in a soil that is toxic to other plants and is manifested by an interaction between a genotype and its environment (Juwarkar et al., 2008; McNair et al., 2000). Metal hyperaccumulation is a phenomenon defined as uptake and sequestration of exceptional concentration of heavy metals in the aboveground parts of a plant under field conditions. The definition is based on comparative surveys, indicating that in metalliferous soils most plants accumulate low concentrations of metal ions in their shoots while a few species, endemic to metalliferous sites, accumulate distinctly high amounts (Jambhulkar and Juwarkar, 2009). For a plant to be identified as a hyperaccumulator, the species can accumulate 0.1% of dry mass [% (d.m.)] of elements such as Ni,

Co or Pb; 1% (d.m.) of Zn and Mn and 0.01% (d.m.) of Cd. Such plants are resistant to certain metal ions suggesting their potential use for cleaning of contaminated soil (Chaney et al., 1997). Use of hyperaccumulators open a new branch of bioremediation technology termed as phytoremediation—an ecofriendly and scientific approach to remove, extract or inactivate metal ions in the soil using plants (Cunningham et al., 1995; Raskin, 1996; Chaney, 1997; Lasat, 2002).

Nearly 450 hyperacumulator plants also known as metallophytes have been described belonging to a wide range of taxa, ranging from annual herbs to perennial, geographically distributed in all continents, both in temperate and tropical environments. Notable centres of distribution are for Ni: New Caledonia, Cuba, SE Asia, Brazil, Southern Europe and Asia Minor; Zn and Pb: NW Europe; and Co and Cu: South-central Africa. Some families and genera are particularly well represented e.g., for Ni: Brassicaceae (Alyssum and Thlaspi), Euphorbiaceae (Phyllanthus), Leucocroton Asteraceae (Senecio, Pentacalia); Zn: Brassicaceae (Thlaspi); Cu and Co: Lamiaceae, Scrophulariaceae (Baker and Brooks, 1989; Chaney et al., 1997; Clemens, 2001; Broadhurst et al., 2004; Gratão et al., 2005; Prasad, 2005). Interestingly 75% of the identified hyperaccumulators accumulate Ni and are termed as nickelophilous plants (Baker and Brooks, 1989; Prasad, 2005). Of the wide range of families of vascular plants the natural hyperaccumulating plant species are well represented by the members of Brassicaceae (Reeves and Baker, 2000; Gratão et al., 2005). Natural hyperaccumulators can grow in their natural habitat alone, have slow growth, low-biomass and very often are selective for an individual metal (Clemens et al., 2002).

9.2.2 Role of Plant Roots Exudates and Interaction with Microbes in Metal Accumulation

Metals exist in various chemical forms (or species). These forms often exist in a complex equilibrium governed by many soil factors and properties. For any given heavy metal, only a fraction is bioavailable and thus potentially it is the only fraction that can be taken up by the plants (Table 9.1). More of the metal could be converted to bioavailable fraction for its gradual uptake and removal by the plant but the extent to which this happens and the kinetics of such processes is not known and are invariably soil specific. Plant roots exude organic acids, for example malic and citric acids, and/or acid phosphatases under P deficiency. This localized enhanced excretion of organic acids increases the effectiveness of exudates for the mobilization of nutrients such as P, Zn, Fe and Mn. The population density and composition of soil microorganisms in the rhizosphere are also known to affect the metal mobility and availability to the plant, through enhanced root exudation and the concentration of organic acids, chelators, and acid phosphatases released as microbial metabolites.

Rhizosphere interactions are based on complex exchanges that evolve around plant roots. Root-based interactions between plants and organisms in the rhizosphere are highly influenced by edaphic factors; however, the belowground biological interactions that are driven by root exudates are more complex than those occurring above the soil surface. The root contains organic acids that bind to metals from highly insoluble forms in the soil and acids like citric acid and malic acids can act as essential ligands for metals. The role of citric acid in regulating Al(III) and Ni(II) detoxification in plants has been clearly demonstrated (Yang et al., 2003). The root exudates are very important agents that form complexes with trace metals and affect their redox behaviour. Root exudates containing organic acid can form complexes with metal compounds, making them available for plant uptake. Studies on the role of organic acids in Cr toxicity in *Lycopersicon esculentum* showed that in the presence of organic acids like carboxylic acid and amino acids, Cr uptake in roots is enhanced (Srivastava et al., 1999). Organic acids like citric acid, aspartic acid and oxalic acid can convert inorganic Cr to organically bound Cr, making it soluble for longer period of time and thereby available to plants. Whether organic acids can play significant role in Cr detoxification is still not completely understood.

As far as production of large plant biomass in the metal-contaminated soil is concerned, in the last few decades, a large array of heterogeneous group of bacteria commonly referred to as plant growth promoting rhizobacteria (PGPR) have been reported to enhance plant growth at contaminated sites to ensure enhanced phytoremediation (Juwarkar et al., 2008; Kumar et al., 2008). These include species of *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus* and *Serratia*. They can be found in the rhizosphere, at root surfaces and in association with roots, which can improve the extent or quality of plant growth directly and/or indirectly.

In the rhizosphere, the processes mediated by roots secretes plant exudates and enzymes. In addition to the compounds that root synthesize and accumulate, a remarkable diversity of micro and macro molecular metabolites is also secreted into the rhizosphere as root exudates. Root exudates play an active and relatively well-documented role in the regulation of symbiotic and protective interactions with microbes. However, the role of root secretions in regulating other rhizospheric interactions is less clear. Through the exudation of a wide variety of compounds, it is suggested that roots can regulate the soil microbial community in their immediate vicinity, withstand herbivory, encourage beneficial symbioses change the chemical and physical properties of the soil, and inhibit the growth of competing plant species. Various mechanical functions have been attributed to root exudation including the maintenance of root-soil contact, lubrication of the root tip, protection of roots from desiccation, stabilization of soil micro-aggregates, and selective adsorption and storage of ions.

Table 9.1: Bioavailability of metals for plant uptake

Category	*Metals*
Readily bioavailable	Cd, Ni, Zn, As, Se, Cu
Moderately bioavailable	Co, Mn, Fe
Hardly bioavailable	Pb, Cr, U

Source: Alkorta et al., 2004

9.3 Molecular Approaches for Detoxification of Toxic Metals in Plants

Heavy metals like Cr, Cd, Zn, Fe, Al, Pb and As are highly reactive and toxic to living cells. Some heavy metals, such as Cu, Zn and Fe, are essential for normal plant growth and development, as they form part of many enzymes and proteins. Elevated contents of both essential and non-essential metals in the soil lead to toxicity symptoms and growth inhibition in plants which might result from binding of metal to SH-group of proteins resulting in the inhibition of their activities or disruption of structure, displacement of an essential element resulting in deficiency effects. In a number of thorough genetic studies, the adaptive metal tolerance has been shown to be governed by a small number of major genes and perhaps contribution of some minor modifier genes (McNair et al., 2000). Perhaps it is this adaptive metal tolerance that gears a plant species for hyperaccumulation. A genetic analysis of copper tolerance with Cu-tolerant and susceptible lines of *Mimulus guttatus* showed that a modifier gene that is active only in presence of the tolerance gene is responsible for the difference in Cu-tolerance in this species. Similar studies with Zn-hyperaccumulator *Arabidopsis halleri* and the non-accumulator *Arabidopsis petrea* suggested that Zn-tolerance is also controlled by a single major gene (McNair et al., 2000).

Chelators contribute to metal detoxification by buffering cytosolic metal concentrations, whereas chaperones specifically deliver metal ions to organelles and metal-requiring proteins. In plants the principal classes of metal chelators include phytochelatins, metallothioneins, organic acids and amino acids. Phytochelatins (PCs) are small metal-binding peptides found in plants and are well documented in literature (Chen et al., 1997). PC formation uses glutathione, homoglutathione, hydroxymethyl-glutathione or -glutamylcysteine. It is catalyzed by phytochelatin synthase (PCS), a constitutive enzyme requiring post-traslational activation by heavy metals and/or metalloids, in particular Cd, Ag, Pb, Cu, Hg, Zn, Sn, As and Au both in vivo and in vitro (Chen et al., 1997).

In vitro experiments have shown that a series of metal-sensitive plant enzymes can tolerate a 10- to 1000-fold concentration of Cd in the form of a PC complex than as free radical ion (Kneer and Zenk, 1992). PC reactivate metal poisoned plant enzymes such as nitrate reductase up to 1000-fold better than chelators such as glutathione (GSH) or citrate, showing again the different

sequestering potential of these peptides. Howden et al. (1995) succeeded in isolating a *cad*1-mutant of *Arabidopsis thaliana* sensitive to Cd ions and deficient in its ability to form Cd-PC complexes. *Arabidpsis* has only single pathway for PC synthesis. The finding of the Cd-sensitive mutant (impaired PC synthesis) confirmed the role of PC in Cd detoxification. The authors concluded that *cad1* gene is the likely structural gene for PC synthase (Howden et al., 1995). As-tolerant *Holcus lanatus* L is shown to synthesize high concentrations of PCs than As-intolerant species (Hartley-Whitaker et al., 2002).

Aside from detoxification, PCs also play a role in homeostasis of heavy metal in plants. On one hand, they complex the metal ions to inactivate and store them in the vacuole and on the other, they transfer the essential metal to the newly synthesized apoenzymes that require Cu^{2+} or Zn^{2+} for catalytic activity or to nucleic acid structures such as Zn-figers. Plants have developed complex mechanisms by which they control the uptake and accumulation of heavy metals (Cobbett and Goldsbrough, 2002). These mechanisms involve chelation and sequestering of metal ions by a particular class of metal binding ligands denominated phytochelatins (PCs) and metallothioneins (MTs) (Cobbett and Goldsbrough, 2002). Metallothioneins (MTs) are ubiquitous low molecular mass cysteine (cys)-rich proteins that bind metal ions in metal thiolate clusters identified in mature embryos of wheat plants as early cys-labeled protein. More than 50 MTs are reported in different plants categorized in four classes of MT proteins (Cobbett and Goldsbrough, 2002). MTs have a possible role in Cr detoxification in plant and it has been reported for sorghum that MT-like proteins are expressed under Cr stress (Shanker et al., 2004). MTs are the products of mRNA translation and are characterized as low molecular weight cystein-rich metal-binding protein. The role of PCs in regulating metal toxicity has been reported in plants. It was suggested that the production of ROS and H_2O_2 as a result of metal exposure might have triggered signals to induce MT mRNA transcription (Shanker et al., 2004). Thus, MTs may have a very important role in metal detoxification in plants, possibly by binding metal ions and making them non-toxic.

9.4 The Potential of Microorganisms for Bioremediation of Metals

9.4.1 Utilization of Microorganisms in Bioremediation

The fate of toxic metal ions in the soil environment depends largely on the interactions of these metals with various inorganic and organic surfaces. One potentially important organic surface which can be exploited in metal remediation is the soil microbial population. Bacteria have a high surface area-to-volume ratio and hence possess a high capacity for sorbing metals from solution. To date, all commercial microbial remediation uses naturally occurring organisms, generally those identified at contaminated sites. Organisms living in contaminated areas develop the ability to metabolize the contaminant, if even

only to a small degree, through a process called adaptation. Microbes evolve the ability to hyperaccumulate metals and non-metals, which gives them a selective advantage within a contaminated environment, allowing them to survive and evolve additional resistance.

Microorganisms can interact with heavy metals via many mechanisms some of which may be used as the basis of potential bioremediation strategies. The major types of interaction are summarized in Figure 9.1. Soil microorganisms have been shown to possess several mechanisms capable of altering metal bioavailability for uptake. For example, microbes have been documented to catalyze redox reactions leading to changes in metal mobility in soil and propensity for uptake. In addition, root mycorrhizal associations have been shown to affect the rate of metal uptake in plants.

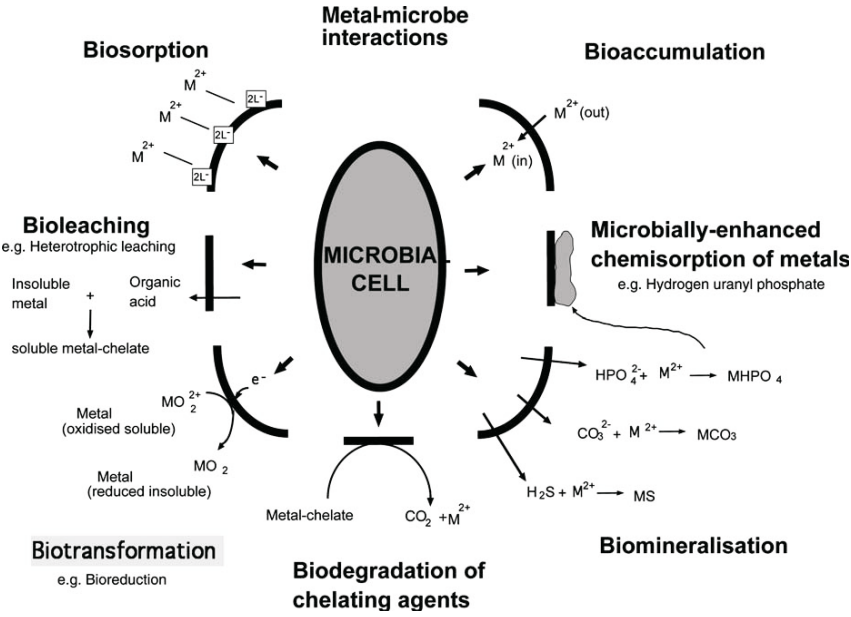

Fig. 9.1: Metal–microbe interactions impacting bioremediation (Tabak et al., 2005).

9.4.2 Metal Resistance Mechanisms in Microorganisms

Toxicity of non-essential metals occurs through the displacement of essential metals from their native binding sites or through ligand interactions (Nies, 1999; Bruins et al., 2000). For example, Hg^{2+}, Cd^{2+} and Ag^{2+} tend to bind to SH groups, and thus inhibit the activity of sensitive enzymes (Nies, 1999). In addition, at high levels, both essential and non-essential metals can damage cell membranes; alter enzyme specificity; disrupt cellular functions; and damage the structure of DNA (Bruins et al., 2000). However, to have any toxic effect most of the metal ions have to enter the cell first. To cope up with the situation

microorganisms have evolved two types of uptake systems for metal ions. One is fast, unspecific, and driven by the chemiosmotic gradient across the cytoplasmic membrane of bacteria (Nies, 1999); the other being an uptake system having high substrate specificity. This system however is slower, often uses ATP hydrolysis as the energy source and is only produced by the cell in times of need, starvation or a special metabolic situation. Cells thus combat the heavy metal ions either intracellularly or extracellularly. There are many mechanisms of heavy metal resistance in microorganisms which involve: energy-dependent efflux of metal, precipitation of metal as insoluble salts, alteration in membrane permeability to metal, immobilization of metal within the cell wall, production of chelating agents and biochemical transformation of metal ions. Microbes resistant to Cd, Zn, Co, Cr, Co, As and Ni are isolated from several contaminated sites and natural deposits and it was found that the resistance-encoding genes appear to be predominantly plasmid-located and probably spread by horizontal transfer (Bender et al., 1990).

9.4.3 Bioaccumulation and Biotransformation of Metals in Microorganisms

There are at least three types of microbial processes that can influence toxicity and transport of metals: biotransformation, biosorption and bioaccumulation, and degradation or synthesis of organic ligands such as siderophore and biosurfactant that affect the solubility of the contaminants (Nair et al., 2007; Juwarkar et al., 2007). Each offers the potential for bioremediation of contaminants in the environment. Metal-reducing microorganisms can reduce a wide variety of multivalent metals that pose environmental problems. The heavy metals subject to enzymatic reduction by microbes include but are not limited to chromium (Cr). Direct enzymatic reduction involves use of the oxidized forms of these contaminants as electron acceptors. The oxidized forms of metals are highly soluble in aqueous media and are generally very mobile in aerobic ground water, while the reduced species are highly insoluble and often precipitate from solution (Nair et al., 2008).

The term ‘bioaccumulation’ is employed to indicate the concomitance of adsorptive and metabolism-dependent mechanisms, in contrast to ‘biosorption’, which does not involve metabolic contribution and can be effected also by non-viable biomass. The relevance of such metal-microbe interactions on the geochemical cycles is widely recognized. Microbial biomass can bind heavy metals either actively, or passively or by a combination of both processes (Nair et al., 2008). However, the passive phenomenon of biosorption has several advantages over the active phenomenon of bioaccumulation. A major disadvantage of bioaccumulation is recovery of the accumulated metal by destructive means whereas in biosorption desorption is accomplished by simple physical methods without damaging the biosorbent’s structural integrity. Moreover biosorption has an edge over bioaccumulation with an easy and

cost-effective procurement of the biomass either as a by-product of large-scale fermentation process or bulk procurement from natural water bodies. Several investigations have shown that relatively large quantities of metallic cations are algae and bioaccumulated bacteria (Strandberg et al., 1981). Metal binding by isolated gram-positive and gram-negative bacterial cell walls has also been evaluated by several investigators (Juwarkar, 1988; Strandberg et al., 1981). In addition to the adsorptive interactions microorganisms are also capable of accumulating metal ions (inspite of their toxicity) by metabolism mediated mechanisms such as metal transport and storage within the cytoplasm; intracellular detoxification systems, such as metal binding protein (metallothioneins) or polyphosphate synthesis (Silver, 1996). Microorganisms highly effective in sequestering heavy metals include bacteria, fungi, algae, and actinomycetes.

Metallothioneins (MTs) are low molecular weight (6-7 kDa), cysteine-rich proteins found in animals, higher plants, eukaryotic microorganisms and some prokaryotes. They are divided into three different classes on the basis of their cysteine content and structure. MTs has been widely exploited due to their role in enhancing the metal tolerance, metal sequestration or accumulation and the high metal-binding capacity. The only prokaryotic MT identified so far is found in a few cyanobacterial strains of the genus *Synechococcus*. Metal-binding peptides and MTs have mainly been exploited to increase the metal tolerance or accumulation of *E. coli* cells. Such metal binding peptides and proteins can be expressed into more environmentally robust bacteria, such as *Pseudomonas* for their potential use in bioremediation (Valls et al., 2000). Recently, Tripathi et al. (2007) reported the modern strategies for the detoxification of arsenic in plant and microbial system (Figure 9.2).

One of the most important metal detoxification mechanisms in microbes is biotransformation in which the metal is converted to less toxic or easily recoverable forms. Microbial transformations have been divided into two broad categories: redox conversions of inorganic forms; and conversions from inorganic to organic form and vice versa, typically methylation and demethylation. Through oxidation of certain metals like iron, sulphur, manganese and arsenic, microbes can obtain energy (Santini et al., 2000). On the other hand, reduction of metals such as arsenic, selenium, chromium and uranium occurs through dissimilatory reduction where microorganisms utilize metals as a terminal electron acceptor for anaerobic respiration (Niggemyer et al., 2001; Stolz and Oremland, 1999). Certain microorganisms may possess reduction mechanisms that are not coupled to respiration, but instead are thought to impart metal resistance; examples include aerobic and anaerobic reduction of Cr(VI) to Cr(III); reduction of Se(VI) to elemental selenium (Lloyd et al., 2001); reduction of U(VI) to U(IV) (Chang et al., 2001); and reduction of Hg(II) to Hg(0) (Wagner-Döbler et al., 2000; Brim et al., 2000). Such biotransformations are important components of biogeochemical cycles of metals and may also be exploited in bioremediation of metal contaminated soils.

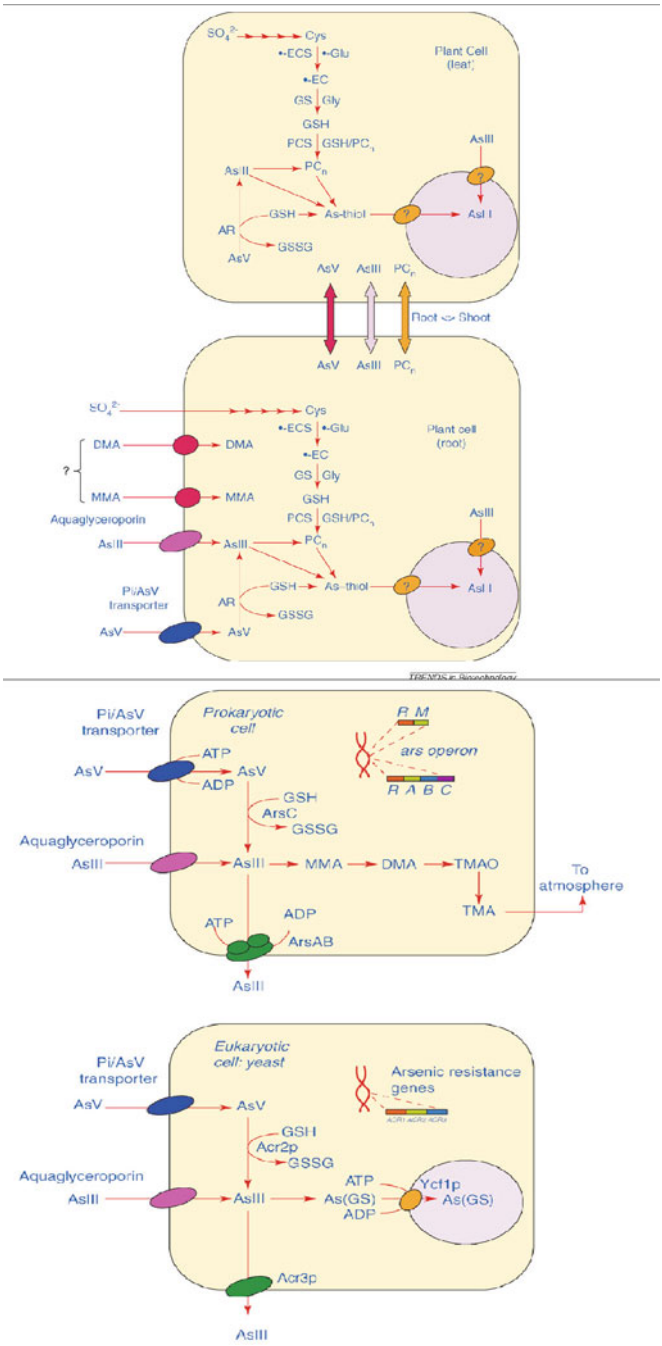

Fig. 9.2: Comparison of the methods for arsenic detoxification in plant (top panel) and bacteria (bottom panel) (Tripathi et al., 2007).

9.5 Advancement of Remediation Technology

9.5.1 Development of Phytoremediator Transgenic Plants for Metal Accumulation

Though several natural hyperaccumulators are known, the plants ideal for green technology to clean up soil should possess multiple traits. They must have deep roots, rapid growth, high biomass, be easily harvested, and must tolerate and accumulate a large range of heavy metals in their aboveground parts. The development of engineered plants (transgenic) harbouring the required traits for bioremediation is perhaps the only alternative. Over-expression and introduction of hyperaccumulating genes into non-hyperaccumulator plant could be a possible way to enhance metal uptake and accumulation, tolerance and detoxification process (Clemens et al., 2002). The over-expression of a gene encoding a rate limiting gene product would be expected to lead a faster overall rate of the pathway and to more efficient phytoremediation. Besides this, the repression of an endogenous gene, by inserting a gene of reverse orientation (antisense technology), can also result in enhanced metal uptake by plants. Several reports on bioengineered plant tolerant to the presence of toxic levels of metals like Se, Cd, As, Zn, Cr, Cu, Pb, etc. have appeared in the literature (Kawashima et al., 2004; Lee et al., 2003). In most of the studies over-expression of the genes encoding for the enzymes of S-metabolism, glutathione, phytochelatin synthase, ACC deaminase, Hg^{2+} reductase, arsenate reductase, aldolase/aldehyde reductase, enzymes of histidine biosynthesis and metallothionein (MT) genes have been carried out. The engineering of transporter genes to manipulate the transport of metal ions inside the cell has also been exploited effectively and a combination of some of these genes in rapidly growing plant species have led to a few promising results (Lee et al., 2003).

A well known example of transgenic metal hyperaccumulator is *Brassica juncea*, which over-express ATP sulphurylase. It shows higher uptake of Se and enhanced Se tolerance compared to wild type when grown in the presence of selenate in either hydroponic conditions or in soil (van Huysen et al., 2004). These transgenic plants can also tolerate Cd, Zn, Cu, Hg and As(III, IV). Transgenic Indian mustard over-expressing cystathione- -synthase (CGS) had low shoot Se concentration with enhanced Se volatilization rate as well as Se tolerance than the wild type plants grown either hydroponically or in soil (Van Huysen et al., 2004). In an attempt to improve the potential for removal of metals using plants, Brewer et al. (1999) created somatic hybrids between *Thlaspi caerulescens* (Zn-hyperaccumulator) and *Brassica napus*. Accumulation of high levels of Zn was observed in hybrids, which otherwise are toxic for *B. napus*. *Arabidopsis* plants transformed with an *E. coli* gene *Znt A* that encodes for Pb^{2+}, Cd^{2+} and Zn^{2+} transport had improved resistance to Pb^{2+} and Cd^{2+} and the *ZntA* was located at the plasma membrane (Lee et al., 2003). Transgenic tomato plants expressing the bacterial gene 1-aminocyclopropane-

1-carboxylic acid (ACC) deaminase showed enhanced metal accumulation and tolerance levels for a range of heavy metals (Cd, Cu, Ni, Mg, Pb and Zn) than untransformed plants (Grichko et al., 2000). The possibilities for enhancing the metal tolerance and phytoremediation potential of higher plants via expression of TcHMA4 hold great potential in metal remediation studies (Papoyan and Kochian, 2004). Arsenic poisoning is a serious problem. Dondon et al. (2005) and Dhankher et al. (2002) examined the effects of co-expressing two bacterial genes for arsenate reductase (arsC) and -glutamylcysteine synthetase (-ECS) in *Arabidopsis* plants. Other than plants the potential model phytoremediators for As now include various genotypes of transgenic trees.

In recent years, the molecular understanding of entry of both essential and non-essential metal ions in plant cells has been greatly advanced. Several plant metal transporters are known and more remain to be identified. The transporters identified so far include ZIP1-4, ZNT1, IRT1, COPT1, AtVramp1/3/4 and LCT1 on the plasma membrane-cytosol interface; ZAT, ABC type, AtMRP, HMT1 and CAX2 seen in vacuoles; and RAN1 seen in Golgi bodies (Table 9.2). Manipulations of these transporters to achieve removal of metal ions from the cell holds great potential.

Fe and Zn uptake is mediated by a group of transporters belonging to the ZIP family i.e. ZRT and IRT related proteins. IRT1 is isolated from *Arabiopsis* and its transcription is induced in *Arabidopsis* roots by iron starvation, which makes this transporter a likely candidate for mediating Fe(II) uptake from soil. IRT1 shows a broad substrate range and also transport Mn^{2+}, Zn^{2+} and possible Cd^{2+} (Korshunova et al., 1999). ZIP-transporters 1-3 confer Zn^{2+} uptake activity (Guerinot and Eide, 1999). Characterization of new metal transporters *Medicago truncatula* with high similarities with the ZIP family (MtZIP) revealed their functions as metal transporters (Lopez-Millan et al., 2004).

The Zn transporter gene ZTP1 is highly similar to the *Arabidopsis* ZAT gene. It probably is an allele of the recently cloned ZNT1 and its close homologue *ZnT2* gene from *Thlaspi caerulescens* (Pence et al., 2000). All three zinc

Table 9.2: Metal ion transporters in plants (Clemens, 2001)

Localization	*Transporter*	*Metal ions*
Cytosol	ZIP1-4	Zn^{2+}, Cd^{2+}
	ZNT1	Zn^{2+}, Cd^{2+}
	IRT1	Fe^{2+}, Mn^{2+}, Zn^{2+}, Cd^{2+}
	COPT1	Cu
	AtVramp1/3/4	Fe^{2+}, Cd^{2+}
	LCT1	Cd^{2+}, Ca^{2+}
Vacuole	ZAT1	Zn^{2+}
	At MRP	Cd-PC
	HMT1	Cd-PC
	CAX2	Cd^{2+}
Golgi	RAN1	Cu complex

transporter genes show increased expression in *T. caerulescens* compared with the non-hyperaccumulator congener *T. arvense*, suggesting an important role in heavy metal hyperaccumulation. The natural resistance associated macrophage proteins (Nramp) family of transporters has been recently characterized from rice and *Arabidopsis*.

Trees are ideal in the remediation of heavy metals as they can withstand and accumulate higher concentration of pollutants owing to their large biomass and size, can reach a huge area and great depths for their extensive rooting and can stabilize an area. Researchers are now trying to extend this technology to cottonwood trees, which are potential effective remediators (Dondon et al., 2005).

9.5.2 Genetically Modified Microorganisms for Metal Accumulation

There may be other unique uses for GMOs in the remediation of sites suffering from a combination of metal and/or organic and radionuclide contamination. Researchers are looking to identify the genes that allow microbes to take up heavy metals or organic contaminants, using these to transform the radioactivity-tolerant species *D. radiodurans*. Radiation resistant strain which could treat mercury contamination has been engineered (Brim, 2000). A group in Spain has engineered a bacterium *Ralstonia eutropha* to contain a gene for mouse metallothionein, a small protein that binds heavy metals (Valls et al., 2000). Such a protein is expressed on the bacteria's outer membrane, enabling it to immobilize heavy metals. The engineering of metal binding peptides on the surface of environmentally acceptable gram-negative bacteria such as *Ralstonia eutropha* and *Pseudomonas putida*, which are already employed in existing systems for heavy metal bioremediation (Diels et al., 1993; Macaskie and Dean, 1990), represents a possible application for enhanced bioaccumulation. Laboratory experiments showed that genetically engineered *R. eutropha* could, indeed, hyperaccumulate cadmium.

However, the commercial potential of GMOs in remediation seems limited. So, there is no need to engineer microorganisms for this purpose, because naturally occurring microbes are effective enough. Moreover, the cost of producing GMOs is high, and with the exception of academic research, a permit from the EPA is needed for field use. Furthermore, the switch in public perception, now more skeptical of the safety of GMOs and opposed to their release into the environment has confounded efforts in this area.

9.6 Future Prospects

Scientists have been able to expand the role of plants in the environment for the metal detoxification using genetic modifications. In order to restore environmental balance the bioremediation technique evidently does indicate several benefits and is one of the most preferred methods to deal with this

problem. However, the efficiency of the method lies in the fact that to implement a specific bioremediation method, a scientific and well formulated strategy must be adopted taking into consideration the type of metal ions, geographical location, biomass of the hyperaccumulator plant, etc. Detailed studies in the field of bioremediation have improved the methods and practice; however, further improvements are required to reduce the limitations of the existing protocols so that they may be utilized with less negative output. The various studies of metal-ion homeostasis in human, yeast and plants suggest that there exists a complex regulated network for metal ion transport, chelation and sequestration. Further identification of mutants for biochemical, molecular and physiological analysis and important molecules involved in tolerance and metal hyperaccumulation in plants is required to develop ideal transgenic hyper-accumulators. Isolation of metal-sensors that sense the metal status of the cell and to elucidate the subsequent regulatory steps in terms of up-regulation and down-regulation of metal-responsive genes, the signal transduction pathway and the responsive transcription factors would also be of immense use for developing effective phytoremediators.

However for effective bioremediation, application of active and growing cells could be a better option due to their ability of self replenishment, continuous metabolic uptake of metals after physical adsorption and the potential for optimization through development of resistant species and cell surface modification. Also, instead of depending on single species for metal removal, a multispecies consortia offers more advantages. Such multispecies consortia can better withstand extreme environmental condition. The rich exopolymer content of the biofilms may also be beneficial for both entrapping dispersed solids and biosorption of dissolved metals. Further, they provide microenvironment (like alkaline pH, high concentrations of CO_2), which could be very beneficial for metal precipitation.

Metal hyperaccumulator plants are relatively rare, often occurring in remote areas geographically and threatened by devastation from mining activities. There is thus an urgent need to collect these materials, bring them into cultivation and establish a germplasm facility for large-scale production of hyperaccumulator species for future research, development and trial work. The cleaning of the metal-contaminated environment might be feasible with a combinatorial approach, i.e. integrate and use different bioremediation methods simultaneously and target the technology both qualitatively as well as statistically. Plant biologists, microbiologists, agronomists and engineers will have to integrate their efforts for the bioremediation of a specific site contaminated with specific heavy metals.

9.7 Conclusion

In view of the above recent information, it may be concluded that plants accumulation and bacterial transformation potential of metals are the key to

phytoremediation and bioremediation technology. Molecular approaches can be used to enhance its applicability more effectively. So, bioaccumulation and biotransformation mechanisms need more attention for further study. Further root-root, root-soil and root-microbes interactions are the important frontier areas which can improve the treatment capabilities of phyto- and bioremediation processes.

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10

Reclamation and Remediation of Solid Waste through Bio-chemical Process

D.S. Ramteke

10.1 Introduction

The amount of hazardous waste generated by manufacturing industries increased tremendously due to industrialization worldwide. This waste is generated at every stage in the production process, use and disposal of manufactured products. Thus, the introduction of many new products for the home and office—computers, drugs, textiles, paints and dyes, plastics—also introduced hazardous waste, including toxic chemicals, into the environment. These, too, must be managed with extreme care to avoid adverse environmental or human health impacts.

India is the second-most populous country, which has about 16% of the world population and 25% of the land area. Rapid industrialization during last few decades has led to the depletion of precious natural resources and pollutes continuously. Further generation of huge quantities of hazardous wastes has further aggravated and creates environmental problems all over the world.

Industrial solid waste management constitutes today a major environmental, economical and social problem worldwide, mainly because the waste volume is growing faster than the world's population. Moreover, as stricter environmental requirements are continuously imposed regarding ground and surface waters, the treatment of landfill leachate becomes a major environmental concern. Hazardous waste management is a new concept for most of the Asian countries including India. The lack of technical and financial resources and the regulatory control for the management of hazardous wastes in the past had led to the unscientific disposal of hazardous wastes all over the world.

M.H. Fulekar (ed.), *Bioremediation Technology: Recent Advances*,
DOI 10.1007/978-90-481-3678-0_10,

The sanitary landfill method for the ultimate disposal of solid waste material continues to be widely accepted and used due to its economic advantages. Comparative studies of the various possible means of eliminating solid waste (land filling, incineration, composting etc.) have shown that the cheapest, in term of exploitation and capital costs, is land filling.

The leachate formation occurs when soluble components are dissolved (leached) out of a solid material by percolating water. Leachates may contain large amounts of organic matter (biodegradable, but also refractory to biodegradation), where humic-type constituents consist an important group, as well as ammonia-nitrogen, heavy metals, chlorinated organic and inorganic salts. The characteristics of landfill leachate depend on the type of industrial waste being dumped, the degree of solid waste stabilization, site hydrology, moisture content, seasonal weather variations, and age of the landfill and stage of the decomposition in the landfill.

If not properly treated, leachate that seeps from a landfill can enter the underlying groundwater, thus posing potentially serious hazards to the surrounding environment and to public health. Therefore it is necessary to treat hazardous industrial solid waste.

Soil is considered to be one of the most important natural resources for human beings. However, organic and inorganic pollutants occur frequently within the soil environment. Heavy metals, Polycyclic Aromatic Hydrocarbons (PAHs) and pesticides are the most common environmental contaminants. Heavy metals pollution has received increasing attention in recent years mainly because of public awareness of environmental concerns that heavy metals are toxic at higher levels in both natural and man-made environment ecosystem. Heavy metals are ubiquitous in nature and hence metals like lead, mercury, chromium and cadmium are well known to be toxic. The contamination are significantly increased due to mining, smelting, chemicals manufacturing, fuel production, fertilizer, sewage and pesticides application and municipal waste generation. Many metals and metalloids play in functions of living organisms as microelements (e.g. Fe, Mn, Mg, Ni, Zn, Cu etc.) serving as components of enzymes, structural proteins and pigments and maintaining the ionic balance and osmotic potential of cell. It has been demonstrated repeatedly that heavy metals adversely affect biological functions in soil, including the size of activity and diversity of the soil microbial community, and the activity of enzyme involved in transformations of C, N, P and S.

Due to the toxic, persistent, bio-accumulative, and synergistic effect of some metals in biota, their cycling and fate in the environment are of great concern. The threat of metal pollution to public and environment health has encouraged interest in developing system that can remove metal contamination from soil and water, or at least neutralize its harmful effects. Most contaminated environments contain mixtures of pollutants, the most troublesome components usually turning out to be metals.

Millions of tons of pesticides are applied annually which is used in modern agriculture to increase production through controlling harmful effects caused by the target organisms including insects, fungi, bacteria, viruses as well as grasses grown in between the economical crops. However, less than 5% of these products are estimated to reach the target organisms. One of the most important problems with the use of pesticides is their possible persistence in the soil environment and therefore, their possible incorporation into the food chain affects ecosystem and all human beings.

Polycyclic aromatic hydrocarbons (PAHs) are a group of common environmental contaminants. PAHs originate from anthropogenic sources such as waste incineration, coal gasification, accidental oil spills, as well as natural processes such as fossil fuel and wood combustion. Polycyclic aromatic hydrocarbons (PAHs) are by-products of the incomplete combustion or pyrolysis of organic materials. They are considered to be priority pollutants in the environment and are of major concern due to their recalcitrance and strong mutagenic/carcinogenic properties. The hydrophobic characteristic and persistence of PAHs result in their accumulation and enrichment in soils. PAHs are widespread and occur at high concentrations of hundreds of mg/kg in soils. The contamination of PAHs in soil is a worldwide environmental problem. So the present study focusses our attention towards eco-friendly solution of these severe environmental problems through phytoremediation.

Reclamation of hazardous wastes disposal site is a necessity of the present era due to the limitation of the land required for disposal, to maintain the aesthetic view through plantation, to establish the pristine eco-system and restrict the pollution load to enter into the surface and groundwaters. Amendment of soil with activated carbon (impregnation) having its large surface area, functional groups, porosity and ability to adsorb most of the heavy metals, organics like high molecular weight compounds (PAHs and pesticides) will be one of the solutions to reduce the organic and inorganic contaminants from the contaminated soil matrix. Moreover, the carbon load through activated carbon and soil may help to degrade the adsorbed materials, rejuvinate the soil condition and convert it into productive soil due to microbial activities in the soil in situ condition through proper conditioning of the amended soil.

Phytoremediation is considered as an innovative, economical and environmentally compatible solution for remediating contaminated sites. It is used for treating many classes of contaminants including petroleum hydrocarbons, heavy metals and pesticides.

The amendment of soil at the hazardous waste disposal site with activated carbon for the adsorption of organics and inorganics from the soil matrix and conversion into productive soil through in situ microbial activities besides exploration of phytoextraction potentials of hyper accumulator plant species which grow in organic and inorganic contaminated sites is the recent trend to be adopted for phytoremediation. Phytoremediation is a general term used to describe various mechanisms by which living plants alter the chemical

composition of the soil matrix in which they are growing. Essentially, it is the use of green plants to arrest and clean-up contaminated soils through natural and exchange phenomenon. The advantages of this technique are evident in that the cost of phytoremediation is much less than traditional in situ and ex situ processes. The phytoremediation is considered an innovative, economical, and environmentally compatible solution for remediating contaminated sites. It is used for treating many classes of contaminants including petroleum hydrocarbon, heavy metals and pesticides.

10.2 Types of Solid Waste

Solid waste can be classified into different types depending on their source: (a) Household waste is generally classified as municipal waste, (b) Industrial waste as hazardous waste, and (c) Biomedical waste or hospital waste as infectious waste.

10.2.1 Municipal Solid Waste

Municipal solid waste consists of household waste, construction and demolition debris, sanitation residue, and waste from streets. This garbage is generated mainly from residential and commercial complexes. With rising urbanization and change in lifestyle and food habits, the amount of municipal solid waste has been increasing rapidly and its composition changing. In 1947 cities and towns in India generated an estimated six million tonnes of solid waste, in 1997 it was about 48 million tonnes. More than 25% of the municipal solid waste is not collected at all; 70% of the Indian cities lack adequate capacity to transport it and there are no sanitary landfills to dispose off the waste. The existing landfills are neither well equipped nor well managed and are not lined properly to protect against contamination of soil and groundwater.

Over the last few years, the consumer market has grown rapidly leading to products being packed in cans, aluminium foils, plastics, and other such nonbiodegradable items that cause incalculable harm to the environment. In India, some municipal areas have banned the use of plastics and they seem to have achieved success. For example, today one will not see a single piece of plastic in the entire district of Ladakh where the local authorities imposed a ban on plastics in 1998. Other states should follow the example of this region and ban the use of items that cause harm to the environment. One positive note is that in many large cities, shops have begun packing items in reusable or biodegradable bags. Certain biodegradable items can also be composted and reused. In fact proper handling of the biodegradable waste will considerably lessen the burden of solid waste that each city has to tackle.

Garbage can be classified into four broad categories.

Organic waste **:** kitchen waste, vegetables, flowers, leaves, fruits.

Toxic waste	:	old medicines, paints, chemicals, bulbs, spray cans, fertilizer and pesticide containers, batteries, shoe polish.
Recyclable	:	paper, glass, metals, plastics.
Soiled	:	hospital waste such as cloth soiled with blood and other body fluids.

10.2.2 Hazardous Waste

Industrial and hospital waste is considered hazardous as they may contain toxic substances. Certain types of household waste are also hazardous. Hazardous wastes could be highly toxic to humans, animals, and plants; are corrosive, highly inflammable, or explosive; and react when exposed to certain things e.g. gases. India generates around seven million tonnes of hazardous wastes every year, most of which is concentrated in four states: Andhra Pradesh, Bihar, Uttar Pradesh, and Tamil Nadu. Household waste that can be categorized as hazardous waste include old batteries, shoe polish, paint tins, old medicines, and medicine bottles.

Hospital waste contaminated by chemicals used in hospitals is considered hazardous. These chemicals include formaldehyde and phenols, which are used as disinfectants, and mercury, which is used in thermometers or equipment that measure blood pressure. Most hospitals in India do not have proper disposal facilities for these hazardous wastes. In the industrial sector, the major generators of hazardous waste are the metal, chemical, paper, pesticide, dye, refining, and rubber goods industries.

10.2.3 Hospital Waste

Hospital waste is generated during the diagnosis, treatment, or immunization of human beings or animals or in research activities in these fields or in the production or testing of biologicals. It may include wastes like sharps, soiled waste, disposables, anatomical waste, cultures, discarded medicines, chemical wastes, etc. These are in the form of disposable syringes, swabs, bandages, body fluids, human excreta, etc. This waste is highly infectious and can be a serious threat to human health if not managed in a scientific and discriminate manner. It has been roughly estimated that of the 4 kg of waste generated in a hospital at least 1 kg would be infected.

Surveys carried out by various agencies show that the health care establishments in India are not giving due attention to their waste management. After the notification of the Bio-medical Waste (Handling and Management) Rules, 1998, these establishments are slowly streamlining the process of waste segregation, collection, treatment, and disposal. Many of the larger hospitals have either installed the treatment facilities or are in the process of doing so.

10.3 Segregation of Municipal Solid Waste

Municipal waste is being generated in ever increasing volumes in the urban areas. The schematic diagram (Fig. 10.1) describes how municipal solid waste is segregated and where it can be used.

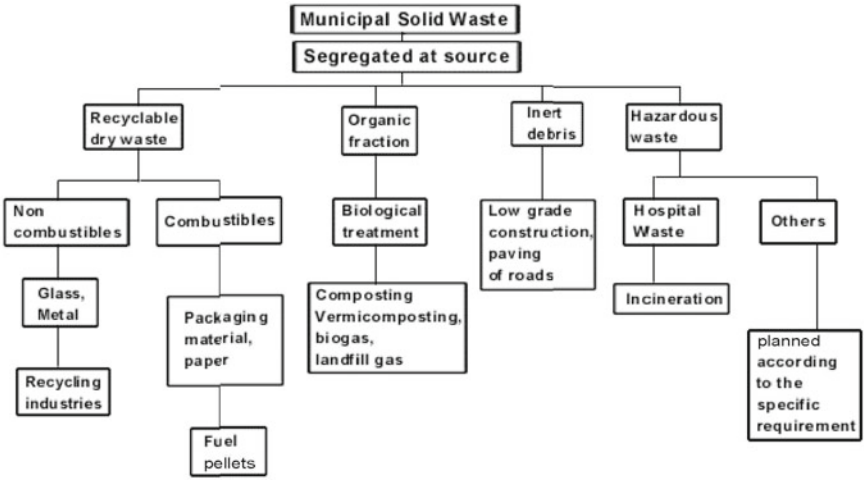

Fig. 10.1: Process of segregation of solid waste.

10.4 Recycling and Reuse

Recycling involves the collection of used and discarded materials, processing these materials and making them into new products. It reduces the amount of waste that is thrown into the community dustbins, thereby making the environment cleaner and the air more fresh to breathe. Surveys carried out by government and non-government agencies in the country have all recognized the importance of recycling wastes. However, the methodology for safe recycling of waste has not been standardized. Studies have revealed that 7-15% of the waste is recycled. If recycling is done in a proper manner, it will solve the problems of waste or garbage. At the community level, a large number of NGOs (Non Governmental Organizations) and private sector enterprises have taken an initiative in segregation and recycling of waste (EXNORA International in Chennai recycles a large part of the waste that is collected). It is being used for composting, making pellets to be used in gasifies, etc. Plastics are sold to the factories that reuse them.

The steps involved in the process prior to recycling include (a) Collection of waste from doorsteps, commercial places, etc. (b) Collection of waste from community dumps, (c) Collection/picking up of waste from final disposal sites.

The schematic diagram (Fig. 10.2) depicts recycling of wastes.

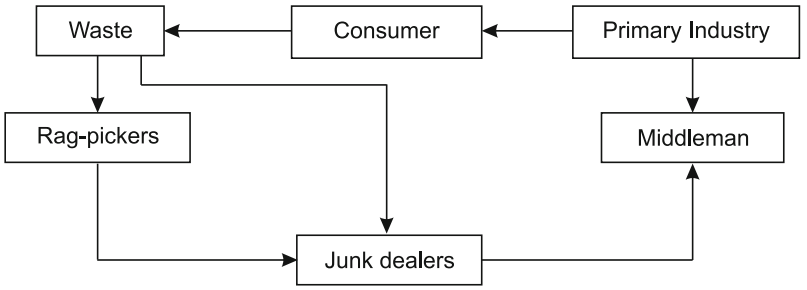

Fig. 10.2: Recycling of wastes.
Source: CPCB Report on Management of Municipal Solid Waste

Waste recycling has some significant advantages. It

- leads to less utilization of raw materials,
- reduces environmental impacts arising from waste treatment and disposal,
- makes the surroundings cleaner and healthier,
- saves on landfill space,
- saves money, and
- reduces the amount of energy required to manufacture new products.

Composting

Organic matter constitutes 35–40% of the municipal solid waste generated in India. This waste can be recycled by the method of composting, one of the oldest forms of disposal. It is the natural process of decomposition of organic waste that yields manure or compost, which is very rich in nutrients. Composting is a biological process in which micro-organisms, mainly fungi and bacteria, convert degradable organic waste into humus-like substance. This finished product, which looks like soil, is high in carbon and nitrogen and is an excellent medium for growing plants. The process of composting ensures that the waste produced in the kitchens is not carelessly thrown and left to rot. It recycles the nutrients and returns them to the soil as nutrients. Apart from being clean, cheap, and safe, composting can significantly reduce the amount of disposable garbage. The organic fertilizer can be used instead of chemical fertilizers and is better, specially when used for vegetables. It increases the soil's ability to hold water and makes the soil easier to cultivate. It helped the soil retain more of the plant nutrients.

10.5 Municipal Solid Waste

Municipal solid waste (MSW), also called urban solid waste, is a waste type that includes predominantly household waste (domestic waste) with sometimes the addition of commercial wastes collected by a municipality within a given area. They are in either solid or semisolid form and generally exclude industrial

hazardous wastes. The term *residual waste* relates to waste left from household sources containing materials that have not been separated out or sent for reprocessing.

The Functional Elements of Solid Waste

- *Waste generation*
  Waste generation encompasses activities in which materials are identified as no longer being of value and are either thrown away or gathered together for disposal.
- *Waste handling and separation, storage and processing at the source*
  Waste handling and separation involves the activities associated with management of waste until they are placed in storage container for collection. Handling also encompasses the movement of loaded containers to the point of collection. Separation of waste components is an important step in the handling and storage of solid waste at the source.
- *Collection*
  The functional element of collection includes not only the gathering of solid waste and recyclable materials, but also the transport of these materials, after collection, to the location where the collection vehicle is emptied. This location may be a material processing facility, a transfer station or a landfill disposal site.
- *Separation and processing and transformation of solid wastes*
  The types of means and facilities that are now used for the recovery of waste materials that have been separated at the source include curbside collection, drop off and buy-back centres. The separation and processing of wastes that have been separated at the source and the separation of commingled wastes usually occur at a materials recovery facility, transfer stations, combustion facilities and disposal sites.
- *Transfer and transport*
  This element involves two steps: (i) the transfer of wastes from the smaller collection vehicle to the larger transport equipment and (ii) the subsequent transport of the wastes, usually over long distances, to a processing or disposal site.
- *Disposal*
  Today the disposal of wastes by landfilling or landspreading is the ultimate fate of all solid wastes, whether they are residential wastes collected and transported directly to a landfill site, residual materials from materials recovery facilities (MRFs), residue from the combustion of solid waste, compost or other substances from various solid waste processing facilities. A modern sanitary landfill is not a dump; it is an engineered facility used for disposing of solid wastes on land without creating nuisances or hazards to public health or safety, such as the breeding of rats and insects and the contamination of ground water.

10.6 Solid Waste Management in India

There has been a significant increase in MSW (municipal solid waste) generation in India in the last few decades. This is largely because of rapid population growth and economic development in the country. Solid waste management has become a major environmental issue in India. The per capita of MSW generated daily, in India ranges from about 100 g in small towns to 500 g in large towns. Although, there is no national level data for MSW generation, collection and disposal, the increase in solid waste generation, over the years, can be studied for a few urban centres. For example, the population of Mumbai grew from around 8.2 million in 1981 to 12.3 million in 1991, registering a growth of around 49%. On the other hand, MSW generated in the city increased from 3200 tonnes per day to 5355 tonnes per day in the same period registering a growth of around 67% (CPCB, 2000). This clearly indicates that the growth in MSW in our urban centres has outpaced the population growth in recent years. This trend can be ascribed to our changing lifestyles, food habits, and change in living standards.

Municipal solid waste in cities is collected by respective municipalities and transported to designated disposal sites, which are normally low lying areas on the outskirts of the city. The limited revenues earmarked for the municipalities make them ill-equipped to provide for high costs involved in the collection, storage, treatment, and proper disposal of MSW. As a result, a substantial part of the MSW generated remains unattended and grows in heaps at poorly maintained collection centres. The choice of a disposal site also is more a matter of what is available than what is suitable. The average collection efficiency for MSW in Indian cities is about 72.5% and around 70% of the cities lack adequate waste transport capacities (TERI, 1998). The insanitary methods adopted for disposal of solid wastes is, therefore, a serious health concern. The poorly maintained landfill sites are prone to groundwater contamination because of leachate production. Open dumping of garbage facilitates the breeding for disease vectors such as flies, mosquitoes, cockroaches, rats, and other pests (CPCB, 2000).

The municipalities in India therefore face the challenge of reinforcing their available infrastructure for efficient MSW management and ensuring the scientific disposal of MSW by generating enough revenues either from the generators or by identifying activities that generate resources from waste management.

10.7 Future Scenario

- *Waste generation and its Impacts*
  In India, the amount of waste generated per capita is estimated to increase at a rate of 1.0-1.33% annually (Shekdar, 1999). Figure 10.3 depicts the rising quantities of municipal solid waste from 1997 to 2047 under the BAU

scenario assuming the daily per capita waste generation in 1995 as 0.456 kg (EPTRI, 1995) and the per capita increase in waste generation as 1.33%. The calculated value of daily per capita waste generation in 1997 is 0.468 kg. From Fig. 10.3 it is evident that the total waste quantity generated in 2047 would be approximately above 260 million tonnes—more than five times the present level. This enormous increase in solid waste generation will have significant impacts in terms of the land required for disposing this waste as well as on methane emissions.

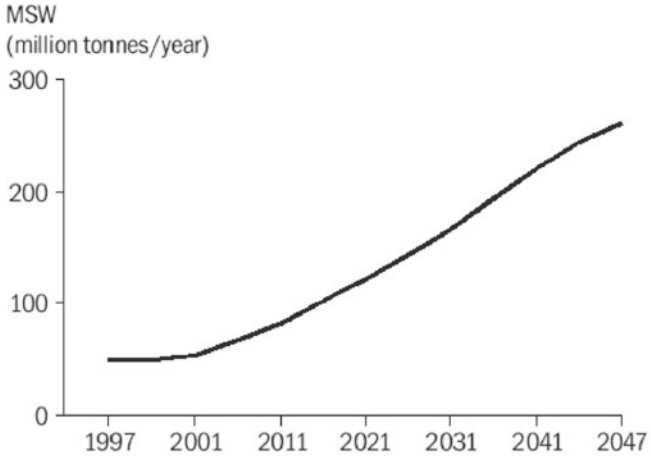

Fig. 10.3: Projected trends in the generation of municipal solid waste (million tonnes/year) according to BAU scenario.

- *Land requirement*

  The burden that the increase in solid waste generation would impose is evident from the fact that the cumulative requirement of land (base year 1997), for disposal of MSW, would amount to around 1400 km^2 by 2047 (Fig. 10.4). The estimates under the BAU scenario are made considering the

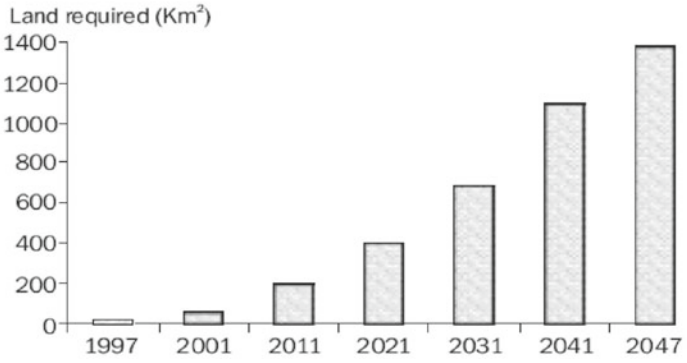

Fig. 10.4: Cumulative land requirement for disposal of municipal solid waste (km^2).

average collection efficiency of waste as 72.5%, average depth of landfill site as 4 m, and average waste density as 0.9 tonne/m^3 (NIUA, 1989). Diversion of land for waste disposal would be physically impossible since areas with the largest concentration of solid waste would also be the areas with serious scarcity of vacant land. The implication, therefore, is that if the current methods of solid waste disposal persist, the waste would have to be carried over long distances, which would require the creation of a great deal of transport facilities and infrastructure. This would involve enormous additional finances.

10.8 Technological Interventions

India has lagged behind in terms of adopting technologies for solid waste management. In particular, collection, treatment and disposal of waste require urgent consideration.

- *Collection of waste*
  The preferred option would be to revamp the existing collection service structure to provide community with waste bins, conveniently placed for the people to deposit domestic waste, and door to door collection of waste. This along with separation of waste, at source, into biodegradable and non-biodegradable components would not only reduce the cost of transportation for final disposal but also provide segregated organic waste stock for waste to energy activities.
- *Treatment and disposal*
  Proper segregation of waste would lead to better options and opportunities for its scientific disposal. Recyclables, for example, could be straightaway transported to recycling units, which, in turn, would pay the corporations for it, thereby increasing their income. Finally, the inert material that will be required to be sent to landfill would be of much lower quantity compared to un-segregated waste, consequently increasing the life of our existing disposal facilities (Singhal and Pandey).

10.9 Solid Waste Management in India: Options and Opportunities

Until recently, environment was not an issue in a third world country like India and solid waste management was definitely not the prime concern of environmentalists and the government, when the awakening to the issue finally did happen. It is only in very recent times, when certain NGO's started working and highlighting the pathetic state of municipal waste services provision in country, that the Indian decision-makers realized the importance of this particular aspect of environmental management.

10.9.1 Background Information about India

Covering an area of 32,87,263 km, India is the seventh largest country in the world. Lying entirely in the northern hemisphere, the Indian mainland extends between latitudes 8° 4′ and 37° 6′ north, longitudes 68° 7′ and 97° 25′ east and measures about 3214 km from north to south between the extreme latitudes and about 2933 km from east to west between the extreme longitudes. It has achieved multifaceted socio-economic progress during the last 50 years of its independence and is now the tenth industrialized nation in the world, with a complete self-sufficiency in field of agriculture. India's population, as in March 1991, stood at 856 million (TERI, 1997), making it the second largest populous country of the world. Assuming an annual growth rate of 1.3%, the estimated figures for 2021 are 1296.8 million (Eduard, 1993). The current per capita GDP figure is Rs. 3197.21 and is projected to increase to Rs. 11,599.7 by 2021 (unpublished data, TERI). The quantum of waste generated in the country is increasing day-by-day on account of its increasing population and increased GDP, though the civic services have not been expanding proportionately and hence are under tremendous pressure.

10.9.2 Waste Transportation Services

Municipal solid waste management is the responsibility of local governments in India. Transportation of waste is carried out by the municipalities employing vehicles like open trucks, tractor-trailers, tipper trucks, dumper trucks and animal drawn carts (mostly in small towns and rural areas). The recent trends in big cities and towns involve the use of container-carriers and dumper-placers, wherein the containers of the vehicles are of the community bin types. The volume of the waste to be transported is generally expressed in terms of cubic metre per million of population. Calculations done on the basis of waste density, waste generated etc. indicate that on an average 320 m^3 of transportation capacity is required for daily transportation of waste generated by population of one million. However, a paper published in 1996 by Bhoyar et al., wherein data of 44 Indian cities has been compiled and analyzed, indicates that 70% of these 44 cities do not have the 320 m^3 per million transport capacities. This percentage might be still higher as the vehicular fleet of most of the cities is several years old and is off the road for a large proportion of the year for want of repairs.

10.9.3 Waste Collection Services

In the absence of modernization and atomization of waste management services, its various components, i.e. collection, transportation and disposal, continue to be labour-intensive activities in India. About 80% of the total budget of all municipal corporations is accounted for by the salaries of sanitation workers engaged in road sweeping and related activities. A survey of 159 cities conducted by the National Institute of Urban Affairs (NIUA,1989) revealed that the waste

collection efficiency in these cities varied from 66% to 77% and the national average was a poor 72.5%, as compared to the developed countries where the waste collection is almost complete except for the most rural areas. Waste collection efficiency is a function of two major factors: manpower availability and transport capacity. Less than 10% of the 157 cities surveyed in 1989 had more than 2800 workers per million population which is an accepted benchmark of optimum workforce requirement, by most of the municipal corporations in India.

10.9.4 Waste Characteristics

The composition of waste depends on a wide range of factors such as food habits, cultural traditions, lifestyles, climate and income etc. The variations due to such factors are found across different countries as well as across different regions within one country. The inter-regional variations are, however, not as marked as those across the countries. Variation also occurs within a region over the years as a consequence of economic and social changes. India is no exception to this, and the data given in Table 10.1 clearly shows the changes in the composition of Indian MSW over a time period of about 25 years. The most remarkable change is in the percentage of recyclables (plastic, metals etc.) which increased from 9.6% in 1971-73 (Bhide, 1983) to 17.2% in 1995 (EPTRI, 1995) owing to changing lifestyles and the increasing consumerist attitude of the common man in the country. This increase has given rise to the phenomenon of rag picking activity especially in the metro cities of the country wherein the recycling units have mushroomed on the peripheral areas providing employment to thousands of unskilled labour. The organic matter has more or less remained the same, whereas ash and fine earth has decreased corresponding to the increase in recyclables. A shift in energy resources consumption from coal and wood to petrochemical-based products could be a plausible explanation for the ash and fine earth percentage decrease.

Table 10.1: Physico-chemical characteristics of Indian MSW

Component	*Percentage on wet weight basis*	
	1971-1973[a]	*1995*[b]
Paper	4.1	5.8
Plastics	0.7	3.9
Metals	0.5	1.9
Glass	0.4	2.1
Rags	3.8	3.5
Ash and fine earth	49.2	40.3
Total compostable matter	41.3	42.5

[a] Bhide and Sundaresan, 1983
[b] EPTRI, 1995

10.9.5 Waste Disposal Practices

In majority of urban centres in India, MSW is disposed off by depositing the same in low-lying areas outside the city. Compaction and levelling of waste and a final covering by earth are rarely observed practices at most of these disposal sites. These low-lying disposal sites, being devoid of a leachate collection system, landfill gas monitoring and collection equipment, can hardly be called sanitary landfills and are more in the nature of dumping sites. Nearly all the Indian cities dispose off their waste by simple dumping and only about 9% practice the environment-friendly way of disposal, namely composting. The Indian municipal waste can be broadly categorized into organic waste, recyclables and ash and fine earth. Of these three, the organic waste component has remained constant over the past many years at approximately 40% and is not expected to change much in absolute terms in the near future. However, the ratio between the other two components has changed in past years and is expected to change further, with the shift occurring in favour of recyclables. At the moment, the country has no policy on segregation of recyclables and hence these too are dumped along with the organic waste.

The municipal corporations being the responsible authority in India for SWM in addition to a wide range of responsibilities related to health and sanitation, have not been very effective as far as SWM services are concerned. Collection, transportation and disposal—all the three components of waste—lack in terms of infrastructure, maintenance and upgradation. However, the weakest link in the chain of waste management in Indian situation is the collection of waste. Proper segregation of waste into different components and their separate collection can definitely lead to remarkable changes in the entire system. One of the immediate measures to revamp the existing collection services structure would involve provision of community waste bins at proper distances for the people to deposit domestic waste. This as the first step will ensure that people do not throw their garbage on the roads and hence do not create open dumpsites. This will enable the sanitation workers to transfer the waste to the transportation vehicle quickly and efficiently with minimum health risk involved and will also help in maintaining the aesthetics of surroundings. The second measure should entail at the source—separation of waste into biodegradable and non-biodegradable components. This would be a long drawn exercise as it involves attitudinal changes in people and will have to be done with careful planning, in a phased manner. The general public will have to be first sensitized towards the whole concept and educated about the need and advantages of doing the segregation. Segregation of waste at the source itself is extremely important as municipal solid waste, which is otherwise environmentally benign on getting mixed with hazardous waste like paints, dyes, batteries, human excrete turns hazardous.

The recyclables like paper and plastic etc. become unsuitable for recycling as these get soiled by the organic matter. Although, it would be more fruitful to sort and place the different kind of recyclables in different receptacles, however

to begin with the waste could be segregated into just two categories. Proper segregation would lead to better options and opportunities for scientific disposal of waste. The recyclables could be straightway transported to recycling units which in turn would pay certain amount to the corporations, thereby adding to their income. This would help in formalizing the existing informal set up of recycling units and this formalization in turn could lead to multi-advantages. To mention a few: enabling technology upgradation, better quality products and saving of valuable raw material resources of country. In addition, a dialogue with the consumer's industry should be undertaken to impress upon them the need for life-cycle assessment of goods manufactured and to reduce the packaging material content associated with every final product. The biodegradable matter could be disposed off either by aerobic composting, anaerobic digestion or sanitary land filling. Depending upon land availability and financial resources either of these disposal methods could be adopted. However, it appears that in this context land filling would continue to be the most widely adopted practice in India in the coming few years, in which case certain improvements will have to be done to ensure that it is sanitary land filling and not merely dumping of waste. The contribution to global warming due to landfill gas release in the atmosphere can be abated if the disposal sites are designed and constructed with the gas collection equipment installations. This will have the dual advantage of minimum greenhouse emissions and also income generation if the gas generated is utilized either directly as a thermal fuel or by conversion into electricity. However, this is a long-term measure and needs to be incorporated at the stage when a disposal site is being designed.

The hazard of groundwater pollution due to leachate percolation, noise pollution etc. can all be minimized by good land filling practices. Aerobic composting is a fast catching option for waste disposal and a beginning in it has already been made. The first aerobic composting plant of the country was set up in Bombay in 1992 to handle 500 tonnes of waste per day by a private company. However, only 300 tonnes/day capacity is being utilized currently due to certain problems but the plant is working very successfully and the compost produced is being sold off at the rate of two rupees/kg. Another plant of 150 tonnes/day capacity has been operating in the city of Vijaywada for about a year now. A similar plant is expected to become operational in the garden city of Bangalore by year end. Calcutta, Thane, Chandigarh, Gwalior, Solan and Delhi too, are towing the same line and have either signed agreements or are in the process of doing so to have the composting facilities very soon. Composting has an edge over sanitary landfilling as it does away with the emission and leachate problems and the end product which is good quality compost can be sold off. Anaerobic digestion, though expensive as compared to sanitary landfilling and aerobic composting too, holds a good promise as a future disposal option. It however needs more research and experimentation before being adopted on a large scale. Incineration is yet another option available for waste disposal. The low calorific value range of 800-110 Kcal/kg of Indian MSW, however rules

out the incineration option for it. Also, the existing trend all over the world is to move away from incineration and India too, can safely opt out of this option except for certain kinds of hazardous waste like hospital waste.

The options and opportunities are many, it is up to the corporations to select and adopt the ones most suitable to them. Constituting a nodal body at the level of Ministry of Urban Affairs with the power of framing guidelines and standards and issuing notifications could come a long way in assisting corporations in their work. This nodal body could also build up and maintain a central database which would contain detailed information about the infra structural, financial and administrative details of all the municipal corporations and councils in the country. Another very good idea for an overall improvement in waste management services could be privatization of one of the aspects of waste management. Once the private sector takes over the certain share of corporations' work, the corporations could probably spend and concentrate more on the rest in terms of financial and administrative resources.

10.10 Soil Bioremediation and Phytoremediation—An Overview

In the 45 years period of 1930-1975, the global human population has increased by approximately two billion to four billion. A further population increase of two billion occurred in the 25 years interval 1975-2000 and population is expected to reach eight billion by 2020. Population growth and the resultant development of large high density urban populations, together with parallel global industrialization, have placed major pressures on our environment, potentially threatening environmental sustainability. This has resulted in the buildup of chemical and biological contaminants throughout the biosphere, but most notably in soils and sediments.

10.10.1 Bioremediation

The historical foundation for modern environmental biotechnology lies in the composting of organic wastes into soil fertilizers and conditioners. In its broadest sense bioremediation includes biological treatment of wastewater, sewage, food and agricultural wastes, contaminated soils and ground water. While its definition is the subject of debate, Prince (1998) defines bioremediation as "the process of judiciously exploiting biological processes to minimize an unwanted environmental impact; usually it is the removal of contaminant from the biosphere". The term "intrinsic bioremediation" essentially involves taking no action but rather monitoring a natural process of contaminant reduction without intervention. Hence, intrinsic bioremediation can hardly be termed as a technology, but it has met with some success as a low cost approach.

Bioremediation can be defined as any process that uses microorganisms, fungi, green plants or their enzymes to return the natural environment altered

by contaminants to its original condition. Bioremediation may be employed to attack specific soil contaminants, such as degradation of chlorinated hydrocarbons by bacteria. An example of a more general approach is the cleanup of oil spills by the addition of nitrate and/or sulfate fertilizers to facilitate the decomposition of crude oil by indigenous or exogenous bacteria.

10.10.1.1 *Monitoring Bioremediation*

The process of bioremediation can be monitored indirectly by measuring the *Oxidation Reduction Potential* or redox in soil and ground water, together with pH, temperature, oxygen content, electron acceptor/donor concentrations, and concentration of breakdown products (e.g. carbon dioxide). Table 10.2 shows the (decreasing) biological breakdown rate as function of the redox potential.

Table 10.2: Biological breakdown rates

Process	*Reaction*	*Redox potential (E_h in mV)*
Aerobic	$O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$	600 ~ 400
Anaerobic denitrification	$2NO_3^- + 10e^- + 12H^+ \rightarrow N_2 + 6H_2O$	500 ~ 200
Manganese (IV) reduction	$MnO_2 + 2e^- + 4H^+ \rightarrow Mn^{2+} + 2H_2O$	400 ~ 200
Iron (III) reduction	$Fe(OH)_3 + e^- + 3H^+ \rightarrow Fe^{2+} + 3H_2O$	300 ~ 100
Sulfate reduction	$SO_4^{2-} + 8e^- + 10\ H^+ \rightarrow H_2S + 4H_2O$	0 ~ −150
Fermentation	$2CH_2O \rightarrow CO_2 + CH_4$	−150 ~ −220

This, by itself and at a single site, gives little information about the process of remediation.

1. It is necessary to sample enough points on and around the contaminated site to be able to determine contours of equal redox potential. Contouring is usually done using specialized software, e.g. using Kriging interpolation.
2. If all the measurements of redox potential show that electron acceptors have been used up, it's in effect an indicator for total microbial activity. Chemical analysis is also required to determine when the levels of contaminants and their breakdown products have been reduced to below regulatory limits.

10.10.1.2 *Advantages of Bioremediation*

Microbial biocatalytic processes and biomineralization generally represent low cost remediation alternatives, for example costing approximately one tenth the cost of incineration (Grommen and Verstraete, 2002). Additionally

biodegradation processes are argued to be flexible and adaptable to variable environmental conditions. Many synthetic chemicals, when first introduced to the environment appear to have evolved which can degrade many of these chemicals (Relebitso et al., 2002). Another claimed advantage of bioremediation has more to do with public perception than with performance; biological processes are perceived as being environmentally benign whereas incineration and more energy and equipment intensive processes are perceived as being more environmentally polluting. Other advantages often offered are that bioremediation processes can be implemented on site, indeed sometimes in situ (without excavation) and that the process is applicable to dilute or widely diffused contaminants (Iwamoto and Nasu, 2001).

The disadvantage most frequently cited for soil bioremediation processes is that they are often very slow and frequently desired end points may not be achieved. Failures occur more frequently in more passive processes. With little intervention, removal of soil contaminants by processes of natural attenuation may occur and acceptable end points may take many years to be reached. There can be many reasons for the slow bioremediation rates and failures principally that the environmental conditions present are sub-optimal for selection and growth promotion of the degrading strains. In addition, the kinetics of microbial growth and biodegradation are such that as contaminant concentrations decline so also do the rates of their further degradation.

10.10.2 Phytoremediation

The phytoremediation method uses various plants to extract, contain, immobilize or dredge contaminants from soil and water. Some plants can remove contaminants from soil by direct uptake, followed by subsequent transformation, transport and accumulation in a non-phytotoxic form. The diverse approaches in phytoremediation include phytodegradation, phytoextraction, phytostabilization, phytovolatilization and rhizofiltration. Phytoremediation is still actively being researched and plant-microbial associations seem to be the key to enhancing removal of inorganic and organic pollutants. Phytoremediation technologies are most appropriate for large areas of low and moderately contaminated soils where the application of conventional remediation technologies would be prohibitively expensive.

A landfill is an extremely variable and heterogeneous environment, as evident from the diversity of refuse composition with respect to location and time. Landfills hold wastes containing a wide range of organic molecules of both natural and xenobiotic origin. In many developed countries, municipal solid wastes (MSW) are dumped in scientifically designed sanitary landfills. In many developing countries, they are dumped in an uncontrolled manner without any precaution to deal with gas emissions and leachate generation, which pose a threat to the environment. Natural or planted vegetation on a landfill has an important role in erosion control and removal of contaminants, besides imparting aesthetic value. Moreover, it may also be used in leachate

treatment (Maurice, 1998). Landfill vegetation often shows signs of damage commonly caused by the presence of landfill gas (LFG) in the root zone. The goal for the reconstruction of a suitable medium for landfill revegetation is to provide a capping that is deep and as favourable to root growth as is necessary to achieve desired plant performance (Vogel, 1987). Although reviews on phytoremediation of sites contaminated with a variety of contaminants are readily available (Siciliano and Germida, 1998a; Lasat, 2002; Schwitzguebel et al., 2002), the applicability of this technology in remediation and rehabilitation of municipal solid waste dumpsites has not been given its due. The present review, an off-shoot of studies on rehabilitation of municipal solid waste dumpsites, attempts to fill this gap by leaning on research findings, especially those reported in the last two decades.

Phytoremediation, collectively referring to all plant-based technologies, uses green plants to remediate contaminated sites (Sadowsky, 1999). This technology draws its inspiration from the myriad of physical, chemical and biological interactions occurring between plants and the environmental media (Fig. 10.5). Phytoremediation is evolving into a cost-effective means of managing wastes, especially excess petroleum hydrocarbons, polycyclic aromatic hydrocarbons, explosives, organic matter, and nutrients.

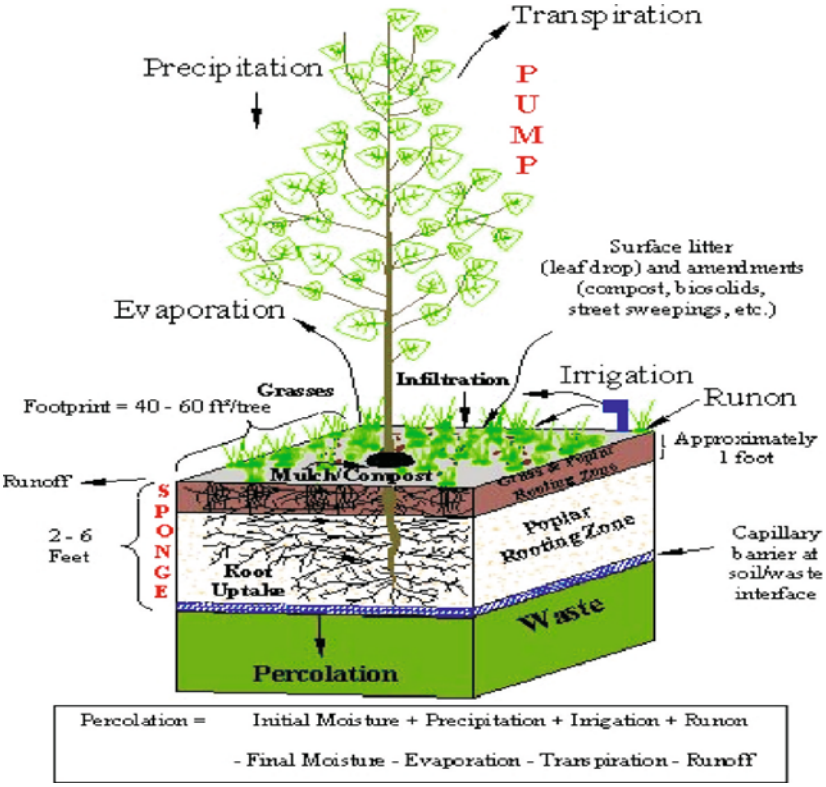

Fig. 10.5: Plant–environment interactions.
Source: Licht and Isebrands (2005)

Applications are being tested for cleaning up contaminated soil, water, and air (McCutcheon and Schnoor, 2003). Several features make phytoremediation an attractive alternative to many of the currently practiced in situ and ex situ technologies. These include: low capital and maintenance costs, non-invasiveness, easy start-up, high public acceptance and the pleasant landscape that emerges as a final product (Boyajian and Carreira, 1997). In the last several decades, phytoremediation strategies have been examined as a means to clean up a number of organic and inorganic pollutants, including heavy metals (Kumar et al., 1995; Salt et al., 1995; Chaney et al., 1997), chlorinated solvents (Walton et al., 1994; Haby and Crowley, 1996), agrochemicals (Anderson et al., 1994; Hoagland et al., 1997; Kruger et al., 1997), polycyclic aromatic hydrocarbons, polychlorinated biphenyls (Brazil et al., 1995; Donnelly and Fletcher, 1995), munitions and radio nuclides. These soluble organic and inorganic contaminants, which move into plant roots or rhizosphere by the mass flow process of diffusion, appear to be most amenable to the remediation process. In several instances, plants and/or their attendant rhizosphere microbes have been shown to transform some chemical compounds to some degree (Walton et al., 1994; Crowley et al., 1996; Siciliano and Germida, 1998b).

Plants are known to sequester, degrade and stimulate the degradation of organic contaminants in soil (Anderson et al., 1993). The sequestration of heavy metals by plants is an effective method of reducing heavy metal contamination in soil (Cunningham et al., 1995). Sequestration of toxicants by plants is an important area of phytoremediation research. Plants are known to accumulate a variety of toxicants from soil and if the toxic chemical is metabolically stable and mobile, it may be transferred via apoplast or symplast compartments, or both, throughout most of the plant as parent compound and stored at highly bioconcentrated levels (McFarlane et al., 1987). However, the mechanisms by which plants stimulate the disappearance of hazardous organics from soil are not fully understood.

In view of its demonstrated potential, phytoremediation has been gaining importance in rehabilitation of contaminated sites including MSW dumpsites. Many types of phytoremediation processes have been described based on the kind of mechanism. These include: phytoextraction, rhizofiltration, phytovolatilization, phytodegradation, rhizosphere biodegradation, hydraulic pumping, phytosorption and phytocapping. Figure 10.6 outlines the common processes involved in phytoremediation. Processes and contaminants dealt with by different phytoremediation processes are presented in Table 10.1. The selection of plant and the type of phytoremediation depends on the type of contaminants to be treated and the nature of the site.

10.10.2.1 *Vegetation at Dumpsites*

Plants are known to increase nutrient availability by secreting cationic chelators, organic acids, or specific enzymes such as phosphatase into the soil systems. Competition of these nutrients by degrading and non-degrading species will

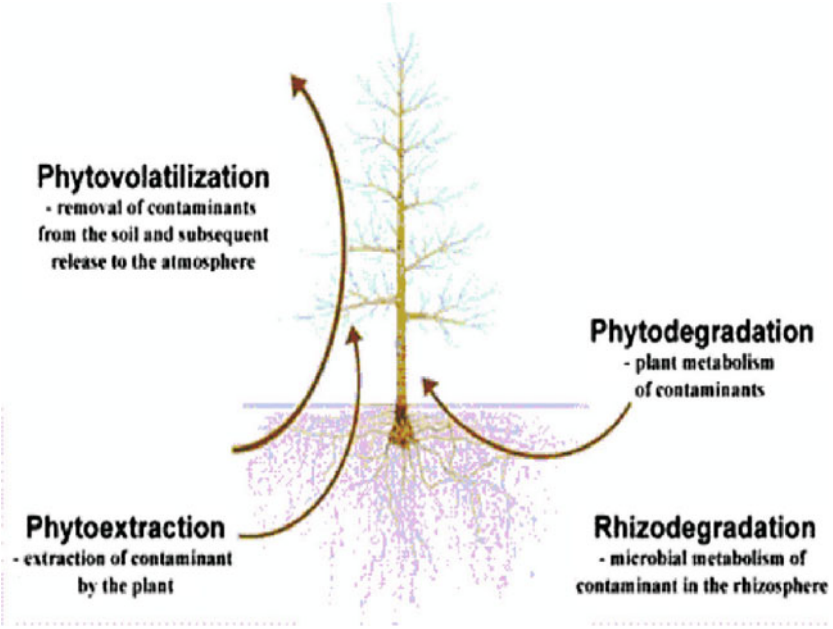

Fig. 10.6: Processes involved in phytoremediation.
Source: http://oldweb.northampton.ac.uk/aps/env/landfillleachate/images/phytorem. jpg.

influence the amount of contaminant degraded (Steffensen and Alexander, 1995). Increase in nutrient availability brought about by plant growth may be one mechanism by which plants stimulate biodegradation.

Reviewing plant species occurring at different landfills facilitates the selection of suitable plant species to deal with a range of contaminants together. It is interesting to note that the species diversity is influenced by the nature of origin of wastes, local flora and the conditions prevailing at the landfill. Hence, a single species cannot be identified as a universal indicator and the plant selection should be based on the climatic conditions and the native plants occurring in a particular landfill.

10.10.2.2 *Landfill Vegetation*

Reclamation of a landfill site must include the objective of containing the material within. This is because the processes that take place after the compaction and the covering of the waste in the site produce products of entirely new characteristics. Many of the products of these processes are toxic to several life forms including plants. The influence of different types of contaminants on vegetation is depicted in Fig. 10.7. Toxic materials in the waste include landfill gases, leachates, heavy metals, organic contaminants and others. Usual landfill conditions and their consequences on the vegetation are presented in Table 10.3. Vegetation in a completed landfill is often poor and damaged.

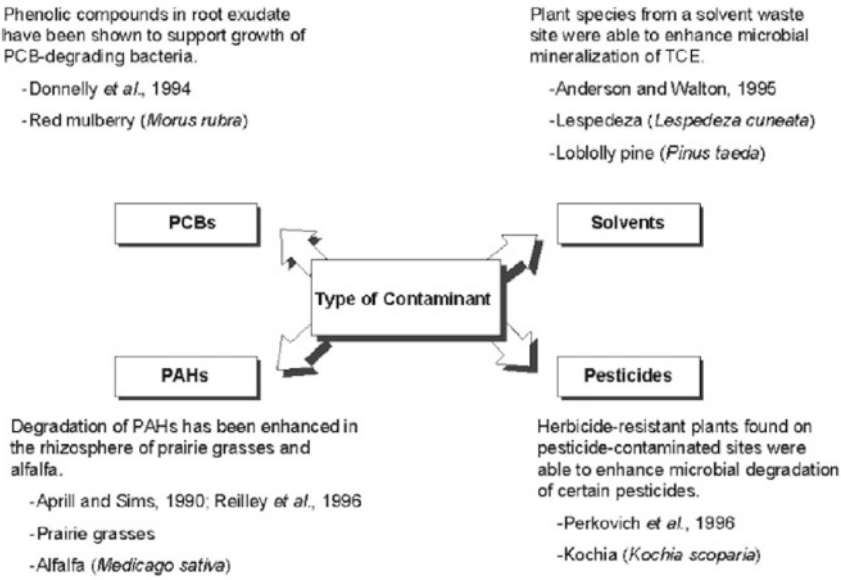

Fig. 10.7: Influence of different types of contaminants on vegetation.

Table 10.3: Landfill conditions and their effects on plants

Landfill conditions	*Consequences for plants*
Good drainage	Risk for dryness
High temperature	Dryness and increase of oxygen demand
Construction above the surface	Exposure to wind, dryness
Infertile top cover soil	Bad growth conditions
Gases	Bad growth conditions
Compact soil	Extra energy required to push root tips

Source: Maurice (1998).

10.10.2.3 *Landfill Capping*

Landfills are usually required to have clay caps and impermeable synthetic membranes to minimize the infiltration of rainfall and generation of leachate. Landfill capping is the most common form of remediation because it is generally less expensive than other technologies and effectively manages the human and ecological risks associated with a remediation site. Considerable research is being done to develop inexpensive and efficient layers. As outlined in Platinum International, Inc. (2002), landfill caps can be used to:

1. minimize exposure on the surface of the waste facility;
2. prevent vertical infiltration of water into wastes that would create contaminated leachate;

3. contain waste while treatment is being applied;
4. control gas emissions from underlying waste; and
5. create a land surface that can support vegetation and/or be used for other purposes.

A vegetative cap is a long-term, self-sustaining cover of plants growing in and/or over materials that pose environmental risk; a vegetative cap reduces that risk to an acceptable level and requires minimal maintenance. A typical landfill cap system is shown in Fig. 10.8.

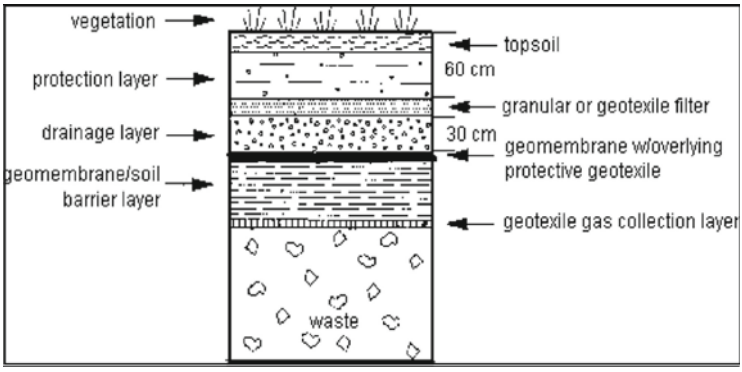

Fig. 10.8: Landfill cap system.

10.10.3 Mycoremediation

Mycoremediation is a form of bioremediation, the process of using fungi to return an environment (usually soil) contaminated by pollutants to a less contaminated state. The term mycoremediation was coined by Paul Stamets and refers specifically to the use of fungal mycelia in bioremediation.

One of the primary roles of fungi in the ecosystem is decomposition, which is performed by the mycelium. The mycelium secretes extracellular enzymes and acids that break down lignin and cellulose, the two main building blocks of plant fiber. These are organic compounds composed of long chains of carbon and hydrogen, structurally similar to many organic pollutants. The key to mycoremediation is determining the right fungal species to target a specific pollutant. Certain strains have been reported to successfully degrade the nerve gases VX and sarin.

In an experiment conducted in conjunction with Thomas, a major contributor in the bioremediation industry, a plot of soil contaminated with diesel oil was inoculated with mycelia of oyster mushrooms; traditional bioremediation techniques (bacteria) were used on control plots. After four weeks, more than 95% of many of the PAH (polycyclic aromatic hydrocarbons) had been reduced to non-toxic components in the mycelial-inoculated plots. It appears that the natural microbial community participates with the fungi to break down contaminants, eventually into carbon dioxide and water.

Wood-degrading fungi are particularly effective in breaking down aromatic pollutants (toxic components of petroleum), as well as chlorinated compounds (certain persistent pesticides; Battelle, 2000).

Advantages

There are a number of cost/efficiency advantages to bioremediation, which can be employed in areas that are inaccessible without excavation. For example, hydrocarbon spills (specifically, petrol spills) or certain chlorinated solvents may contaminate groundwater, and introducing the appropriate electron acceptor or electron donor amendment, as appropriate, may significantly reduce contaminant concentrations after a lag time allowing for acclimation. This is typically much less expensive than excavation followed by disposal elsewhere, incineration or other ex situ treatment strategies, and reduces or eliminates the need for "pump and treat", a common practice at sites where hydrocarbons have contaminated clean groundwater.

10.10.4 Remediation of Solid Waste

On-site remediation of soils and media contaminated with petrochemicals, pesticides, explosives or hazardous organic materials can be an expensive and time-consuming process. There are also serious legal and regulatory issues to consider when decontaminating soils on-site—issues that can jeopardize the viability of a construction or redevelopment project.

Waste Management, North America's largest solid waste services company, has developed a collection of innovative off-site remediation technologies to help companies deal effectively with contaminated soils. These bioremediation services include:

- **TOSS^{SM} (Two-Step Static System):** TOSS^{SM} is a two-stage, solid-phase bioremediation technology that involves both anaerobic and aerobic treatment stages. In the first stage, explosives-contaminated soil is combined with a carbon source, an inoculums, vitamins and water to achieve anaerobic conditions. The resulting mixture is formed into a static pile or placed in a bermed construction or box to facilitate the chemical reduction of nitroaromatic and nitramine explosives. In the second stage, the anaerobically treated soil is combined with yard waste compost and built into an aerated biopile. The biopile may be aerated by forced air conveyed through perforated piping buried within the pile or by turning the pile with a compost turner. Previous testing of TOSS^{SM} has demonstrated TNT removal efficiencies of greater than 99 percent.
- **The BioSite^{SM} System:** The BioSite^{SM} System is Waste Management's proprietary system for the large-scale bioremediation of soils contaminated with:
  - petrochemicals including, but not limited to: acetone, alcohols, benzene, ethylbenzene, methyl ethyl ketone (MEK), methyl isobutyl ketone

(MIBK), petroleum hydrocarbons, toluene, two- and three-ring PAHs, and xylene

- other contaminants, including: aliphatic chlorinated hydrocarbons (e.g., trichloroethylene), spent molecular sieve from packing towers, chemical manufacturing wastes, and pesticides

Regulated compounds including underlying hazardous constituents (UHC) are screened prior to acceptance. Soils co-contaminated with metals may be accepted depending on their concentration.

- **Bio-In-A-Box^{SM}:** Bio-In-A-Box^{SM} is based on the same principles as both BioSite^{SM} and TOSS^{SM}, but is designed to operate indoors on a relatively smaller scale. Instead of being formed into long earthen mounds, the contaminated soil is moistened, mixed with nutrients and custom-grown microorganisms, and then placed in enclosed containers called "solid phase bioreactors" for incubation. These containers may or may not be linked to aeration and vacuum pipes, depending on the contaminants being processed. In just a few weeks, the decontaminated soil will be ready for landfill disposal or reintroduction into the environment.

10.10.5 Composition for Solid Waste Remediation—United States Patent

Solid waste contaminated with cationic metals is remediated with a composition that reduces the mobility of the metals. A recycled product is formed having improved structural properties for possible use as engineered fill such as road base material. Said composition comprising an emulsion of asphalt or tall oil pitch in water and a chemical fixing agent.

The invention relates to the treatment and remediation of solid waste that contains cationic metals. More particularly, this invention relates to the formulation of organic based emulsions that contain chelating or complexing agents or agents to form insoluble metal compounds. The emulsions are then used to serve two functions: 1) immobilize the cationic metal as determined by EPA's TCLP test; and 2) create a recycled product with improved structural properties as compared to the untreated solid waste. The remediated solid waste is then reused onsite or transferred offsite for commercial use.

10.11 Landfills and Dumpsites

Landfills and dumpsites used for disposal of municipal solid wastes require occasional rehabilitation, especially in the context of upgrading such facilities in developing economies. Rehabilitation measures and strategies must ensure that at all stages of the exercise, environmental concerns including groundwater contamination resulting from migration of leachate, transmigration of pollutants and aesthetics are not overlooked. Phytoremediation offers viable solutions to many environmental problems related to landfill rehabilitation and has several applications as summarized in Table 10.4.

Table 10.4: Application of phytoremediation

Sr. No.	*Application*	*Description*	*Contaminants*	*Types of plants*
Soils				
1.	Phytotransformation	Sorption, uptake and transformation of contaminants	Organics, including nitroaromatics and chlorinated aliphatics	Trees and grasses
2.	Rhizosphere biodegradation	Microbial biodegradation in the rhizosphere stimulated by plants	Organics: e.g. PAHs, petroleum hydrocarbons, TNT, pesticides	Grasses, alfalfa, many other species including trees
3.	Phytostabilization	Stabilization of contaminants by binding, holing soils, and/or decreased leaching	Metals, organics	Various plants with deep or fibrous root system
4.	Phytoextraction	Uptake of contaminants from soil into roots or harvestable shoots	Metals, inorganics, radionuclides	Variety of natural and selected hyperaccumulators e.g., *Thalaspi, Alyssum, Brassica*
Water/gram water				
5.	Rhizofiltration	Sorption of contaminants from aqueous solutions onto or into roots	Metals, radionuclides, hydrophobic organics	Aquatic plants (e.g., duckweed, pennywort), also *Brassica*, sunflower
6.	Hydraulic control plume capture/ phytotrans	Removal of large volumes of water from aquifers by trees	Inorganics, nutrients, chlorinated solvents	Poplar, willow trees
7.	Phytovolatilization	Uptake and volatilization from soil water and groundwater; conversion of Se and Hg to volatile chemical species	Volatile organic compounds, Se, Hg	Trees for VOCs in ground water, *Brassica,* grasses, wetlands plants for Se. Hg in soil/sediments
8.	Vegetative caps	Use of plants to retard leaching of hazardous compounds from landfills	Organics, inorganics, wastewater, landfill leachate	Trees such as poplar, plants (e.g., alfalfa) and grasses
9.	Constructed wetlands	Use of plants as part of a constructed ecosystem to remediate contaminants from aqueous waste streams	Metals, acid mine drainage, industrial and municipal wastewater	Free-floating, emergent, or submergent vegetation, reeds, cattails, bamboo

Source: Schnoor (2002)

10.12 Overview and Applications

Naturally occurring bioremediation and phytoremediation have been used for centuries. For example, desalination of agricultural land by phytoextraction has a long tradition. 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 the removal of the contaminated material to be treated elsewhere. Some examples of bioremediation technologies are bioventing, land farming, bioreactor, composting, bioaugmentation, rhizofiltration, and biostimulation.

Bioremediation can occur on its own (natural attenuation) or can be spurred on via the addition of fertilizers to increase the bioavailability within the medium (biostimulation). Recent advancements have also proven successful via the addition of matched microbe strains to the medium to enhance the resident microbe population's ability to break down contaminants (bioaugmentation).

Not all contaminants, however, 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

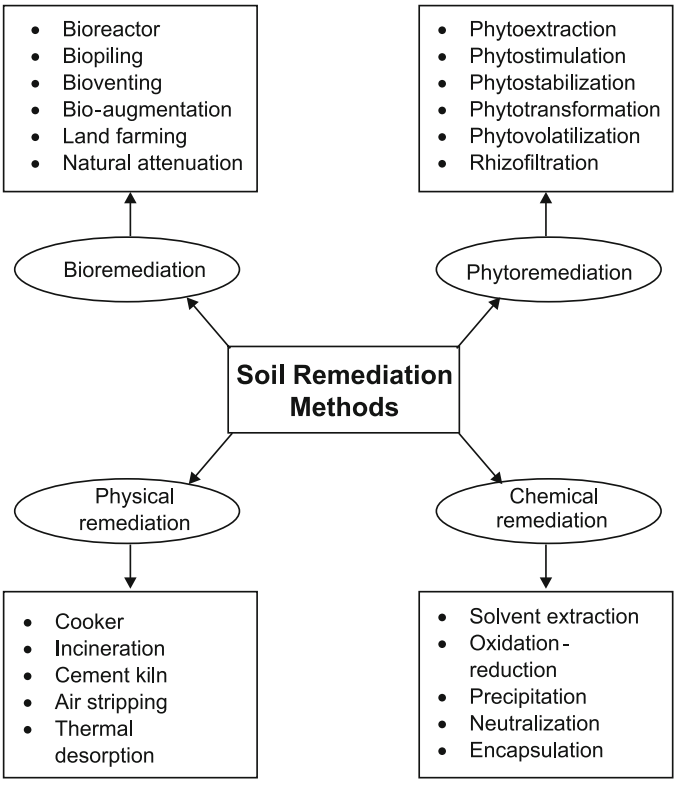

Fig. 10.9: Soil remediation methods.

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 then harvested for removal. The heavy metals in the harvested biomass may be further concentrated by incineration or even recycled for industrial use.

The elimination of a wide range of pollutants and wastes from the environment requires increasing our understanding of the relative importance of different pathways and regulatory networks to carbon flux in particular environments and for particular compounds and they will certainly accelerate the development of bioremediation technologies and biotransformation processes.

Soil bioremediation processes may be implemented using a variety of different engineered configurations ranging from in situ subsurface (unexcavated) processes to application of completely mixed soil slurry reactor systems for treatment of excavated soils. The technology is interdisciplinary, involving microbiology, engineering, geology, ecology chemistry and perhaps other disciplines.

Various biological and non-biological methods used in soil remediation are shown in Fig. 10.9. While there are many aspects of soil-related biodegradation and bioremediation, this article will primarily focus on remediation of chemical contaminants in soil and will not address the issues of biowaste recycling.

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11

Phytoremediation of Low Level Nuclear Waste

M.H. Fulekar and Anamika Singh

11.1 Introduction

Waste, by definition, is any material (solid materials such as process residues as well as liquid and gaseous effluents) that has been or will be discarded as being of no further use. Radioactive waste is a waste product containing radioactive material. It is usually the product of a nuclear process such as nuclear fission. However, industries not directly connected to the nuclear industry may also produce radioactive waste. The majority of radioactive waste is "low-level waste", meaning it contains low levels of radioactivity per mass or volume. The radiation from this type of waste is protected by use of protective clothing, but still dangerous radioactive contamination occurs in a human body through ingestion, inhalation, absorption, or injection.

The issue of disposal methods for nuclear waste is one of the most pressing current problems being faced by the international nuclear industry when trying to establish a long-term energy production plan; yet there is a hope that it could be safely solved. A report giving the nuclear industry's perspective on this problem is presented in a document from the IAEA (The International Atomic Energy Agency) published in October 2007. It summarizes the current state of scientific knowledge on whether waste could find its way from a deep burial facility back to soil and drinking water and threaten the health of human beings and other forms of life. In the United States, DOE acknowledges progress in addressing the waste problems of the industry, and successful remediation of some contaminated sites; still some uncertainty and complications in handling the issue properly, cost effectively, and in the projected time frame is persisting. In other countries with lower ability or will to maintain environmental integrity the issue would be even more problematic.

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In the United States alone, the Department of Energy states there are "millions of gallons of radioactive waste" as well as "thousands of tons of spent nuclear fuel and material" and also "huge quantities of contaminated soil and water." Despite copious quantities of waste, the DOE has stated a goal of cleaning all presently contaminated sites successfully by 2025. For example, Ohio site had 31 million pounds of uranium product, 2.5 billion pounds of waste, 2.75 million cubic yards of contaminated soil and debris, and a 223 acre portion of the underlying Great Miami Aquifer had uranium levels above drinking standards. The United States has at least 108 sites designated as areas that are contaminated and unusable, sometimes many thousands of acres. DOE wishes to clean or mitigate many or all by 2025; however the task can be difficult and it acknowledges that some may never be completely remediated. In just one of these 108 larger designations, Oak Ridge National Laboratory had at least 167 known contaminant release sites with three subdivisions of the 37,000-acre (150 km^2) site. Some of the U.S. sites were smaller in nature; however, cleanup issues were simpler to address, and DOE has successfully completed cleanup, or at least closure, of several sites.

11.2 Characteristics of Nuclear Waste in India

Table 11.1: Characteristics of liquid and solid waste generated in India

Source	*Liquid waste*		*Solid waste*	
	Average annual generation (m^3)	*Specific activity (Bq/ml)*	*Average annual generation (m^3)*	*Radiation field (mCi/l)*
Research reactor	16,000	1-3	20-25	0.01-1000
Power reactor				
BWR	26,800	50-100	80	0.05–1000
PHWR	26,800	0.1-1	100	0.01–1000
Fuel-reprocessing facility	34,300	4-20	130	0.01–500
R&D lab	12,000	1-4	50	0.01–7000

Source: Rao, 2001.

Radioactive waste typically comprises a number of radioisotopes which are unstable configurations of elements that decay, emitting ionizing radiation which can be harmful to human health and to the environment.

11.3 Pharmacokinetics

Exposure to high levels of radioactive waste may cause serious harm or death. Treatment of an adult animal with radiation or some other mutation-causing

effect, such as a cytotoxic anti-cancer drug, may cause cancer in the animal. In humans it has been calculated that a 5 sievert dose is usually fatal, and the lifetime risk of dying from radiation induced cancer from a single dose of 0.1 sieverts is 0.8%, increasing by the same amount for each additional 0.1 sievert increment of dosage. Ionizing radiation causes deletions in chromosomes. If a developing organism such as an unborn child is irradiated, it is possible a birth defect may be induced, but it is unlikely this defect will be in a gamete or a gamete forming cell. The incidence of radiation-induced mutations in humans is undetermined, due to flaws in studies done to date.

Depending on the decay mode and the pharmacokinetics of an element (how the body processes it and how quickly), the threat due to exposure to a given activity of a radioisotope will differ. For instance iodine-131 is a short-lived beta and gamma emitter but because it concentrates in the thyroid gland, it is more able to cause injury than caesium-137 which, being water soluble, is rapidly excreted in urine. In a similar way, the alpha emitting actinides and radium are considered very harmful as they tend to have long biological half-lives and their radiation has a high linear energy transfer value. Because of such differences, the rules determining biological injury differ widely according to the radioisotope, and sometimes also the nature of the chemical compound which contains the radioisotope.

The main objective in managing and disposing or destruction of radioactive (or other) waste is to protect people and the environment. This means isolating, diluting, or destroying (transmutating) the waste so that the rate or concentration of any radionuclide returned to the biosphere is harmless. To achieve this, the preferred technology to date has been deep and secure burial for the more dangerous wastes; transmutation, long-term retrievable storage, and removal to space have also been suggested.

In spite of the present treatment technology and protective measures, the waste containing radioactive material enter into the environment (Fig. 11.1).

11.4 Sources of Waste

Radioactive wastes come from a number of sources. The majority of waste originates from the nuclear fuel cycle and nuclear weapons reprocessing. However, other sources include medical and industrial wastes, as well as naturally occurring radioactive materials (NORM) that can be concentrated as a result of the processing or consumption of coal, oil and gas, and some minerals.

11.5 Types of Radioactive Waste

Although not significantly radioactive, uranium mill tailings are waste. They are byproduct material from the rough processing of uranium-bearing ore. They

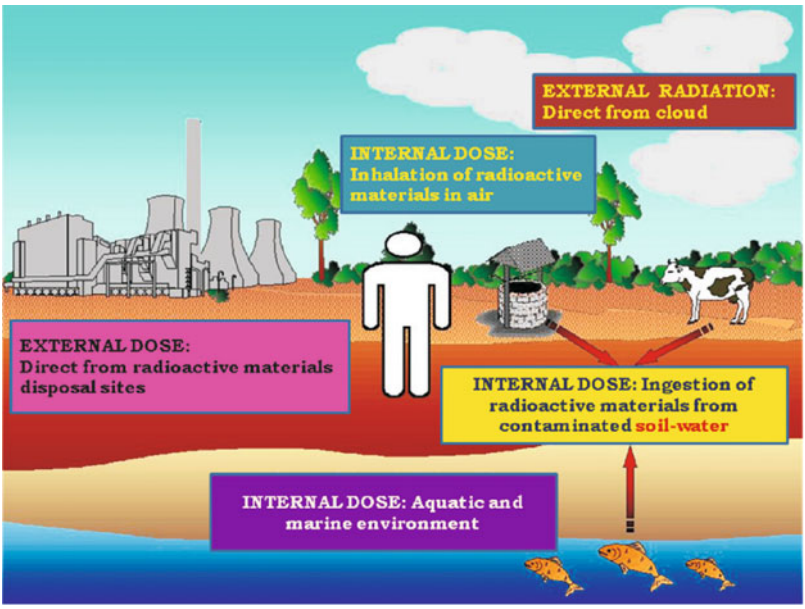

Fig. 11.1: The exposure of human being to radioactive material.

are sometimes referred to as 11(e)2 wastes, from the section of the U.S. Atomic Energy Act that defines them. Uranium mill tailings typically also contain chemically-hazardous heavy metals such as lead and arsenic. Vast mounds of uranium mill tailings are left at many old mining sites, especially in Colorado, New Mexico, and Utah.

11.5.1 Low Level Waste (LLW)

Basically all radioactive waste that is not high-level radioactive waste or intermediate-level waste or transuranic waste is classified as low-level radioactive waste. Volume-wise it may be larger than that of high level radioactive waste or intermediate-level radioactive waste or transuranic waste, but the radioactivity contained in the low-level radioactive waste is significantly less and made up of isotopes having much shorter half lives than most of the isotopes in high-level radioactive waste or intermediate-level waste or transuranic waste. Large amounts of waste contaminated with small amounts of radionuclides, such as contaminated equipment (glove boxes, air filters, shielding materials and laboratory equipment), protective clothing, cleaning rags, etc. constitute low-level radioactive waste. Even components of decommissioned reactors may come under this category (after part decontamination procedures). The level of radioactivity and half-lives of radioactive isotopes in low-level waste are relatively small. Storing the waste for a period of 10 to 50 years will allow most of the radioactive isotopes in low-level waste to decay, at which point the waste can be disposed off as normal refuse.

It may come as a surprise that several investigations have shown that exposure of mammals to low levels of radiation may indeed be beneficial, including, increased life span, greater reproductive capacity, better disease resistance, increased growth rate, greater resistance to higher radiation doses, better neurological function, better wound healing and lower tumour induction and growth (Devaney, 1998). Beneficial effects on plants include accelerated growth and development and increased harvests.

11.5.2 Intermediate Level Waste (ILW)

It contains higher amounts of radioactivity and in some cases requires shielding. ILW includes resins, chemical sludge and metal reactor fuel cladding, as well as contaminated materials from reactor decommissioning. It may be solidified in concrete or bitumen for disposal. As a general rule, short-lived waste (mainly non-fuel materials from reactors) is buried in shallow repositories, while long-lived waste (from fuel and fuel-reprocessing) is deposited in deep underground facilities. U.S. regulations do not define this category of waste; the term is used in Europe and elsewhere.

High Level Waste flasks are transported by train in the United Kingdom. Each flask is constructed of 14 in (360 mm) thick solid steel and weighs in excess of 50 tons.

11.5.3 High Level Waste (HLW)

High-level radioactive waste is conceptualized as the waste consisting of the spent fuel, the liquid effluents arising from the reprocessing of spent fuel and the solids into which the liquid waste is converted. It consists, generally, material from the core of a nuclear reactor or a nuclear weapon. This waste includes uranium, plutonium and other highly radioactive elements created during fission, made up of fission fragments and transuranics. (Note that this definition does not specify the radioactivity that must be present to categorize as high-level radioactive waste.) These two components have different times to decay. The radioactive fission fragments decay to different stable elements via different nuclear reaction chains involving α, β and γ emissions to innocuous levels of radioactivity, and this would take about 1000 years. On the other hand, transuranics take nearly 500,000 years to reach such levels. Heat output lasts over 200 years. Most of the radioactive isotopes in high-level waste emit large amounts of radiation and have extremely long half-lives (some longer than 100,000 years), creating long time-periods before the waste will settle to safe levels of radioactivity. As a thumb-rule one may note that volumes of low level radioactive waste and intermediate-level waste greatly exceed those of spent fuel or high-level radioactive waste. In spite of this ground reality, the public concerns regarding disposal of high-level radioactive waste is worldwide and quite controversial.

11.5.4 Transuranic Waste (TRUW)

As defined by U.S. regulations, without regard to form or origin, it is waste contaminated with alpha-emitting transuranic radionuclides with half-lives greater than 20 years, and concentrations greater than 100 nCi/g (3.7 MBq/kg), excluding High Level Waste. Elements that have an atomic number greater than uranium are called transuranic ("beyond uranium"). Because of their long half-lives, TRUW is disposed more cautiously than either low level or intermediate level waste. In the U.S. it arises mainly from weapons production, and consists of clothing, tools, rags, residues, debris and other items contaminated with small amounts of radioactive elements (mainly plutonium).

Under U.S. law, Transuranic waste is further categorized into "contact-handled" (CH) and "remote-handled" (RH) on the basis of radiation dose measured at the surface of the waste container. CH TRUW has a surface dose rate not greater than 200 mrem per hour (2 mSv/h), whereas RH TRUW has a surface dose rate of 200 mrem per hour (2 mSv/h) or greater. CH TRUW does not have the very high radioactivity of high level waste, nor its high heat generation, but RH TRUW can be highly radioactive, with surface dose rates up to 1000,000 mrem per hour (10,000 mSv/h). The United States currently disposes off TRUW generated from nuclear power plants and military facilities permanently at the Waste Isolation Pilot Plant.

11.5.5 Accidents Involving Radioactive Waste

A number of incidents have occurred when radioactive material was disposed off improperly, shielding during transport was defective, or when it was simply abandoned or even stolen from a waste store. In the Soviet Union, waste stored in Lake Karachay was blown over the area during a dust storm after the lake had partly dried out. At Maxey Flat, a low-level radioactive waste facility located in Kentucky, containment trenches covered with dirt, instead of steel or cement, collapsed under heavy rainfall into the trenches and filled with water. The water that invaded the trenches became radioactive and had to be disposed off at the Maxey Flat facility itself. In other cases of radioactive waste accidents, lakes or ponds with radioactive waste accidentally overflowed into the rivers during exceptional storms. In Italy, several radioactive waste deposits let material flow into river water, thus contaminating water fit for domestic use. In France, in the summer of 2008 numerous incidents happened; in one, at the Areva plant in Tricastin, it was reported that during a draining operation liquid containing untreated uranium overflowed out of a faulty tank and about 75 kg of the radioactive material seeped into the ground and, from there, into two rivers nearby; in another case, over 100 staff were contaminated with low doses of radiation. (http://en.wikipedia.org/wiki/Radioactive_waste)

Scavenging of abandoned radioactive material has been the cause of several other cases of radiation exposure, mostly in developing nations, which may have less regulation of dangerous substances (and sometimes less general

education about radioactivity and its hazards) and a market for scavenged goods and scrap metal. The scavengers and those who buy the material are almost always unaware that the material is radioactive and it is selected for its aesthetics or scrap value. Irresponsibility on the part of the radioactive material's owners, usually a hospital, university or military, and the absence of regulation concerning radioactive waste, or a lack of enforcement of such regulations, has been significant factors in radiation exposures.

11.6 Waste Disposal

Radioactive waste management involves minimizing radioactive residues, handling waste-packing safely, storage and safe disposal in addition to keeping sites of origin of radioactivity clean. Poor practices lead to future problems. Hence choice of sites where radioactivity is to be managed safely is equally important in addition to technical expertise and finance, to result in safe and environmentally sound solutions. The International Atomic Energy Agency (IAEA) is promoting acceptance of some basic tenets by all countries for radioactive waste management. These include:

(i) Securing acceptable level of protection of human health;
(ii) Provision of an acceptable level of protection of environment;
(iii) While envisaging (i) and (ii), assurance of negligible effects beyond national boundaries;
(iv) Acceptable impact on future generations; and
(v) No undue burden on future generations.

There are other legal, control, generation, safety and management aspects also.

The following options have been aired sometime or the other. Each one of the options demands serious studies and technical assessments:

- Deep geological repositories
- Ocean dumping Seabed burial
- Sub-seabed disposal
- Subductive waste disposal method
- Transforming radioactive waste to non-radioactive stable waste
- Dispatching to the Sun.

11.7 Radioactive Waste Management in India

Waste disposal is discarding waste with no intention of retrieval. Waste management means the entire sequence of operations starting with generation of waste and ending with disposal.

Just as per capita consumption of electricity is related to the standard of living in a country, the electricity generation by nuclear means can be regarded

as a minimum measure of radioactive waste that is generated by a country and hence the related magnitude of radioactive waste management. On the scale of nuclear share of electricity generation, India ranks fourth from the bottom in about 30 countries. As of the year 2000, India's share of nuclear electricity generation in the total electricity generation in the country was 2.65% compared to 75%, 47%, 42.24%, 34.65%, 31.21%, 28.87%, 19.80%, 14.41% and 12.44% of France, Sweden, the Republic of Korea, Japan, Germany, UK, USA, Russia and Canada, respectively. The reactors in operation produce in net gigawatts (one billion (10^9) watts) (E) in the latter countries nearly 63, 9, 13, 44, 21, 13, 97, 20 and 10, respectively; India's reactors in operation yield 1.9 on this scale (both data are as per IAEA Report of 2000). Hence the magnitude of radioactive waste management in India could be miniscule compared to that in other countries, especially when one takes into account the nuclear arsenal already in stockpile in the nuclear weapons countries. As more power reactors come on stream and as weaponization takes deeper routes the needs of radioactive waste management increase and in this context the experience of other countries would provide useful lessons.

Radioactive waste management has been an integral part of the entire nuclear fuel cycle in India. Low-level radioactive waste and intermediate-level waste arise from operations of reactors and fuel reprocessing facilities. The low-level radioactive waste liquid is retained as sludge after chemical treatment, resulting in decontamination factors ranging from 10 to 1000. Solid radioactive waste is compacted, bailed or incinerated depending upon the nature of the waste. Solar evaporation of liquid waste, reverse osmosis and immobilization using cement matrix are adopted depending on the form of waste. Underground engineered trenches in near-surface disposal facilities are utilized for disposal of solid waste; these disposal sites are under continuous surveillance and monitoring. High efficiency particulate air (HEPA) filters are used to minimize air-borne radioactivity. Over the past four decades radioactive waste management facilities have been set up at Trombay, Tarapore, Rawatbhata, Kalpakkam, Narora, Kakrapara, Hyderabad and Jaduguda, along with the growth of nuclear power and fuel-reprocessing plants. Multiple barrier approach is followed in handling solid waste (Rao, 2001).

11.8 Phytoremediation of Heavy Metals and Radionuclides: Case Studies

The research studies carried out on the remediation of heavy metals and radionuclides and decontamination of LLNW from the contaminated environment using recent technique, phytoremediation, are cited.

Nuclear waste generated through chemical processing in nuclear industry or nuclear weapons program have enhanced the level of hazardous environmental contaminants. The waste generated from nuclear industry generally contains radionuclides (U, Pu, Tc, Cs and Sr), heavy metals such as Cr, Pb, Hg, Zn and

Cd along with myriads of toxic organics (e.g. BTEX, Trichloroethylene, TBP, DBP etc.). The conventional methods like physico-chemical treatments are not being practiced to decontaminate the Low Level Nuclear Waste (LLNW) due to the presence of low concentration and large volumes of contaminants. The organics as well as inorganic chemicals present in the nuclear waste find their way in soil-water causing environmental pollution. Recent studies have shown that nuclear waste contaminants can have both lethal and sub lethal effects on a wide variety of living organisms when exceeding the concentrations. Some heavy metals are being subjected to bioaccumulation and biotransformation and may pose risk to human health when transferred through food chain. Phytoremediation of heavy metals with in vitro cultured plants in fertile soil-water environment is a recent advancement to remediate heavy metals from contaminated sites.

Phytoremediation technology uses green plants to reduce, remove, degrade or immobilize environmental pollutants/toxins from soil and water with an aim of restoring area sites to a condition useable for intended purpose (Cunningham et al., 1995; Flathman and Lanza, 1998; Salt et al., 1998; Weber et al., 2001; Wendy et al., 2006). The effectiveness of phytoremediation technology depends on the selection of appropriate plants or plant species. Plants are unique organisms equipped with remarkable metabolic and adsorption capabilities, as well as transport systems that can take up nutrients or contaminants selectively from the soil and water (Fulekar, 2005). The phytoremediation technology for remediation of radionuclides is demonstrated in Fig.11.2.

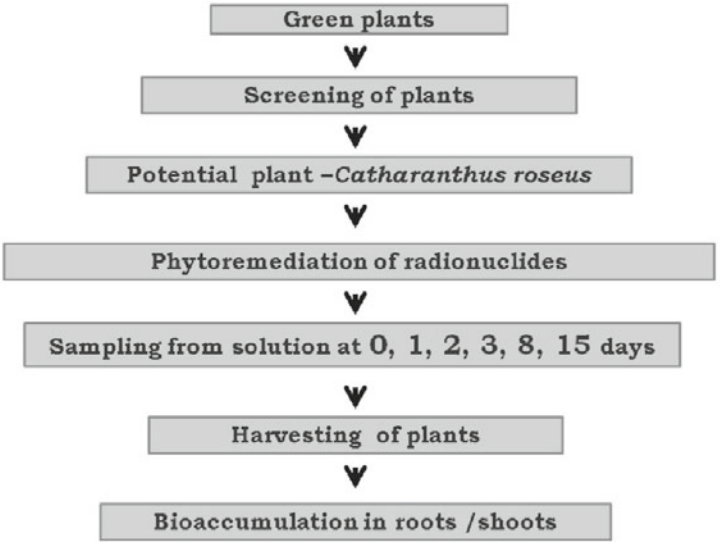

Fig. 11.2: Phytoremediation of radionuclides.

11.8.1 Phytoremediation of cadmium, lead and zinc by *Brassica juncea* L. Czern and Coss

The remediation of heavy metals in aquatic environment depends on the concentration of metals, plant species, pH and the nutrients available for the growth. The potential use of a plant in remediation can be assessed by exposing it to the toxic metal in the presence of nutrient medium. In the present research study, suitable nutrient medium, i.e. Steinberg medium has been taken for proper growth of the plants and spiked with varying concentrations of metals (Cd, Pb and Zn) viz. 5, 10, 20, and 50 μg ml^{-1} in each experimental set up. The metal concentration (in each setup) from the aquatic environment assessed at an interval of 0^{th}, 1^{st}, 3^{rd} 7^{th}, 14^{th} and 21^{st} days and plant growth was continued upto a period of 21 days. After the completion of phytoremediation, *B. juncea* plants were harvested and Cd, Pb and Zn metals concentration were measured in the roots and shoots of plant separately. The present study highlights the potential of *Brassica juncea* (Indian mustard) to take up heavy metals such as cadmium, lead and zinc from aquatic environment.

The study investigated remediation of heavy metals from aquatic environment using green plant *B. juncea*. After 21 days of uptake, the heavy metals were depleted from the growth solution indicating successful absorption of cadmium, lead and zinc by *B. juncea* (Anamika et al., 2008).

The reduction in concentration of these metals in the medium was attributed to their uptake by the plants. Figures 11.3 (a, b and c) demonstrate the depletion of metals from the aquatic solution between the 1st and the 21st days showing

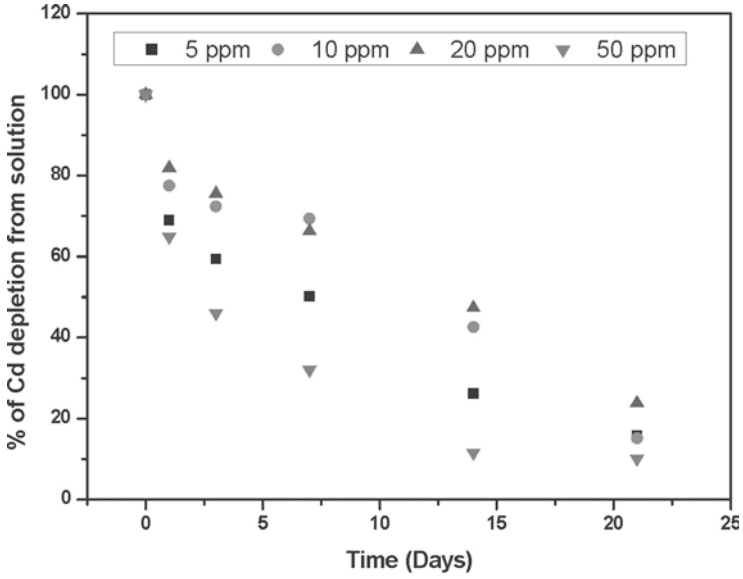

Fig. 11.3a: Depletion of cadmium from solution during 21 days of uptake by *Brassica juncea*.

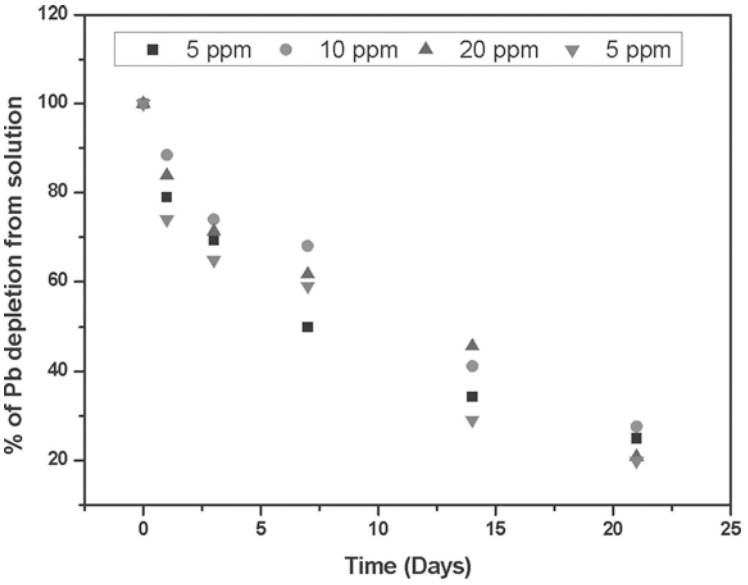

Fig. 11.3b: Depletion of lead from solution during 21 days of uptake by *Brassica juncea*.

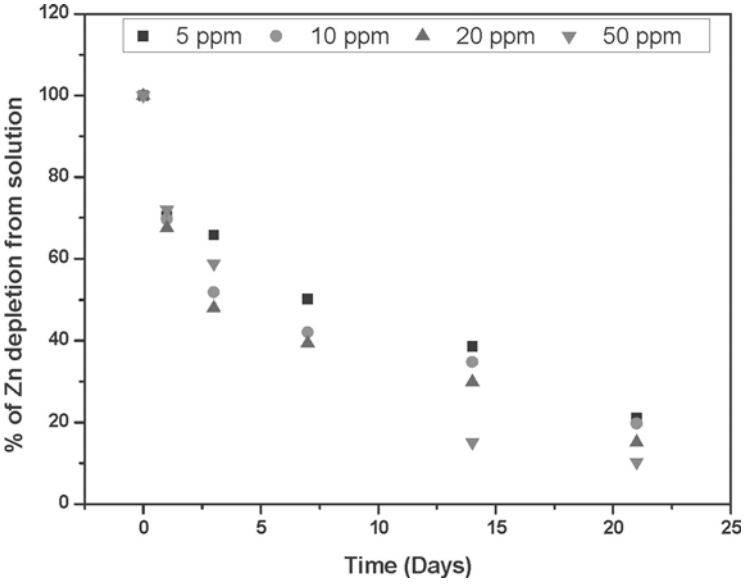

Fig. 11.3c: Depletion of zinc from solution during 21 days of uptake by *Brassica juncea*.

cadmium (35.2-88.9%), lead (26-80.1%), and zinc (30-89.8%), respectively. The metals were taken up in amounts directly proportional to their concentration in the solution proportionately over the entire period of 21 days (Figs 11.3a, 11.3b

and 11.3c). Zinc uptake by *B. juncea* was found to be highest as compared to cadmium and lead.

The plant biomass was determined with respect to uptake of cadmium, lead and zinc by *B. juncea*. There were no significant differences in the biomass of *B. juncea* when exposed to cadmium, lead and zinc at various concentrations ranging from 0 to 50 μg ml^{-1} (Table 11.2). This shows that *B. juncea* is tolerant and has potential to grow in contaminated environments and to efficiently take up heavy metals.

Table 11.2: Biomass of *B. juncea* after 21 days of exposure to the heavy metals contaminated Steinberg nutrient solution*

Metal	*Concentration* ($\mu g\ ml^{-1}$)	*Dry Weight (g)*	
		Roots	*Shoots*
Cd	Control	0.011±0.002	0.080±0.017
	5	0.008±0.004	0.076±0.019
	10	0.005±0.002	0.055±0.006
	20	0.007±0.003	0.063±0.014
	50	0.009±0.004	0.079±0.009
Pb	Control	0.037±0.007	0.124±0.007
	5	0.022±0.003	0.064±0.009
	10	0.014±0.004	0.072±0.013
	20	0.017±0.002	0.095±0.017
	50	0.028±0.005	0.107±0.014
Zn	Control	0.020±0.008	0.063±0.012
	5	0.016±0.003	0.057±0.007
	10	0.014±0.002	0.056±0.003
	20	0.013±0.004	0.057±0.008
	50	0.015±0.004	0.061±0.009

*Values are averages of three replicates ± S.D.

Table 11.3 demonstrates accumulation of zinc, cadmium and lead at varying concentrations in root and shoots of Indian mustard (*B. juncea)*. Results show that metals were accumulated more in roots than shoots. The increase level of metal content was observed with increasing concentration of metal upto 50 μg ml^{-1} (ppm). Bioaccumulation coefficient has also been analyzed.

In the harvested plant biomass, the metals showed an increasing trend as their concentration in the nutrient solution increased (Table 11.3). Heavy metals were efficiently taken up mainly by the roots of *B. juncea* plants at all the evaluated concentrations. Similar findings were reported by Jadia and Fulekar (2008) for uptake of heavy metals (Cd, Cu, Ni, Pb and Zn) by fibrous

Table 11.3: Metal accumulation in roots and shoots of *B. juncea* and their bioaccumulation coefficient (compared to control treatment)*

Metal	*Concentration* ($\mu g\ mL^{-1}$)	*Metal uptake* ($\mu g\ g^{-1}$)		*Bioaccumulation coefficient*
		Roots	*Shoots*	
Cd	Control	ND	ND	ND
	5	2230±111.97	344±27.42	514.8±24.35
	10	5850±409.26	629±81.87	647.8±18.35
	20	13927±345.22	1449±319.92	767.86±38.89
	50	18149±465.55	3349±391.96	435.36±28.68
Pb	Control	ND	ND	ND
	5	1258±173.78	242±42.64	300.6±30.83
	10	4520±349.50	267±77.10	478.7±41.47
	20	7356±170.88	881±48.56	411.85±20.03
	50	12264±991.13	2477±344	294.82±22.87
Zn	Control	ND	ND	ND
	5	2250±464.30	471±35.48	544.2±30.83
	10	4243±680.26	707±88.73	495±35.92
	20	12665±331.52	1004±53.86	683.45±38.77
	50	26517±718.71	2582±142.79	582.04±43.98

*Values are averages of three replicates ± S.D. ND= Not detected.

root grass. Once metal ions are absorbed, they can be accumulated in the roots or be exported to the shoots via the transpiration stream (Ximenez-Embun et al., 2001). Our study showed higher accumulation of all metals (cadmium, zinc and lead) in the roots than in the shoots. The maximum accumulation of cadmium (at 50 $\mu g\ ml^{-1}$) in roots and shoots of *B. juncea* were 18.42 mg gm^{-1} and 3.35 mg gm^{-1}, respectively, while the highest accumulations of Pb (at 50 $\mu g\ ml^{-1}$) averaged 12.26 mg gm^{-1} and 2.47 mg gm^{-1} respectively, in roots and shoots. Zinc concentration in root and shoot tissues reached its highest values of 26.52 mg gm^{-1} and 2.58 mg gm^{-1} respectively, at 50 $\mu g\ ml^{-1}$ concentration. Metal accumulation in *B. juncea* was found to be 5.4, 4.9 and 5.96 times higher in roots as compared to the shoots in case of cadmium, lead and zinc, respectively. The data showed heavy metals accumulation by *B. juncea* in the order: Zn>Cd>Pb.

Research has shown that metal concentration in plant tissues is a function of the heavy metals content in the growing environment (Cui et al., 2004), and that the uptake and accumulation of different metals may vary from plant to plant species. Kim et al. (2003) suggested that such discrepancies arise due to variation in type of heavy metals, its concentration, form of metal present and plant species. Different metals are differently mobile and within a plant, cadmium and zinc are more mobile than lead and copper (Greger, 2004).

Cadmium is one of the most dangerous heavy metals due to its high mobility and the small concentration at which its effects on the plants begin to show (Vazquez et al., 1992). The bioaccumulation coefficient (BC) of cadmium varied from 514.8 to 435.36 as exposure increased from the minimum (5 μg ml^{-1}) to the higher rate (50 μg ml^{-1}); lead BC varied from 300.6 to 294.82 at exposure from 5 to 50 μg ml^{-1}, while BC for zinc ranged from 544.2 to 582.04 between 5 μg ml^{-1} and 50 μg ml^{-1}. The BC of different metals by *B. juncea* was found to be in the order of Pb<Zn<Cd at almost all concentrations tested.

The translocation factor which can describe the movement and distribution of heavy metals in plants was also determined in order to assess the uptake of heavy metals by roots of *B. juncea* from the hydroponics solution and their translocation from roots to the shoots. After 21 days of exposure, only 15.38% of cadmium (at 50 μg ml^{-1}), 16.80% of lead (at 50 μg ml^{-1}) and 17.30% of zinc (at 5 μg ml^{-1}) was translocated from roots to shoots (Fig. 11.4). The translocation factor was found in the order of Pb<Zn<Cd. It was observed that lead accumulated in roots of *B. juncea* in higher concentrations as compared to cadmium and zinc. Zinc being a micronutrient was translocated proportionately in root and shoot; whereas cadmium uptake from root to shoot is similar to that of zinc. Transport across root cellular membrane is an important process which initiates metal absorption in plant tissues. Tanhan et al. (2007) have studied the uptake and accumulation patterns of Pb, Cd and Zn in *Chromolina odorata* and reported that accumulation increased in order of Cd<Zn<Pb, while the BC increased in order of Cd< Pb<Zn. Similar translocation pattern was reported in *Polygonum thunbergii* but with a different accumulation pattern i.e. Cd< Pb<Zn<Cu (Kim et al., 2003).

The cadmium and zinc uptake were found to be higher in shoot as compared to lead. Zinc and cadmium have many physical and chemical similarities as they both belong to Group II of the periodic table. They are usually found together in the ores and compete with each other for various ligands. Thus the

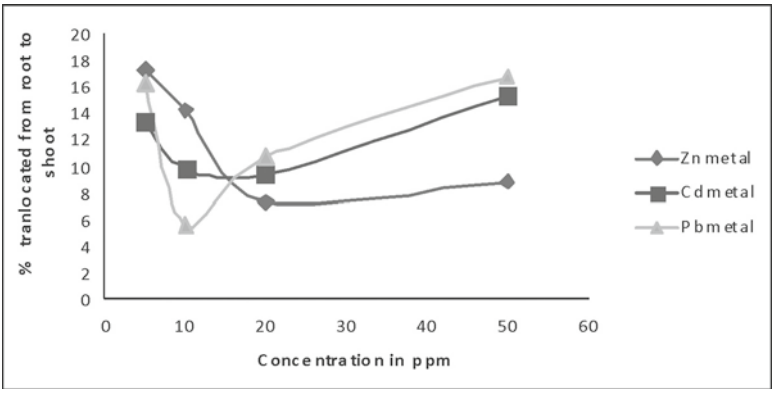

Fig. 11.4: Translocation factor to shoot part in *B. juncea.* The percentage translocated to the shoot is high in case of cadmium metal.

interaction between zinc and cadmium in the biological system is likely to be similar. The fact that cadmium is a toxic metal and zinc is an essential element makes this association interesting as it raises the possibility that the toxic effects of cadmium may be preventable or treatable by zinc (Chaudhury and Chandra, 1987). Our results further showed that lead is accumulated more in roots as compared to the other two metals (zinc and cadmium). Lead uptake studies in plants have demonstrated that roots have an ability to take up significant quantities of lead whilst simultaneously greatly restricting its translocation to aboveground parts (Lane and Martin, 1977).

Roots of plants act as a medium for heavy metal translocation and there may be a potential tolerance mechanism comparison data of metal uptake revealed that only small amount of lead is translocated from root to shoot, as compared to zinc and cadmium. Liu et al. (2000) have reported that *B. juncea* has considerable ability to remove lead from solutions and accumulate it in roots. Nandakumar et al. (1995) have also reported the higher accumulation of lead in roots of sorghum species, with indications that lead can be found on the outer surface of plant roots, as crystalline or amorphous deposits, and could be deposited in the cell walls or in vesicles. The findings of the present phytoremediation study have demonstrated the potential use of *B. juncea* for remediation of heavy metals from contaminated aquatic environment. This plant can be grown in fields that are contaminated with heavy metals and thereafter be removed, burnt and buried for safe disposal.

11.8.2 Remediation of radiostrontium (^{90}Sr) and radiocaesium (^{137}Cs) by *Catharanthus roseus* (L.) G. Don in Aquatic Environment

The soil-water environment could be contaminated with radionuclide wastes from various sources including natural sources, global fallout from nuclear weapon testing, discharge from nuclear installations, disposal of nuclear waste and nuclear accidents. The accidental and routine releases of radionuclides from the nuclear industry are inevitable and can cause local, regional and even global environmental contamination (Fulekar, 2005; 2009). Radionuclides in the environment can eventually be passed on to human beings through food chains and may represent an environmental threat to the health of local population (Howard et al., 1991; Robinson and Stone, 1992). The proper care and handling of the material containing nuclear waste even in traces is of outmost importance.

The radionuclides such as ^{137}Cs and ^{90}Sr are the common contaminants found in the soil-water environment at the site of waste related with low level nuclear waste. The radiostrontium (^{90}Sr) and radiocaesium (^{137}Cs) have long-term radiological and health impacts due to their long half lives and chemical similarities with two essential elements required for plant growth, Ca^{2+} and K^{+}, respectively (Shaw and Bell, 1991). The various techniques and methods are evolved for environmental cleanup. The recent technology involves the use of

green plants for phytoremediation, an eco-friendly, cost-effective, aesthetically pleasing removal method of radionuclides from contaminated environment (Salt et al., 1998; Negri and Hinchman, 2000; Andrade et al., 2002; Eapen et al., 2003).

In India Bhabha Atomic Research Centre is a regulatory authority that has prescribed the standard for treating the radioactive waste containing radionuclides. In the radioactive material process, radionuclides like ^{137}Cs and ^{90}Sr are produced by nuclear fission with half lives of 30 years and 29 years, respectively. Cs poses a health hazard from both beta and gamma radiation while Sr emits only beta radiation. ^{137}Cs and ^{90}Sr have no role in plant nutrition but they can be taken up by the plants. The capability of the plant to accumulate radionuclides in their aerial parts determines their potential for phytoremediation.

In the present research study *Catharanthus roseus* plants have been screened and found to have high potential compared to other plants for uptake of radionuclides. Researchers have not yet looked for the potential of *C. roseus* for remediation of radionuclides. Anamika et al. (2009) have studied the uptake of ^{90}Sr and ^{137}Cs in liquid solutions under in vitro conditions to evaluate the potential of plant for the radionuclides from contaminated environment. The exposure treatment was carried out upto a period of 15 days for remediation of radionuclides from artificially contaminated aquatic environment in a laboratory setup. The bioaccumulation of these radionuclides in roots and shoots has been studied using translocation factor. Research highlights: the plant *Catharanthus roseus* grown in the vicinity of BARC has the efficiency to uptake the radionuclides with special reference to radiocesium and radiostrontium. Research findings are beneficial to other radionuclides which could be remediated effectively from aquatic environment.

The present research study investigated the potential of *C. roseus* for remediation of radionuclides in aquatic environment.

The plants of *C. roseus* were exposed to three different activity concentrations, viz. 3.7×10^2 kBqL^{-1}, 3.7×10^3 kBqL^{-1} and 3.7×10^4 kBqL^{-1} of ^{137}Cs and ^{90}Sr separately for a period of 15 days. *C. roseus* plants have shown the potential for remediation of radionuclides ^{137}Cs and ^{90}Sr. Result shows that the percentage of radionuclides activity decreased with the increasing initial concentration of ^{137}Cs and ^{90}Sr and increased with increasing period of exposure treatment (Figs 11.5 and 11.6). *C. roseus* plants were found to remove 70, 48 and 45% of ^{90}Sr and 73, 59 and 51% of ^{137}Cs from the 3.7×10^2 kBqL^{-1}, 3.7×10^3 kBqL^{-1} and 3.7×10^4 kBqL^{-1} activity concentrations respectively. The transfer factor for Cs uptake was found as 0.70, 0.48 and 0.45 and for Sr 0.73, 0.59 and 0.51 at 3.7×10^2 kBqL^{-1}, 3.7×10^3 kBqL^{-1} and 3.7×10^4 kBqL^{-1}, respectively after 15 days of the treatment period.

Figures 11.7 and 11.8 demonstrate the accumulation of radionuclides in the roots and shoots of plant. The present research study shows that the accumulation of ^{137}Cs in roots of *C. roseus* was found to be 2.16 kBq g^{-1},

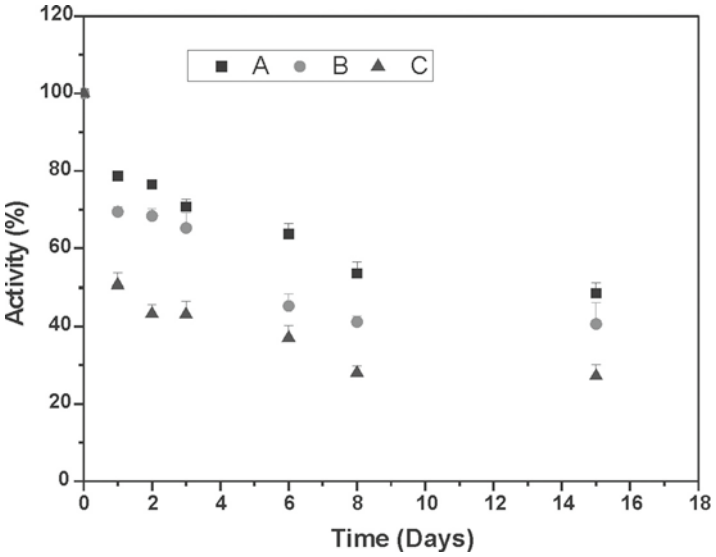

Fig. 11.5: Activity of ^{137}Cs in solutions after incubation with *C. roseus* plants for different time intervals. All the values are the mean of three replicates ±S.E. Initial activities of ^{137}Cs were A: 3.7×10^4 kBqL^{-1}, B: 3.7×10^3 kBqL^{-1} and C: 3.7×10^2 kBqL^{-1}.

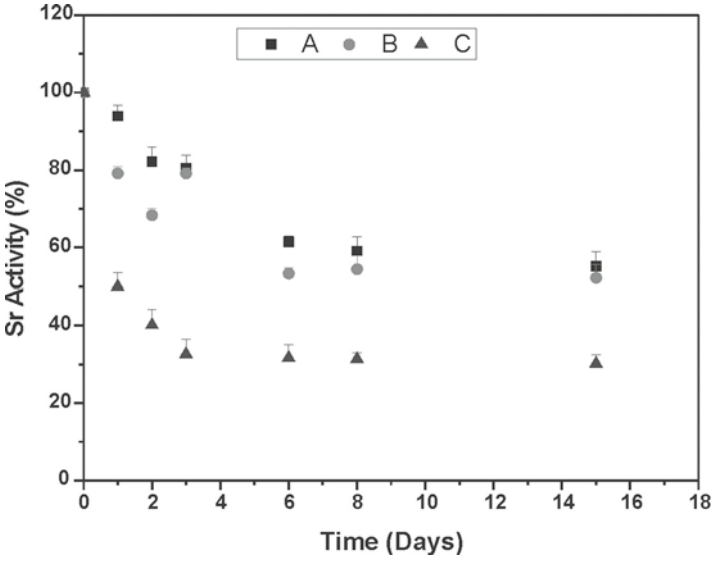

Fig. 11.6: Activity of ^{90}Sr in solutions after incubation with *C. roseus* plants for different time intervals. All the values are the mean of three replicates ±S.E. Initial activities of ^{90}Sr were A: 3.7×10^4 kBqL^{-1}, B: 3.7×10^3 kBqL^{-1} and C: 3.7×10^2 kBqL^{-1}.

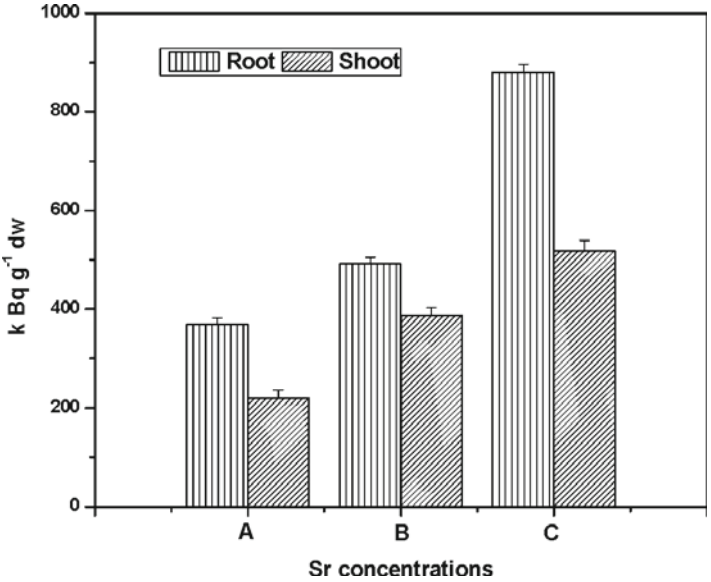

Fig. 11.7: Distribution of ^{90}Sr in roots and shoots of *C. roseus*. All the values are the mean of three replicates ±S.E. Initial activities of ^{90}Sr were A: 3.7×10^2 kBqL^{-1}, B: 3.7×10^3 kBqL^{-1} and C: 3.7×10^4 kBqL^{-1}.

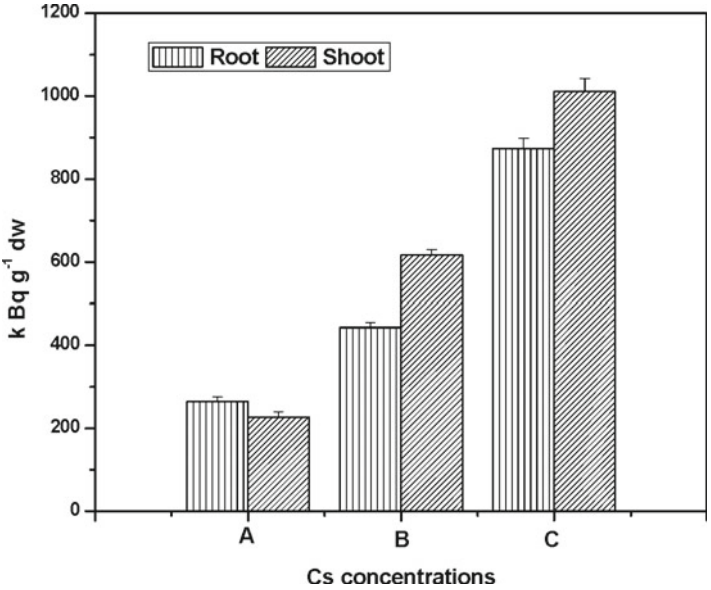

Fig. 11.8: Distribution of ^{137}Cs in roots and shoots of *C. roseus*. All the values are the mean of three replicates ±S.E. Initial activities of ^{90}Sr were A: 3.7×10^2 kBqL^{-1}, B: 3.7×10^3 kBqL^{-1} and C: 3.7×10^4 kBqL^{-1}.

17.74 kBq g^{-1} and104.73 kBq g^{-1}, when exposed to 3.7×10^2 $kBqL^{-1}$, 3.7×10^3 $kBqL^{-1}$ and 3.7×10^4 $kBqL^{-1}$ activity concentrations, respectively; whereas the accumulation of ^{137}Cs in shoot was found 2.61 kBq g^{-1} at 3.7×10^2 $kBqL^{-1}$, 22.15 kBq g^{-1} at 3.7×10^3 $kBqL^{-1}$ and 198.93 kBq g^{-1} at 3.7×10^4 $kBqL^{-1}$ activity level. The accumulation of ^{137}Cs in the shoots is found 1.9 times higher than roots at higher activity concentration (3.7×10^4 $kBqL^{-1}$) and this has proved the efficiency of *C. roseus* plants to remediate the ^{137}Cs from the aquatic-waste streams. Similarly, the study conducted for the phytoremediation of ^{90}Sr indicates that the exposure of ^{90}Sr at 3.7×10^2 $kBqL^{-1}$, 3.7×10^3 $kBqL^{-1}$ and 3.7×10^4 $kBqL^{-1}$ activity concentrations accumulated 2.23 kBq g^{-1}, 13.68 kBq g^{-1} and 53.70 kBq $g^{-1}$of ^{90}Sr in the roots, respectively. The values of this radionuclide (^{90}Sr) determined in the shoots are 2.72 kBq g^{-1} at 3.7×10^2 $kBqL^{-1}$, 15.26 kBq g^{-1} at 3.7×10^3 $kBqL^{-1}$ and 61.65 kBq g^{-1} at 3.7×10^4 $kBqL^{-1}$ activity level. In case of ^{90}Sr, accumulation is found 1.14 times higher in shoots than the roots at higher activity concentration (3.7×10^4 $kBqL^{-1}$).

The bioaccumulation of radionuclides in the shoot of *C. roseus* has been demonstrated in Fig. 11.7. The data indicates ^{137}Cs and is bioaccumulated more in shoot as compared to the ^{90}Sr at 3.7×10^2 $kBqL^{-1}$, 3.7×10^3 $kBqL^{-1}$ and 3.7×10^4 $kBqL^{-1}$activity levels. Similarly, the accumulation of these radionuclides in the root of *C. roseus* demonstrate that ^{137}Cs is hyper accumulative than ^{90}Sr (Fig. 11.8). *Catharanthus roseus* has shown uptake of radionuclides as a hyperaccumulator plant and accumulated 3.22 and 1.95 times higher ^{137}Cs than ^{90}Sr in shoots and roots at high level of activity, respectively. Eapen et al. studied the phytoremediation of radionuclides by using *Calotropis gigantean* plants. However, in that study it has been reported that Cs was less remediated by the plants in comparison to Sr. The plants *Eichornia crassipes* have been reported for higher uptake for ^{90}Sr as compared to ^{137}Cs (Jayaraman and Prabhakar, 1982; Eapen et al., 2006). The results of the present research show that *C. roseus* has remediated higher amount of Cs as compared to Sr and therefore this weed plant *C. roseus* is a good candidate for remediation of caesium contaminated environment.

Phytoremediation exploits the natural ability of vascular plants to take up a variety of chemical elements through the root system, deliver these elements to vascular tissues, and transport and compartmentalize radioactive elements in the aboveground biomass. Caesium and strontium are mobile radionuclides and can be readily absorbed by plant roots from the solution and translocated to the aboveground parts (Zhu and Smolder, 2000). Strontium is chemically similar to calcium and caesium to potassium and hence, similar uptake mechanism may be applicable for these radionuclides in the plants. Aboveground biomass loaded with radionuclides is harvested, processed for volume reduction and further radionuclide concentration, and disposed off as radioactive waste.

The transfer factor describes the movement and distribution of radionuclides from root to shoot of the plant. Transfer factor is found more in case of caesium as compared to strontium. Soudek et al. (2006) have studied the uptake and

distribution of ^{137}Cs than ^{90}Sr by *Helianthus annuus* plants in a hydroponic medium and also assessed the relationship between caesium and potassium or ammonium ions and strontium and calcium ions.

Our research finding shows that *C. roseus* has high uptake potential for radiocaesium as compared to the radiostrontium and can serve as a good candidate for remediation of ^{137}Cs from the contaminated environment. The treated plants could be removed, incinerated and the ashes disposed off in a suitable dumping ground to protect health and environment.

The case studies presented here are based on the research work done and documented under the BRNS research project. The research facilities provided for the research studies are gratefully acknowledged.

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12

Improving Plants for Zinc Acquisition

Prachy Dixit and Susan Eapen

12.1 Introduction

Plants require essential metals for their growth and development and uptake of mineral elements from soil is a part of mineral nutrition. The plant roots are in direct contact with soil and are involved in uptake of metal ions which are later translocated to different aerial parts, where they are subsequently used for growth, development and reproduction. Concentration of metal ions in soil and their chemical speciation change with the location and environment and plants have tightly regulated mechanisms for uptake, transport and sequestration of metals (Kramer et al., 2007). Essential metals at elevated levels and contaminant non-essential metals can be toxic to plant cells and a highly regulated network of metal homeostasis mechanisms operate for control of metal uptake, trafficking and storage (Clemens, 2001).

Zinc (Zn) is a transition metal of atomic number 30 and is the 23rd most abundant element on Earth. It is the second-most abundant transition metal in organisms after iron and is present in all six enzyme classes EC-1-6 (oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases) (Broadley et al., 2007). Zinc acts as a functional, structural or regulatory cofactor in over 300 enzymes (Marschner, 1988; Coleman, 1998). Some of the enzymes which contain zinc include alcohol dehydrogenase, Cu-Zn superoxide dismutase, carbonic anhydrase and RNA polymerase. Zinc is taken up as a divalent cation (Zn^{2+}) by plant roots and in plants, zinc is not oxidized or reduced, but the functions are based on its properties as a divalent cation with a strong tendency to form tetrahedral complexes (Clarkson and Hanson, 1980). The largest class of zinc binding proteins is the zinc finger domain containing proteins, which can regulate transcription directly through their effects on DNA/RNA binding, through regulation of chromatin structure, RNA metabolism and

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protein-protein interactions (Klug, 1999; Engelbrecht et al., 2004). Zinc is also known to stabilize biomembranes (Chvapil, 1973) and plays a crucial role in transcription factors (Kramer and Clemens, 2006, Hershfinkel, 2006). When zinc is in short supply, a range of deficiency symptoms occur and plant growth is reduced (Marschner, 1988).

Alteration of plant metabolism to improve zinc acquisition will help in phytoremediation of zinc from contaminated sites and in biofortification of foods. Zinc (Zn) is an essential component of food, which is deficient in diet of people of developing countries and needs to be fortified in food. Zinc, although a component of thousands of proteins in plants, is toxic when present in excess. A better understanding of Zn acquisition will help in routing Zn to the edible part of crop plants for biofortification. Zinc is released into the environment by mining and smelting activities and phytoremediation can be used to remove the excess Zn from contaminated soil. The physiological and molecular information gained from zinc hyperaccumulator plants such as *Thlaspi caerulescens* and *Arabidopsis halleri* will help in designing strategies for enhanced acquisition of zinc. Powerful genetic and molecular techniques have now identified a range of gene families such as Heavy Metal ATPases (HMA), Nramps, Cation Diffusion family and ZIP family of transporters involved in zinc uptake, translocation and sequesteration. Overexpression of the genes or heterologous gene transfer may pave the way for development of plants with enhanced zinc acquisition, which will have potential application for phytoremediation/biofortification.

12.2 Phytoremediation and Biofortification

Phytoremediation deals with the use of plant's ability to take up excess metals from soil and solutions to clean up contaminated soils and solutions. Biofortification is the process of increasing the natural content of micronutrients in the edible parts of crop plants. Improving uptake of minerals from soil and enhancing the movement to edible parts with the objective of bioavailability on consumption will improve human nutrition. Both phytoremediation and biofortification operate through the same uptake and transport systems in plants and are like two sides of the same coin.

12.2.1 Importance of Zinc in Human and Animal Nutrition

Zinc deficiency ranks fifth as health risk factor in developing countries (World Health Organization, 2002) and is known to affect more than 25% of the world population (Maret and Sandstead, 2006). Although zinc is known to improve crop productivity (Cakmak, 2008), excess zinc causes toxicity. Hence, plants have tightly controlled homeostatic mechanisms to take up and maintain the right concentrations of zinc in the cellular compartments. For effective utilization of zinc for biofortification, it has to accumulate in the edible parts of crop plants.

12.2.2 Zinc in Soils

Zinc is present in soils as a result of chemical and physical weathering of rocks. Zinc in soil can occur in three primary fractions: (1) water soluble zinc, (2) adsorbed and exchangeable zinc in colloidal fraction and (3) insoluble zinc complexes and minerals (Lindsay, 1979; Barber, 1995). Mining and smelting activities, fossil fuel combustion, discharge of mine waste, addition of phosphate fertilizers, sewage sludge, agrochemicals, manure, particles from galvanized surfaces and rubber mulches have enhanced the levels of zinc in soils and solutions (Alloway and Steinnes, 1999).

12.2.3 Zinc Uptake and Transport into Roots of Plants

Zinc is taken up by root epidermal cells and transported across the plasma membrane into the symplasm. Subsequent transport from epidermal and cortical cells of root to root xylem can occur through the cytoplasmic continuum of cells connected by plasmodesmata. Zinc finally enters the stelar apoplast. This pathway is mediated by membrane transporters. Zinc can also enter apoplastically and enter xylem through areas where casparian strip is not well developed.

Once the metal enters the cytoplasm of cell, the metal can be bound to phytochelatins, which use their thiol moieties for binding with the metal (Clemens, 2006) or metallothionein (Zhou and Goldsbrough, 1994; Clemens, 2001) or can get bound to nicotianamine (von Wiren et al, 1996; Schaef et al., 2003). Root cells contain vacuoles and if the metal is targeted to vacuoles in the root cells, the translocation of the metal to the shoot cells gets limited. The metal has to move symplastically through plasmodesmata from root epidermal cells to cortex cells and finally cross the endodermis, which has characteristic casparian strips and cross the xylem parenchyma and enter the apoplastic xylem vessels. In general, there is a tendency for reduced uptake of ions as the distance of root from apex increases probably because of heavy suberinization of casparian cell strip in root tissues away from root apex. Since apical cells of root have a nutrient requirement for development and differentiation, the contribution of apical zones of roots for translocation to shoot can be low. There are two parallel pathways for radial transport of metal ions in roots: one passing through the apoplast and the other passing from cell to cell in symplast through plasmodesmata, which bypass the vacuole. The apoplastic transport of metal ions is stopped at the casparian strip.

12.2.4 Xylem Loading and Transport

The release of ions into xylem vessels involves a retransfer from symplast to apoplast. In *Arabidopsis*, xylem parenchyma cells have a plasma membrane proton pump AHA4 (Vitart et al., 2001). Zinc pumps are required for the movement of zinc from xylem parenchyma symplast to the apoplast of xylem vessels. HMA Zn pumps in root may be involved in this step. Enhanced

expression of HMA4 observed in the metal hyperaccumulator *A. halleri* is attributable to a combination of modified cis regulatory sequences and copy number expansion in comparison with *A. thaliana* (Hanikenne et al., 2008).

Metals to be transported to shoots should not be stored in root vacuoles. Members of the Nramp family (Natural resistance associated macrophage protein) are involved in efflux of heavy metals from vacuoles. For movement of zinc from cell to cell in the root, a cytosolic binding component such as nicotianamine may be involved. Movement of zinc through the xylem may also be achieved by binding to low molecular weight ligands (Suzuki et al., 2008).

12.2.5 Unloading of Zinc from Xylem into Mesophyll Cells

For unloading of zinc, the metal has to move from the xylem elements in the shoot to the xylem parenchyma symplast and move symplastically from cell to cell in the leaf cell and finally enter the last destination in the leaf cell, where it will be deposited and stored.

12.2.6 Loading of Zinc into Seeds

For biofortification of zinc in cereals and legumes, it is necessary for the essential metal to be translocated to the edible part i.e., the seeds. For this, the metal ion which has reached the shoot through xylem will be unloaded and will move symplastically from cell to cell until they reach the mesophyll cells. When the shoot meristem is converted into floral meristems or inflorescences bearing flowers, seed setting starts after fertilization. The supply of minerals to the developing grain can occur either through direct uptake and transport from soil or through remobilization of minerals as the leaves senescence during the grain filling stage. YSL transporters may play a role in zinc mobilization. The only vascular tissue to reach cereal grain is the phloem. Inorganic elements have to leave the xylem after the long distance transport, move into leaf cells, move from leaf cells into phloem and finally get transported to the seed. Inside the phloem, metal ions are likely to form complexes with phytochelatins. For the metal ion to reach the embryo and endosperm, the metal ion has to exit the phloem. Phloem unloading is not yet well understood. More information is required on specific transporters needed for transport to the seeds.

12.2.7 Distribution of Metals in Seeds

The seed has testa and pericarp, which are diploid and are part of the mother plant. In cereals, most of the food is stored in the triploid endosperm and embryo is covered on three sides by the endosperm. Aleurone layer, which is part of the endosperm, surrounds the internal starchy part of endosperm. Depending on where they are stored, zinc has to move into the seed. Most of the zinc in the cereal grain is stored in the embryo and scutellum compared to the endosperm. Hence zinc, which is unloaded from phloem, has to move several cell layers to

reach its final destination. Zinc is stored alongwith phytate, since it is a strong chelator of divalent cations. Storage of zinc-phytate compounds takes place in protein storage vacuoles.

12.3 Hyperaccumulators of Zinc: Understanding the Mechanism

Brooks et al. (1977) first coined the term "hyperaccumulator" for plants which accumulate high concentrations of metals in the shoot biomass when grown under natural environment. Threshold values for different metals vary and for zinc, it is 10,000 μg g^{-1}dwt. (1%) (Baker and Brooks, 1989), while non-hyperaccumulator plants have a zinc concentration of 30-100 μg^{-1} dwt. Concentration above 300 μg^{-1} is considered to be toxic to plants (Marschner, 1995). Zinc hyperaccumulators include about 15 species (Baker, 1995; Brooks et al., 1994), most of which are confined to calamine soil. *Thlaspi* genera have 11 species; *Arabidopsis* has one species; while a few species belong to other families (Baker and Brooks, 1989). *Thlaspi caerulescens* and *Arabidopsis halleri* (Lombi et al., 2002), which are zinc hyperaccumulators also hyperaccumulate cadmium, which is a toxic element. A better understanding of zinc hyperaccumulation will lead to a desired breakthrough for designing strategies for biofortification (Grusak, 2002) and phytoremediation. An understanding of the physiological mechanism on how the cells maintain such high levels of intracellular metals and what are the transport mechanisms involved and finally what are the genes involved in such uptake, transport and sequestration will help in developing novel strategies.

12.3.1 *Arabidopsis halleri* and *Thlaspi caerulescens*

The physiological mechanisms underlying enhanced metal uptake by *Arabidopsis halleri* and *Thlaspi careulescens* are being studied. There are several steps in the process such as mobilization in the rhizosphere, metallophilic root proliferation, uptake, chelation and compartmentalization in roots, xylem loading, xylem unloading and chelation and compartmentalization in leaf cells (Assuncao et al., 2003).

12.3.2 Conventional Breeding for Improved Zinc Acquisition

Conventional breeding can be used for improving zinc acquisition. Selection, hybridization and mutation breeding can be used for improving zinc uptake, translocation and sequestration.

12.4 Improving Plants for Zinc Accumulation Using Transgenic Approach

Transgenic approaches for enhancing Zn acquisition rely on improving the phytoavailability of mineral elements in the soil, their uptake from the

rhizosphere, translocation to the shoot and accumulation in plant tissues (White and Broadley, 2005). A transgenic approach to increase the Zn accumulation in plants might involve the manipulation of transporters involved in Zn translocation (Ramesh et al., 2004). With respect to Zn uptake, translocation and deposition, a predominant role is played by members of the ZIP family (Ramesh et al., 2003) and MTP family (Drager et al., 2004). Furthermore, with respect to root-to-shoot allocation of Zn, the Zn pump HMA4 seems to be a major player (Hussain et al., 2004). Transgenic plants have been developed with the objective of enhancing Zn uptake and accumulation. Van der Zaal et al. (1999) overexpressed a ZAT gene and the plants showed enhanced resistance to Zn and also accumulated Zn in roots. Tomato plants transformed with a mouse metallothionein gene showed enhanced Zn content in the leaves (Sheng et al., 2007). Expression of *Arabidopsis* phytochelatin synthase gene in Indian mustard resulted in enhanced tolerance to Zn and Cd. Introduction of a defensin gene (Ah PDF-1) from Zn hyperaccumulator *Arabidopsis halleri* to *Arabidopsis thaliana* resulted in transgenic plants more tolerant to Zn than control plants (Mirouze et al., 2006). Overexpresssion of an *Arabidopsis* Zn transporter gene in *Hordeum vulgare* increased the content of Zn in seeds (Ramesh et al., 2004). Transgenic tobacco overexpressing glyoxalase pathway genes was shown to accumulate more Zn in roots compared to control plant (Mirzoue et al., 2006). Transgenic tobacco overexpressing glyoxylase pathway genes (glyoxylase I and glyoxylase II) was shown to accumulate Zn in the roots (Singla-Pareek et al., 2006). Expression of *Arabidopsi*s phytochelatin synthase in Indian mustard resulted in enhancement in tolerance to Cd and Zn (Gasic and Korban, 2007). Major bottlenecks for biofortification of Zn are due to root to shoot (grain) translocation of the metal. Ectopic overexpression of AtHMA4 gene was shown to have enhanced levels of Cd and Zn in leaves (Verret et al., 2004). Table 12.1 lists the various transgenic plants developed for Zn acquisition.

It is essential to study whether transgenic lines with enhanced nicotianamine concentrations will have enhanced ability to accumulate Zn in shoot. For remobilization of metals from leaf and transport to seeds, other genes such as YSL protein genes have to be overexpressed. Hence a transgenic approach to increase Zn content in seeds will involve manipulation of several genes in translocation. Thus ZIP family genes, MTP family, HMA have to be overexpressed in plants for improving Zn uptake, translocation, sequestration and remobilization. Since many of the Zn transporters transport Cd also, it is essential to introduce Zn transporters which are specific to Zn and not cadmium. We have introduced a highly specific zinc transporter gene from *Neurospora crassa* into tobacco plants and were shown to accumulate zinc in roots (Eapen et al., 2009). It will be worthwhile adding specifically Cd exporters in the roots to remove Cd (Lee et al., 2003).

Table 12.1: Genetically transformed plants for Zn tolerance/acquisition

S. No.	*Gene source*	*Transgenic plants*	*Result*	*Reference*
1.	*Arabidopsis* ZAT genes	*Arabidopsis*	Tolerance to Zn and accumulation of Zn in soils	Van der Zaal et al., 1999
2.	*AtZIPI*	*Hordeum vulgare*	Higher Zn uptake rate after Zn deprivation	Ramesh et al., 2004
3.	Glyoxylase I & II	*N. tabacum*	Accumulation of Zn in roots	Singla-Pareek et al., 2006
4.	Defensin *(AhPDF1)* from *A. halleri*	*A. thaliana*	Zn tolerance	Mirouze et al., 2006
5.	Mouse MT1	*Lycopersicon esculentum*	High Zn and antioxidant activity	Sheng et al., 2007
6.	*Arabidopsis* phytochelatin synthase gene	*Brassica juncea*	Accumulation of Zn and Cd	Gasic and Korban, 2007
7.	*BjCET2*	*Brassica juncea*	Tolerance and accumulation of Zn/Cd	Xu et al., 2009
8.	*OsIRT1*	*Oryza sativa*	Increased Fe and Zn accumulation in rice seeds	Lee and An, 2009
9.	*Zif1*	*Arabidopsis thaliana*	Increased Zn tolerance and accumulation	Hayden and Cobbett, 2007
10.	*HMA1*	*Arabidopsis thaliana*	Zn transport from chloroplast to cytoplasm	Kim et al., 2009
11.	*OsMT1a*	*Oryza sativa*	Zn homeostasis and drought tolerance	Yang et al., 2009

12.5 Conclusions

Contamination of heavy metals such as zinc and cadmium and its remediation using plants is a challenging problem, which requires efforts from researchers from different disciplines. For Zn biofortification, Zn has to be translocated from root to shoot and sequestered and redistributed in grain tissues. Although significant progress has been made in understanding the mechanisms involved

in Zn acquisition in plants, more work needs to be focussed in this area. Specifically for Zn biofortification, accumulation of toxic metals such as Cd, which share the same transporters as essential metals needs to be controlled. We still have to learn a lot on the plant ionome and the regulatory mechanisms. Combining powerful tools currently being used to study gene function with genetics, comparative genomics, high density 'omics' and integral mechanisms that undertake the coordinate regulation of ionome at cellular, organellar and tissue level will pave the way for a better understanding of Zn acquisition in plants.

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13

Environmental Nanotechnology: Nanoparticles for Bioremediation of Toxic Pollutants

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13.1 Introduction

The size dependent behaviour of any particle relates to some of its unique properties. This gave rise to rapidly growing field of Nanosciences. Nanotechnology has attracted considerable interest of both scientific and industrial community in the past few years. Nanotechnology inter-relates various research areas and applied sciences like physics, chemistry, biology, electronics and material sciences. It is often described as an emerging technology which is truly capable of revolutionizing our approaches to common problems. Nanotechnology involves the design, characterization, production and application of structures/particles by controlling their size and shape at nanoscale. An important challenge in nanotechnology is to engineer particles with desired optical and electronic properties by controlling their size and shape. This can be achieved by chemical processes and also by biological agents. Utilization of microorganisms for intracellular/extracellular synthesis of nanoparticles with different chemical composition, size, shapes and controlled monodispersity (of similar size and shape) can be a novel biological, economically viable and eco-friendly means for biosynthesis of nanoparticles. Nanotechnology holds promise in improving various aspects of life ranging from medicine to industrial materials. Also, Nanotechnology has much more to offer to environmental protection, reduction and clean-up of pollution, energy production and conservation. This chapter is an attempt to introduce the readers to the nanoworld and its use for bioremediation of toxic pollutants in

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the environment. This discussion also raises some of the important questions like what will be the fate of nanomaterials released in the environment and their consequent impact on the ecosystem.

13.2 What are Nanoparticles?

The word 'Nano' derived from the Greek word "nanos", which means dwarf or extremely small. The nanoscale (1 nm=10^{-9} m) is a billionth of a metre. Generally dimensions of nano particles ranges from 0.1-100 nm. A nanometre (nm) is one billionth of a metre, or roughly the length of three atoms kept side by side. A DNA molecule is 2.5 nm wide, a flu virus about 100 nm and human hair is approximately 10,000 nm thick (Fig. 13.1). A nanoparticle is a microscopic particle with at least one dimension less than 100 nm. Nano-sized particles have large surface areas relative to their volumes and thus have enhanced chemical and biological reactivity. Owing to their small dimensions nanoparticles can be tailored possessing novel properties for specific applications not exhibited by the particles of the same material at micro or macroscale. Nanostructures exist in various shapes and sizes commonly being the spheres, rods, wires and tubes.

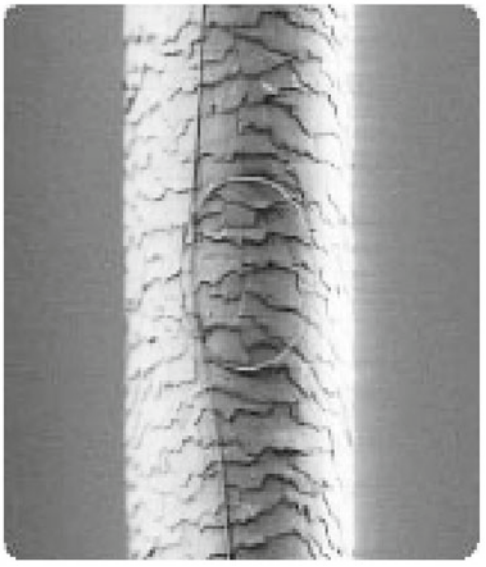

Fig. 13.1: Micrograph of looped nanowire against the backdrop of a human hair (Mazur Group, Harvard University, 2008).

13.3 Nanoparticles in Nature

Nanoparticles can be grouped into three major categories: natural, incidental and engineered. Naturally occurring nanomaterials such as volcanic ash, ocean spray, magnetosomes present in magnetotactic bacteria, mineral composites etc.

exist ubiquitously in our environment. Incidental nanoparticles, also known as anthropogenic nanoparticles, arise from industrial processes like diesel exhaust, coal combustion, welding fumes etc. The third category of nanoparticles is engineered nanoparticles, the real contribution of nanotechnology. Engineered nanoparticles are subclassified by the type of basic material and/or their use: metals, semiconductors, metal oxides, nanoclays, nanotubules, and quantum dots. Both natural and incidental nanoparticles may have irregular or regular shapes. Engineered nanoparticles most often have regular shapes, such as tubes, spheres, rings, etc. Table 13.1 summarizes the various categories of nanoparticles.

Table 13.1: Major groups of nanoparticles

Natural nanoparticles	*Incidental nanoparticles*	*Engineered nanoparticles*
Volcanic ash	Combustion products	Carbon nanotubes
Ocean spray	Frying, cooking	Quantum dots
Biogenic magnetite Magnetosomes in magnetotactic bacteria, meteorite dust	Sand blasting Mining Metal working Biomaterial degradation	Sunscreen pigments (Titanium dioxide nanoparticles for white coloration)
Forest fire smoke		Fullerenes
Mineral composites		Semiconductor wires
Ferritin (12.5 nm) lipoprotein particles (1-75 nm, plasma)		
Clouds		

Quantum dots are unique class of semiconductors having very small size, ranging from 2-10 nanometres (10-50 atoms) in diameter discovered by Louis E. Brus, a professor of chemistry at Columbia University. The term "Quantum dots" was coined by Mark A. Reed. These are a special class of materials known as semiconductors, which are generally composed of elements belonging to periodic groups II-VI, III-V or IV-VI. At these small sizes materials behave differently, giving quantum dots unprecedented tunability and enabling never before seen applications in science and technology. Within each category the shapes, sizes, and surface coatings further determine structure and function of these molecules.

The best example of use of natural and incidental nanoparticles is the *Kaajal* which the Indian women have been using as eye-liner without knowing that it is a product of advanced technology. Scientists at the Indian Institute of Technology (IIT), Kanpur have shown that *kaajal* actually contains Carbon Nanotubes (CNTs). These objects, much thinner than human hair, have strange properties and are considered the building block of ultra-small (nano) electronic and other devices. While researchers worldwide are trying to develop newer and

better methods for producing CNTs, the age-old practice of making *kaajal* by burning mustard oil is the simplest, fastest and the cheapest one. Synthesis of *kaajal* is well known and its use is even mentioned in epics like the *Ramayana* and the *Mahabharata*.

13.4 Nanoparticles and their Role in Bioremediation

Detoxification of a wide variety of common environmental contaminants, such as chlorinated organic solvents, organochlorine pesticides, poly-chlorinated hydrocarbons (PCH), toxic metal compounds using microorganisms and/or their products is known as bioremediation. Recently, the emerging field of nanotechnology has also contributed significantly in remediating these common soil and water pollutants in environment friendly manner. Some of noteworthy applications of nanomaterials in remediation of toxic environmental pollutants are highlighted below.

13.4.1 Degradation of PCHs

Polychlorinated hydrocarbons, class of organic compounds having several chlorine atoms attached to benzene rings are major recalcitrant environmental pollutants. PCHs/PCBs are widely used for many applications, especially as dielectric fluids in transformers, capacitors and coolants. Due to their inherent PCB's toxicity and classification as persistent organic pollutants, PCB production was banned by the United States Congress in 1976 and by the Stockholm Convention on Persistent Organic Pollutants in 2001.

Bimetallic nanoparticles have been used in place of granular zero-valent metal nanoparticles in environmental clean up of chlorinated hydrocarbon contaminants because of the following advantages:

(i) Small size enables the nanoparticles to penetrate or diffuse in contaminated area where micro sized particles fail to reach.
(ii) Nanosized particles have higher reactivity to redox-amenable contaminants.

Studies have shown that oxide-coated Fe^0 can form weak and outer sphere complexes with organic contaminants such as carbon tetrachloride (CTC). By electron transfer it can be broken down into methane, carbon monoxide or formate. Organic contaminants like benzoquinone, trichloroethene and several other chlorinated aliphatic hydrocarbons can be converted to compounds having less toxicity (Nurmi et al., 2005).

TiO_2 nanotubes have been used in labscale degradation of pentachlorophenol (PCP) through a photoelectrocatalytic reaction (Quan et al., 2005).

The use of nanoparticles as biocatalyst for reductive dechlorination of organic contaminants has also been demonstrated by De Windt et al., 2005. In one of the study palladium Pd(0) are deposited on the cell wall and inside the cytoplasm of *Shewanella oneidensis* and charged with H* radicals by using

various substrates such as hydrogen, acetate and formate as electron donors in a bioreductive assay containing Pd(II). When these charged Pd(0) bearing cells are brought in contact with chlorinated compounds, the H* radical on Pd(0) catalyse the dechlorination of the chlorinated compounds (De Windt et al., 2005).

13.4.2 Degradation of Hydrophobic Compounds

Nanoparticles are also being used to increase the bioavailability of hydrophobic organic compounds for their enhanced bioremediation. Polymeric nanoparticles prepared from a Poly(ethylene) glycol- Modified Urethane Acrylite (PMUA) precursor was applied to enhance the availability of polynuclear aromatic hydrocarbons (PAHs) in soil and aqueous solutions. Due to the hydrophobicity of interior regions of PMUA there is increased affinity between PAHs and outer hydrophilic surfaces of PMUA. This promotes particle mobility; increases amount of PAHs released into the aqueous phase and enhances the rate of mineralization (Tungittiplakorn et al., 2004, 2005). Subsequently the released PAHs can be treated by natural attenuation or pump-and-treat process in which polymeric nanoparticles can be recovered and recycled after microbial degradation of PAHs.

Further nanoparticles can also be used to immobilize bacterial cells which are capable of degrading specific toxic compound or to biorecover certain compounds. In one of the study, magnetic nanoparticles (Fe_3O_4) were functionalized with ammonium oleate and coated on the surface of *Pseudomonas delafieldii*. On application of external magnetic field to the microbial cells, the nanoparticle coated cells concentrate on particular site of the reactor wall separating them from the whole solution and enabling recycling of the cells for the treatment of the same compound. These coated cells were applied for the desulfurization of organic sulfur from the fossil fuel (i.e., dibenzothiophene) in a bioreactor and were observed to be as efficient as the non-nanoparticle coated microbial cells (Shan et al., 2005).

13.5 Nanoparticles for Water Purification

Progress in nanoscale sciences may provide solution to many of the current problems involving water quality. The use of nanosorbents, nanocatalysts, bioactive nanoparticles, nanostructured catalytic membranes and nanoparticle enhanced filtration products and processes resulting from the development of nanotechnology would greatly help to get potable drinking water. Innovations in the development of novel technologies to desalinate water are among the most exciting and promising. The development of novel nanoscale materials and processes for treatment of surface water, ground water and industrial wastewater contaminated by toxic metal ions, radionuclides, organic and inorganic solutes like pesticides, bacteria and viruses would be the major environmental contribution of nanotechnology.

Recent studies prove that many of the issues involving potable water quality could be resolved using nanoparticles, nanofiltration or other nanomaterials. Innovative use of nanoparticles for treatment of industrial wastewater is another potentially useful application of nanomaterials as many industries generate large amounts of wastewater contaminated with toxic and non-biodegradable effluents. Removal of contaminants and recycling of the contaminated water would provide significant reductions in cost, time, and labour for the industries and increase their eco-friendliness.

Aquifer and groundwater remediation are also critical issues, becoming more important as potable water supply is steadily decreasing. Most of the remediation technologies available at present, while being effective, very often are costly and time consuming, particularly pump-and-treat methods. The ability to remove toxic compounds from subsurface and other environments are very difficult to access in situ, and doing so rapidly, efficiently and within reasonable costs is the ultimate goal of nanotechnology. Some of the nanomaterials used for water purification are discussed in the following paragraphs.

Nanosorbents

Nanomaterials have two important key properties that make them effective sorbents:

(i) Due to their small size nanoparticles have larger surface areas.
(ii) These can also be functionalized with various chemical groups to increase their affinity towards the target compound/compounds.

Efforts are being made by several research groups to exploit the unique properties of nanoparticles to develop high capacity and selective sorbents for metal ions and anions which are common water contaminants. Li et al. (2003) have reported the sorption of Pb(II), Cu(II) and Cd(II) onto multiwalled carbon nanotubes (MWCNTs). The maximum sorption capacities of 97.08 mg/g for Pb(II), 24.49 mg/g for Cu(II) and 10.86 mg/g for Cd(II) at room temperature, pH 5.0 and metal ion equilibrium concentration of 10 mg/l. It was also found that the metal-ion sorption capacities of the MWCNTs was 3–4 times higher than that of powder activated carbon and granular activated carbon which are two commonly used sorbents in water purification. Qi & Xu (2004) have evaluated the sorption of Pb(II) onto chitosan nanoparticles (40–100 nm) prepared by ionic gelation of chitosan and tripolyphosphate. The phosphate-functionalized chitosan nanoparticles have a maximum Pb(II) sorption capacity of 398 mg/g. Peng et al. (2005) have recently developed a novel sorbent with high surface area (189 m^2/g) consisting of cerium oxide supported on carbon nanotubes (CeO_2-CNTs). It was observed that the CeO_2-CNT particles are effective sorbents for As(V). As(V) is very toxic metalloid and proven carcinogen found in the ground waters of some districts (Midnapur, 24-Parganas etc.) of West Bengal, India and also in Bangladesh. Interestingly, Peng et al. (2005) found that the addition (from 0 to 10 mg/l) of two divalent cations [Ca(II) and Mg(II)]

resulted in a substantial increase of the amount of sorbed As(V) from 10 to 82 mg/g. Deliyanni et al. (2003) have also synthesized and characterized a novel As(V) sorbent consisting of akaganeite [β-FeO(OH)] nanocrystals. In addition, Lazaridis et al. (2005) have shown that nanocrystalline akaganeite is also an effective sorbent for Cr(VI).

Zeolites are effective sorbents and ion-exchange media for metal ions. NaP1 zeolites ($Na_6Al_6Si_{10}O_{32}$, $12H_2O$) have a high density of Na ion exchange sites. These can be inexpensively synthesized by hydrothermal activation of fly ash with low Si/Al ratio at 150°C in 1.0–2.0 M NaOH solutions (Moreno et al., 2001). NaP1 zeolites have been used as ion exchange media for the removal of heavy metals from acid mine wastewaters (Moreno et al., 2001). Alvarez-Ayuso et al. (2003) reported the successful use of synthetic NaP1 zeolites to remove Cr(III), Ni(II), Zn(II), Cu(II) and Cd(II) from metal electroplating wastewaters. Self-assembled monolayers on mesoporous supports (SAMMS) made via surfactant templated synthesis of mesoporous ceramics are more effective sorbents for toxic metal ions (Yantasee et al., 2003), anions (Kelly et al., 2001) and radionuclides (Fryxell et al., 2005). This produces nanoporous ceramic oxides with very large surface areas (1000 m^2/g) and high density of sorption sites that can be functionalized to increase their selectivity toward target pollutants.

Carbonaceous nanomaterials have been proved as high capacity and selective sorbents for organic solutes in aqueous solutions. Mangun and coworkers (2001) have synthesized nanoporous activated carbon fibres (ACFs) with an average pore-size of 1.16 nm and surface areas ranging from 171 to 483 m^2/g. The sorption of benzene, toluene, p-xylene and ethylbenzene onto the ACFs at 20°C was quite effective. Peng et al. (2003) have evaluated the sorption of 1, 2-dichlorobenzene (DCB) onto CNTs. They reported that it takes only 40 min for DCB sorption onto the CNTs to reach equilibrium with a maximum sorption capacity of 30.8 mg/g. Li et al. (2004) observed that MWCNTs were better sorbents of volatile organic compounds than carbon black in aqueous solutions. Fugetsu et al. (2004) have successfully encapsulated MWCNTs inside cross-linked alginate vesicles. The caged MWCNTs showed high sorption capacity and selectivity for four water-soluble dyes (acridine orange, ethidium bromide, eosin bluish and orange G). Zhao and Nagy (2004) have synthesized hybrid inorganic-organic nanosorbents by incorporation of sodium dodecyl sulfate (SDS) into magnesium-aluminum layered double hydroxides (LDHs). They reported that the SDS functionalized Mg/Al LDHs had higher sorption capacity for chlorinated alkenes [tetrachloroethylene (PCE) and trichloroethylene (TCE)] in aqueous solutions than organoclays. Fullerenes can also serve as sorbents for polycyclic aromatic compounds (PAHs) such as naphthalene (Cheng et al., 2004). The amphiphilic polyurethane nanoparticles can also sorb PAHs (e.g., naphthalene) and increase their bioavailability in aqueous solutions (Tungittiplakorn et al., 2004, 2005).

13.6 Nanocatalysts and Redox Active Nanoparticles

Nanoparticles have great potential as water-purification catalysts and redox active media. This feature is due to their large surface areas and their size and shape dependent optical, electronic and catalytic properties. During the past few years, titanium dioxide (TiO_2) nanoparticles have emerged as promising photocatalysts for water purification (Adesina, 2004). They can serve both as oxidative and reductive catalysts for organic and inorganic pollutants. Chitose et al. (2003) reported enhanced removal of total organic carbon from waters contaminated with organic wastes by the addition of TiO_2 nanoparticles in the presence of ultraviolet light. Kabra et al. (2004) documented the successful use of TiO_2 nanoparticles to (i) degrade organic compounds (e.g. chlorinated alkanes and benzenes, dioxins, furans, PCBs, etc.) and (ii) to reduce toxic metal ions [e.g., Cr(VI), Ag(I) and Pt(II)] in aqueous solutions under UV light. One of the most cited studies by Asahi et al. (2001) in which they reported the synthesis of N-doped TiO_2 nanoparticles capable of photodegrading methylene blue under visible light. Bae and Choi (2003) have synthesized visible light-activated TiO_2 nanoparticles based on TiO_2 modified by ruthenium-complex sensitizers and Pt deposits. The $Pt/TiO_2/RuIIL_3$ nanoparticles drastically enhanced the rate of reductive dehalogenation of trichloroacetate and carbon tetrachloride in aqueous solutions under visible light.

Nanoscale zero valent iron (Fe^0) and bimetallic Fe^0 particles are also effective redox media for the detoxification of organic and inorganic pollutants in aqueous solutions. These nanomaterials have larger surface areas and reactivity than bulk Fe^0 particles (Schrick et al., 2002; Zhang, 2003; Nurmi et al., 2005). Zhang (2003) has reported the synthesis, characterization and use of nanoscale Fe^0 particle and Fe^0/Pd^0, Fe^0/Pt^0, Fe^0/Ag^0, Fe^0/Ni^0 and Fe^0/Co^0 in environmental remediation. These nanoparticles can reduce various organic pollutants (e.g., chlorinated alkanes and alkenes, chlorinated benzenes, pesticides, organic dyes, nitro aromatics, PCBs) and inorganic anions (e.g., nitrates) in aqueous solutions to less toxic by-products. Fe^0 and bimetallic Fe^0 nanoparticles have been efficiently used to reduce redox active metal ions such as Cr(VI) to less toxic and mobile Cr(III) (Zhang, 2003). Immobilized metalloporphyrinogens in soil-gel matrices have also been effectively used to prepare redox and catalytically active nanoparticles for the reductive dehalogenation of chlorinated organic compounds (PCE, TCE and carbon tetrachloride) in aqueous solutions (Dror et al., 2005).

13.7 Nanostructured and Reactive Membranes

Ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO) are emerging as key components of advanced water purification and desalination technologies (US Bureau of Reclamation, 2003). Van der Bruggen and Vandercasteele (2003) have discussed the use of nanofiltration to remove cations, natural organic

matter, biological contaminants, organic pollutants, nitrates and arsenic from ground water and surface water. Favre-Reguillon et al. (2003) demonstrated that nanofiltration can be used to remove minute quantities of U(VI) from seawater. Mohsen et al. (2003) have used nanofiltration to desalinate water and found that nanofiltration in combination with reverse osmosis could effectively render brackish water potable. Srivastava et al. (2004) reported the successful use of carbon nanotube filters which were effective in removing bacteria (*Escherichia coli* and *Staphylococus aureus*) and *Poliovirus sabin 1* from contaminated water. The carbon nanotube filters can be easily cleaned by ultrasonication and autoclaving. DeFriend et al. (2003) reported the successful fabrication of alumina UF membranes using alumina (A-alumoxanes) nanoparticles (7–25 nm). The new UF membranes, which have molecular weight cut-off (MWCO) between 1000 and 10,000 Da and average pore diameter of 4 nm, showed selectivity toward a number of synthetic dyes (e.g., Direct Red 81, Direct Blue 71 and Direct Yellow 71). The selectivity and permeate flux through the UF membranes can be increased by doping the alumina nanoparticles with Fe, Mn and La. Stanton et al. (2003) have fabricated novel NF membranes by deposition of 4.5–5.0 layer pairs of poly(styrene sulfonate)/poly(allylamine hydrochloride) onto porous alumina. The new NF membranes exhibit high water flux, high retention of divalent cations [Ca(II) and Mg(II)] and (Cl/SO_4^{-2}) selectivity ratios up to 80. Meyer et al. (2004) successfully prepared reactive membranes by incorporation of bimetallic Fe^0/ Pt^0 nanoparticles into cellulose acetate films. The embedded metal domains of the membranes have an average diameter of 24 nm and were very effective in reducing TCE with ethane as the only observed by-product.

13.8 Bioactive Nanoparticles

Strong oxidants (e.g., chlorine) are usually applied as disinfectants for pathogens (e.g., bacteria and viruses) in water treatment. These compounds generate toxic disinfection byproducts such as trihalomethanes, haloacetic acids and aldehydes. Thus, alternative disinfectants are needed to comply with the Stage 1 Disinfection Byproduct Rule of the 1996 Safe Drinking Water Act Amendments (USEPA, 1998b). Disinfectants such as chlorine inactivate water-borne pathogens by (1) impairment of pathogen cellular function by destruction of major constituents (e.g., cell wall), (2) interference with the pathogen cellular metabolic processes, and (3) inhibition of pathogen growth by blockage of the synthesis of key cellular constituents (e.g., DNA, coenzymes and cell wall proteins). Nanomaterials are also providing unparalleled opportunities to develop chlorine-free biocides. Stoimenov et al. (2002) demonstrated that MgO nanoparticles are very effective biocides against Gram-positive and Gram-negative bacteria (*Escherichia coli* and *Bacillus megaterium*), as well as against spores of *Bacillus subtillus*. Studies by atomic force microscopy (AFM), transmission electron microscopy (TEM), and laser confocal microscopy

showed considerable changes in the integrity of the cell membranes when the nanoparticles interact with the bacteria, resulting in their death in most cases. Ag(I) and silver compounds have been used as antimicrobial compounds in various biomedical products and applications. Sondi and Salopek-Sondi (2004) have prepared stable Ag nanoparticles of narrow size distribution by reducing silver nitrate solutions with ascorbic acid in the presence of Daxad 19 (sodium salt of a high molecular weight naphthalene sulfonate formaldehyde condensate) as stabilizing agent. They found that Ag nanoparticles were effective biocides against *Escherichia coli*. Son et al. (2004) reported that cellulose acetate (CA) fibres with embedded Ag nanoparticles were effective against Gram-positive and Gram-negative bacteria including *Staphylococcus aureus, Escherichia coli, Klebsiella pneumonia* and *Pseudomonas aeruginosa.*

13.9 Dendrimer Enhanced Ultrafiltration

Advances in macromolecular chemistry such as the invention of dendritic polymers are providing effective UF processes for purification of water contaminated by toxic metal ions, radionuclides, organic and inorganic solutes, bacteria and viruses. The name dendrimer is derived from Greek words *dendron* meaning "tree" and *meros* meaning "part". Major difference between linear polymers and dendrimers is that a linear polymer consists of long chains of molecules, like coils, crisscrossing each other (Fig. 13.2). A dendrimer consists of molecular chains that branched out from a common centre, and there is no entanglement between each dendrimer molecules. Dendritic polymers, which

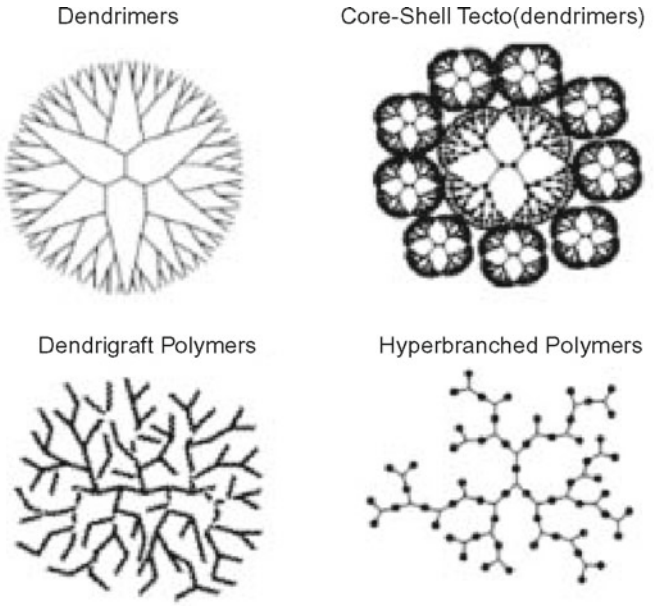

Fig. 13.2: Stucture of Dendrimers (Diallo et al., 2005).

include random hyperbranched polymers, dendrigraft polymers, dendrons and dendrimers, are relatively monodispersed and highly branched macromolecules with controlled composition and architecture consisting of three components: a core, interior branch cells and terminal branch cell (Fre´chet and Tomalia, 2001). Dendritic polymers have many features that make them attractive as functional materials for water purification. These 'soft' nanoparticles (1–20 nm) can be used as high capacity and recyclable watersoluble ligands for toxic metal ions, radionuclides and inorganic anions (Ottaviani et al., 2000; Birnbaum et al., 2003). Dendritic polymers are also reported to be used in (i) recyclable unimolecular micelles for recovering organic solutes from water (Arkas et al., 2003) and (ii) scaffolds and templates for the preparation of redox and catalytically active nanoparticles (Niu et al., 2005).

Dendrimer-enhanced ultrafiltration (DEUF) process for recovering metal ions from aqueous solutions was successfully developed by Diallo (2004). Diallo et al. (2005) tested the feasibility of using DEUF and poly (amidoamine) (PAMAM) dendrimers with ethylene diamine (EDA) core and terminal NH_2 groups to recover Cu(II) ions from aqueous solutions. On a mass basis, the Cu(II) binding capacities of the PAMAM dendrimers are much better and more sensitive to solution pH than those of linear polymers with amine groups. Separation of the dendrimer-Cu(II) complexes from solutions can be achieved simply by UF membranes with the appropriate MWCO. Used dendrimers can be regenerated by decreasing the solution pH to 4.0 (Diallo et al. 2005); thus enabling the recovery of the bound Cu(II) ions and recycling of the dendritic polymer. Advantage of PAMAM dendrimers is that they have low tendency to foul as compared to commercially available regenerated cellulose (RC) membranes (Diallo et al., 2005). Lesser operating pressure and energy consumption could be achieved with dendritic polymers in tangential/cross-flow UF systems typically used in water purification because of their smaller intrinsic viscosities than linear polymers with the same molar mass. For further details readers may refer to interesting reviews by Theron et al. (2008) and T. Pradeep and Anshup (2009).

13.10 Limitation of Nanomaterials for Water Purification

A key bottleneck to the applications of nanotechnology to water purification will be the availability of suppliers that can provide large quantities of nanomaterials at economical cost. A recently completed study on the nanomaterials industry was done by Freedonia in 2005. These forecasts anticipate that most nanomaterials will be nanoscale versions of established products such as silica, titanium dioxide, clays, metal powders, polymers and chemicals (Freedonia, 2005). Larger quantities of carbon nanotubes, fullerenes and dendrimers will also be available as these nanomaterials become key components of electronic products, drug delivery systems, batteries, fuel cells, water purifiers etc.

The integration of nanomaterials into existing water purification systems is another key challenge for their efficient use in water purification. Membrane processes such as RO, NF and UF are becoming the 'standard' water purification technologies for public utilities and industry because they are flexible, scalable, modular and relatively easy to operate and maintain. The environmental fate and toxicity of a material are critical issues in materials selection and design for water purification. Not much is known about the environmental fate, transport and toxicity of nanomaterials. Clean water is essential to human health and is a critical feedstock in a variety of key industries including electronics, pharmaceuticals and food. There is rising demand of clean water due to the decrease in available supplies of freshwater as a result of (i) extended droughts, (ii) population growth, (iii) more stringent health-based regulations, and (iv) competing demands from users. Nanomaterials have a number of key physicochemical properties that make them particularly attractive as separation media for water purification and holds promise for their extensive use for water purification in future.

13.11 Biogenic Synthesis of Nanoparticles and Metal Detoxification

Microbes are emerging as highly efficient nanofactories for nanoparticle synthesis. Microorganisms can survive and grow even at high metal ion concentrations. Microorganisms play an important role in remediation of metals through reduction of metal ions. They are often exposed to extreme environmental conditions, forcing them to resort to specific defense mechanisms to combat the toxicity of foreign metal ions or metals present in the microenvironment. The toxicity of metal ions is reduced or eliminated by changing the redox state of the metal ions and/or precipitation of the metals intracellularly/extracellularly thus, forming the basis of the synthesis of nanoparticles.

13.12 Magnetotactic Bacteria—Nature's Marvel

The first peer-reviewed article on magnetotactic bacteria appeared in the year 1975 in *Science* by Richard Blakemore, a microbiologist at the Woods Hole Oceanographic Institution. Blakemore observed that these microorganisms were following the direction of Earth's magnetic field, thus coined the term "magnetotactic" bacteria. Interestingly, Alivisatos in 2001 reported the presence of inorganic crystals in magnetotactic bacteria of the size between 35 and 120 nm. These crystals were of iron compounds (magnetite or greigite) which help to align bacteria with the external magnetic field. In natural environments, this magnetotactic behaviour enables the bacteria to navigate with respect to the earth's magnetic field towards their ideal environment in the upper microaerobic sediments of ponds, streams, oceans.

Toxic metal compounds can be transformed by microorganisms very efficiently to nanoparticles in the process of their detoxification. In the following paragraphs biosynthesis of nanoparticles of As, Se, Te and CdS are discussed which are major soil and water pollutants.

13.13 Biogenesis of Arsenic Nanoparticles

Arsenic is very toxic metalloid as it targets ubiquitous enzyme reactions and affects nearly all organ systems. Arsenic is strongly associated with lung and skin cancers. Pigment changes and palmoplantar hyperkeratosis are characteristic effects of chronic arsenic exposure. Arsine gas causes a hemolytic syndrome.

Several microorganisms have been isolated from the arsenic rich environments which can detoxify toxic arsenic compounds but very few have been reported to produce arsenic nanostructures during this reduction process. In one study Ji-Hoon Lee et al. (2007) studied the production of an extensive extracellular network of filamentous, arsenic-sulfide (As-S) nanotubes (20–100 nm in diameter by ~30 μm in length) by the dissimilatory metal-reducing bacterium *Shewanella* sp. HN-41. The As-S nanotubes, formed by the reduction of As(V) and $S_2O_3^{2-}$, were initially amorphous As_2S_3 but evolved with increasing incubation time toward polycrystalline phases of the chalcogenide minerals realgar (AsS) and duranusite (As_4S). The As-S nanotubes behaved as metals and semiconductors in terms of their electrical and photoconductive properties. In another study it was found that under anaerobic conditions Fe(III)-reducing microorganisms can couple the reduction of solid phase Fe(III) hydroxides with the oxidation of organic carbon. Nutrients and trace metals, such as arsenic, associated with Fe(III) hydroxides may be mobilized through microbially-mediated surface reduction. Arsenic mobilization mechanisms was studied using a series of controlled microcosm experiments containing aggregated arsenic-bearing ferrihydrite nanoparticles and an Fe(III)-reducing microorganism, *Geobacter metallireducens*. The phase distribution of iron and arsenic was determined through filtration and ultracentrifugation techniques. Biotic activity resulted in changes in nanoparticle surface potential and caused deflocculation of nanoparticle aggregates. Arsenic mobilized over time in the biotic trials was found to be exclusively associated with the nanoparticles. As arsenic contamination of natural waters due to mobilization from mineral surfaces is a significant route of human arsenic exposure, improved understanding of the biologically-mediated mechanisms that partition arsenic between solid and solution phases is required for development of effective treatment and remediation strategies.

13.14 Biogenesis of Selenium and Tellurium Nanoparticles

Selenium is an essential trace element, used particularly in the glutathione peroxidase enzyme system which protects intracellular structures against

oxidative damage. Substantial toxicity can occur with excessive selenium consumption or exposure. Selenium, like arsenic, inactivates the sulphydral groups of amino acids. Chronic oral exposure of selenium results in disease called selenosis. Toxicity has been associated with a garlicky odour in the breath (caused by methylated selenium), fatigue, gastrointestinal disturbances, and transverse lines on the nails, hairloss, alopecia and peripheral neuropathy.

The potential use of microorganisms to biotransform metals has led to another new dimension of exploring the biological mechanisms towards generation of zero-valent elements, bi/multi elemental quantum dots, and metal-containing nanoparticles. The biogenesis of selenium nanomaterials was demonstrated by Oremland et al. (2004) and Baesman et al. (2007) who reported effectively reduction of two chalcogenide oxyanion species viz., selenite/nate and tellurite/rate, to elemental selenium and tellurium by two anaerobic bacteria, *Bacillus selenireducens* and *Sulfurospirillum barnesii*. In the case of selenium, extracellular granules formed consisting of stable, uniform nanospheres (diameter ~300 nm) of Se^0 and having monoclinic crystalline structures. In the case of tellurium, *B. selenireducens* formed initially as nanorods (~10 nm) that cluster together forming larger rosettes (~1000 nm) composing of numerous individual shards. In contrast, *S. barnesii* forms extremely irregular shaped nanospheres (diameter <50 nm) that coalesce into large composite aggregates. The microbial synthesis of Se^0 nanospheres results in unique, complex, compacted nanostructural arrangements of Se atoms. These arrangements probably reflect a diversity of enzymes involved in the dissimilatory reduction that are ingeniously different in different microbes. Remarkably, these conditions cannot be achieved by current methods of chemical synthesis reported in the literature.

13.15 Biogenesis of CdS Nanoparticles

Chronic cadmium exposure primarily affects the kidneys and secondarily the bones. Acute inhalation of fumes containing cadmium affects the lungs. The well known "Itai-itai" or ouch-ouch disease due to cadmium was first described in post-menopausal Japanese women exposed to excessive levels of cadmium over their lifetimes. These women were exposed through their diet because the region of Japan in which they resided was contaminated with cadmium (Watanabe et al., 2000). Symptoms and signs of "itai-itai" disease are severe osteoporosis and osteomalacia with simultaneous severe renal dysfunction, normochromic anemia and low blood pressure sometimes also occur (Alfven et al., 2002), and average urinary cadmium level in these patients is 20-30 μg/g-creatinine of cadmium in urine (Ezaki et al., 2003).

Only a few microorganisms have been reported to synthesize cadmium nanoparticles. Among the first reports of intracellular semiconductor nanoparticle synthesis, Sweeney et al. demonstrated that *Escherichia coli*, when incubated with cadmium chloride ($CdCl_2$) and sodium sulfide (Na_2S), spontaneously formed cadmium sulfide (CdS) semiconductor nanocrystals. They showed

that the formation of nanocrystals was markedly affected by physiologic parameters. Indeed, the entry into stationary phase increased the yield by 20-fold. Cunningham and Lundie found that *Clostridium thermoaceticum* precipitates CdS at the cell surface as well as in the medium when exposed to $CdCl_2$ in the presence of cysteine hydrochloride as a source of sulfide in the growth medium. *Rhodopseudomonas palustris*, a photosynthetic bacteria, forms cadmium sulfide nanoparticles of an average size of 8.01 ± 0.25 nm. The cadmium sulfate solution incubated with *R. palustris* biomass changed to a yellow colour from 48 h onward, indicating the formation of cadmium sulfide nanoparticles.

Electron diffraction pattern confirmed the face-centered cubic (fcc) crystalline structure of cadmium sulfide. Furthermore, it was observed that the cysteine desulfhydrase producing S^{2-} in the *R. palustris* was located in cytoplasm, and the content of cysteine desulfhydrase depending on the growth phase of cells was responsible for the formation of CdS nanocrystal, while protein secreted by the *R. palustris* stabilized the cadmium sulfide nanoparticles. In addition, *R. palustris* was able to efficiently transport CdS nanoparticles out of the cell (Bai et al., 2009). The yeasts *Schizosaccharomyces pombe* and *Candida glabrata* were successfully cultured in a fed-batch process at cadmium levels up to 100 mg l^{-1}. *S. pombe* incorporated 20 mg Cd g^{-1} dry biomass within 24 h. *Candida glabrata* accumulated 8 mg Cd g^{-1} dry biomass in 24 h. The higher Cd uptake from *S. pombe* cells correlate with the elevated glucose concentrations during and at the end of the cultivation. Analysis of the cells with energy-filtering transmission electron microscopy-element specific imaging (EFTEM-ESI) revealed that cadmium is not precipitated outside the cells or at the cell wall but evenly distributed inside the cell plasma. Cd is immobilized by an intracellular detoxification mechanism. Size exclusion chromatography showed that Cd is associated to a protein fraction between 25 and 67 kDa which corresponds to the theoretical molecular weight of CdS nanoparticles of 35 kDa coated with phytochelatins.

13.15.1 Pt Nanoparticles

Platinum as a metal is not very dangerous, but platinum salts can cause several health effects, such as DNA alterations, cancer, allergic reactions of the skin and the mucous membrane, damage to organs such as intestines, kidneys and bone marrow and hearing damage. A danger of platinum is that it can cause potentiation of the toxicity of other toxic chemicals in the human body, such as selenium.

Using green chemistry approach metal ion-reducing bacterium *Shewanella algae* was used to form platinum nanoparticles. Resting cells of *S. algae* in presence of lactate as electron donor were able to reduce aqueous $PtCl_6^{2-}$ ions into elemental platinum at room temperature and neutral pH within 60 minutes. Biogenic platinum nanoparticles of about 5 nm were formed and found to be located in the periplasm—a preferable, cell surface location for easy recovery of biogenic nanoparticles (Konishi et al., 2007).

A consortium of sulphate-reducing bacteria was used to study the enzymatic mechanism for the total bioreduction of platinum(IV) into platinum(0) nanoparticles. It was established that two different hydrogenase enzymes were involved. First the platinum(IV) was reduced to platinum(II) by a two-electron bioreduction using an oxygen-sensitive novel cytoplasmic hydrogenase. Second the platinum(II) ion was reduced to platinum(0) nanoparticle by another two-electron bioreduction involving an oxygen-tolerant/protected periplasmic hydrogenase. No exogenous electron donors were necessary as endogenous production of hydrogen/electrons, via the oxidation of metabolites, was generated in situ by the cytoplasmic hydrogenase (Riddin et al., 2009).

13.15.2 Other Metallic Nanoparticles e.g. Gold, Silver

Pure metallic (elemental) gold and silver are non-toxic and non-irritating when ingested. Soluble compounds (gold salts) such as gold chloride are toxic to the liver and kidneys. Common cyanide salts of gold such as potassium gold cyanide, used in gold electroplating, are toxic both by virtue of their cyanide and gold content. In recent years the syntheses of gold nanoparticles have been the focus of intense interest because of their emerging applications in a number of areas such as bioimaging, biosensors, biolabels, and biomedicines. Numerous microorganisms summarized in Table 13.2 are reported to biosynthesize gold and silver nanoparticles by NADPH-dependent reductase enzymes that reduce metal salts to nanoparticles through electron shuttle enzymatic metal reduction process (Kaushik et al., 2010).

Table 13.2: Biogenic synthesis of gold and silver nanoparticles by various microorganisms

Microbe	*Location*	*Size range (nm)*
Silver (Ag) nanoparticles		
Bacteria		
Pseudomonas stutzeri	Intracellular	~ 200
Morganella sp.	Extracellular	20-30
Lactobacillus strains	Intracellular	-
Plectonema boryanum (Cyanobacteria)	Intracellular	1-10, 1-100
Klebsiella pneumonia	Extracellular	5-32
Stenotrophomonas maltophila		
Yeast		
MKY3	Extracellular	2-5
Fungi		
Phoma sp. 3.2883	Extracellular	71.06-74.46
Verticillium	Intracellular	25±12

(*Contd.*)

Aspergillus fumigates	Extracellular	5-25
Trichoderma asperellum	Extracellular	13-18
Phaenerochaete chrysosoporium	Extracellular	50-200
Gold (Au) Nanoparticles		
Bacteria		
Lactobacillus strains	Intracellular	-
Shewanella alga	Intracellular, pH 7	10-20
	Extracellular, pH 1	1-50
Escherichia coli DH5α	Intracellular	25-33
Thermomonospora sp.	Extracellular	8
Rhodococcus sp.	Intracellular	5-15
Pseudomonas aeruginosa	Extracellular	15-30
Fungi		
Fusarium oxysporum	Extracellular	20-40
Algae		
Sargassum wightii	Extracellular	8-12
Chlorella vulgaris	-	9-20

Adapted with some modification from Thakkar et al., 2009.

13.16 Case Studies

Nanomaterials have been applied to a number of contaminated sites with some preliminary success. These sites discussed below are contaminated with hazardous chlorinated hydrocarbons, hydrophobic recalcitrant compounds which are among the major environmental pollutants. Nanomaterials have also been used to remediate contaminated groundwater. The use of metallic nanomaterials as remediating agent is based on the redox (oxidation-reduction) reaction concept in which a neutral electron donor (usually a metal) reduces an electron acceptor (contaminant). This property of metals leads to their potential use as reducing agents for site remediation.

13.16.1 Nease Chemical Site, Ohio

The Nease Chemical Superfund site is 44 acres land, two and a half miles northwest of Salem on the Columbiana-Mahoning county line. From 1961 to 1973, Nease Chemical produced various household cleaning compounds, fire retardants and pesticides—some of which included an uncommon chemical called mirex which is used to control fire ants and as flame retardant in plastic, rubber, paints, paper and electric goods. It was banned in US in 1978. Mirex

degrades very slowly and remains in the environment for years. The company used unlined ponds to treat waste from its manufacturing process. Hazardous substances seeped into the soil and ground water from these ponds. Here the soil is contaminated with mirex and ground water is contaminated by a group of chemicals called volatile organic compounds (VOCs). Surface water runoff from the waste treatment ponds flowed into nearby Feeder Creek tributaries that run through the site causing pollution in the Middle Fork of Little Beaver Creek. The remediation plan and site description specifies the use of NZVI (Nano-ZeroValent Iron) technology employing palladium-coated colloidal iron. The reactive medium was administered through injection wells reaching deep ground water containing a plume of volatile organic compounds (VOCs).

13.16.2 RCRA Site, Alaska

NZVI was also used for remediation purposes in an Alaskan facility regulated under EPA's Resource Conservation and Recovery Act (RCRA) programme. The site contaminants include VOCs constituents of diesel fuel, which was released during an earlier tanker spill, in addition to perchloroethylene (PCE) and trichloroethylene (TCE). Studies indicate that ZVI (Fig. 13.3) may effectively reduce the chlorinated compounds in groundwater plumes as well as soil. Field-scale tests were conducted in both shallow and deep treatment zones. If field-scale tests yield the anticipated positive results, the project will be expanded to full-scale as was reported in 2006.

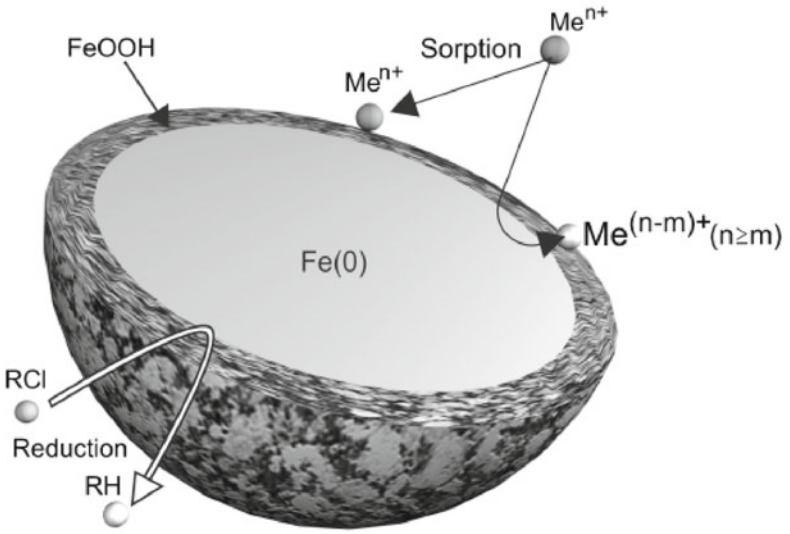

Fig. 13.3: The core shell model of Zero-Valent Iron (nZVI) nanoparticle. The core consists of mainly metallic iron and the shell consists of iron oxides and hydroxides.

13.16.3 Manufacturing Plant, Trenton, New Jersey

The site of a manufacturing plant in Trenton, New Jersey, was selected for testing of emulsified zero-valent iron (EZVI) technology to decontaminate plume that had migrated downgradient from the facility. The soil and ground water at this site was contaminated with PCE (Perchloroethylene), TCE (Trichloroethylene), c-DCE (cis-1,2-dichloroethene), vinyl chloride, chloroform, carbon tetrachloride, and 1,1-DCE. The test employed ZVI in the form of nanoscale particles suspended in a hydrophobic fluid, thus providing micelles to act as a means of transporting NZVI to non-aqueous phase liquid (NAPL). The micelle membranes are bound by a surfactant that allows cell transport through the groundwater plume (Fig. 13.4a). EZVI was injected in a water-based slurry through an injection well ("DGC-15") which also served as a monitoring well. Three multi-depth monitoring wells, or piezometers (PZ-1, PZ-2, and PZ-3) also were installed (Fig. 13.4b). After the injection process, researchers noted significant trichloroethylene TCE reductions in ground water of the second monitoring well but little concentration change in the third. According to *Environmental Science & Technology* journal article, the primary purpose of this field study was to obtain NZVI "proof of concept," i.e., evidence that ZVI administered in the slurry was nanoscale in dimension. The following redox reaction was provided by the authors to show how BNP are manufactured on the nanoscale through the process of reductive deposition:

$$Fe^0 + Pd^{2+} \rightarrow Fe^{2+} + Pd^0$$

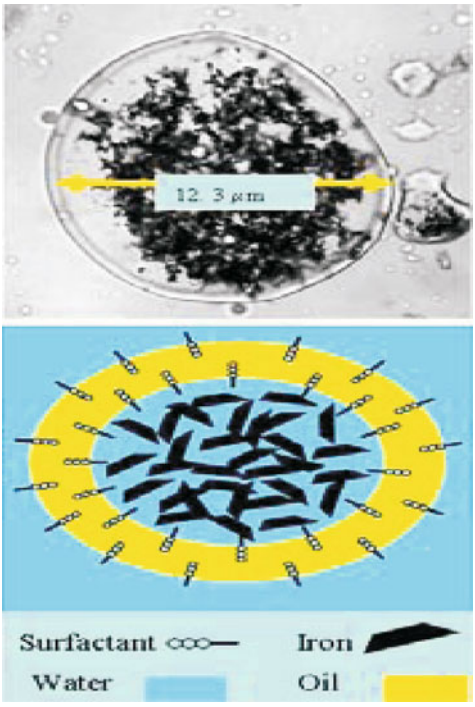

Fig. 13.4a: EZVI agglomerated particle.

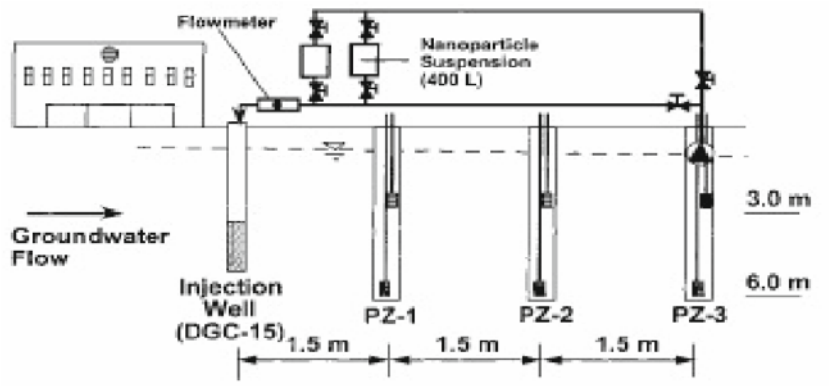

Fig. 13.4b: Injection setup at Trenton manufacturing plant.

Reduction of a chlorinated compound can then proceed via the following reaction:

$$C_2HCl_3 + 4Fe^0 + 5H^+ \rightarrow C_2H_6 + 4Fe^{2+} + 3Cl$$

The success of the operation was revealed when up to 96% reductions in TCE concentrations were observed.

13.16.4 Klockner Road Site, Hamilton Township, New Jersey

The area of contamination covers approximately four acres of uncultivated and vacant property historically used as a disposal area for various industrial wastes. Geologically, the site exhibits a perched water aquifer 2-8 feet below ground surface and a bedrock formation 130-160 feet. The major contaminants in ground water of this site were TCE, DCE, TCA and DCA with concentration as high as 400-1600 ppb. Administration of ZVI reactive agent Nanoiron slurry (NanoFe Plus) was performed through a 20-point injection system on a square grid. Reduction in dissolved chlorinated contaminants up to 90% was observed. The operation was carried out in two phases: Phase I of 20 days and Phase II of 10 days. Chemical reduction of contaminants occurred through the following reactions:

$$3Fe^0 + 3Fe^{2+} + 6e^- \rightarrow CCl_3CH_3 + 6e^- + 3H^+ C_2H_6 + 3Cl$$

$$Fe^0 + 2H_2O \rightarrow Fe^{2+} + H_2 + 2OH$$

$$CCl_3CH_3 + 3H_2 \rightarrow C_2H_6 + 3HCl$$

13.16.5 NASA Launch Complex 34, Cape Canaveral, Florida

This site was famous for its use as a launch pad for shuttle craft and other space-bound vehicles using rocket propulsion in the 1960s. The use of rocket fuel resulted in contamination of site's ground water, soil, and sediment with chlorinated compounds such as TCE. Three methods were employed for administering NZVI as alternatives to the direct-injection method to remediate

the site: pressure pulsing, pneumatic fracturing, and hydraulic fracturing. First, pressure pulsing involved forced administration of NZVI particles to various and relatively predictable subsurface depths. Second, pneumatic fracturing employs compressed air to create subsurface crevices and small pathways that facilitate distribution of the reactive medium. Similar to pneumatic fracturing, third method of hydraulic fracturing uses high-pressure liquids to enhance reagent distribution in the subsurface. These methods were expected to show reductions in groundwater contaminants.

13.17 Fate of Nanoparticles in the Environment and their Toxicity

Although nanoparticles have wide applications in various fields but what will be effect of these nanoparticles released in the environment is an important issue to be addressed. It is likely that the waste generated during nanaoparticle synthesis and their substantial use and subsequent release in the environment will also be of concern in the coming years. Exposure of humans to nanoparticles can be through inhalation, dermal adsorption and ingestion. Various studies have shown potential adverse effects of nanoparticles on human health and the environment (Auffan et al., 2009; Mortimer et al., 2010). In some studies the cytotoxicity of QDs (Quantum dots) of CdSe on different cell lines has been highlighted. It was found that cadmium hepatotoxicity (binding of thiol groups in mitochondria) correlates well with the release of free Cd^{2+} ions inside the cells due to the degradation of CdSe core. The degradation of the CdSe core is also affected by the chemistry of the surface modification of water soluble QDs. The studies further revealed that the extent of cell viability is influenced by oxidation, UV exposure time and dose of QDs (Derfus et al., 2004; Kirchner et al., 2005). It is minimized if QDs are properly coated with shell materials such as ZnS. Also nanoparticles can be easily absorbed on to the cell membrane and degraded leading to their cytotoxic effects. Nanowires and nanotubes are comparable to microneedles which can damage cell walls and thus impair cell growth and antimicrobial activity of silver nanoparticles against *E. coli*. These particles were shown to be an effective bactericide. Scanning and transmission electron microscopy (SEM and TEM) confirmed that the treated *E. coli* cells were damaged, showing formation of "pits" in the cell wall of the bacteria, while the silver nanoparticles were found to accumulate in the bacterial membrane. A membrane with such morphology exhibits a significant increase in permeability, resulting in death of the cell. These nanomaterials, which can be prepared in a simple and cost-effective manner, may be suitable for the formulation of new types of bactericidals (Sondi and Salopek-Sondi, 2004). Several types of nanoparticles have proven to induce oxidative stress, suggesting that particles of different types could initiate and aggravate inflammation and tissue damage leading to neurodegenerative and cardiovascular disease, carcinoma, and other disorders (Biswas et al., 2005; Wu et al., 2005; Scalbert et al., 2005).

The potential toxic effects of the nanoparticles in the natural environment and organisms which are still unknown will be revealed in the coming years. Thus, there is urgent need to understand the potential toxic effects of nanoparticles on human health and the other organisms before there is massive contamination of the environment by these nanoparticles. Extensive research is now needed to understand the fate and transport of free nanoparticles in the environment, the degree of the persistence and their toxicological effects on various biological system including human beings.

13.18 Conclusion and Future Trends

Nanoparticles exhibit unique properties in terms of photoemission, electrical conductivity, heat conductivity and catalytic activity. Owing to their unique properties these particles are being applied in different biological studies including medicine like biomolecule detection, separation, purification, concentration of sample, substrate coding, signal transduction, amplification, drug delivery in medicine to mention a few. Nanoparticles are also being widely applied in environmental research in degradation/transformation and recovery of toxic/nontoxic chemical compounds. Understanding potential environmental hazards of nanomaterials will require the ability to determine not only the chemical nature of the nanoparticles but also their size, shape, and number released in the environment. It is well known that chemicals can have different effects in plants and animals than in humans. The additive effect, or potential synergism, of exposure to different nanoparticles to human beings is also matter of investigation. As discussed earlier the immediate question of concern in the scientific world is whether we have understood the physicochemical properties of nanomaterials with the existing sophisticated instruments and techniques well enough to effectively use the materials for remediation purposes resulting in their subsequent release in the environment. This is an important question which will definitely find some answers in the coming years as we progress with nanotechnology. In conclusion, the field of nanotechnology is at an exciting stage of development. Timely understanding of the impacts with ongoing studies is essential before widespread adoption of nanotechnology in different fields particularly in bioremediation. The benefits of environmental nanotechnology are immense and with effective scientific studies, it can be ensured that the technology has minimal deleterious impacts on the environment and human beings.

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