T. Satyanarayana
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Anil Prakash *Editors*

Microorganisms in Environmental Management

Microbes and Environment

Springer

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Foreword

India has a large coastal line, huge interwoven riverine system and other water bodies with variegated mountains leading to complex and diverse ecological niches. The trajectory growth of industrialization, urbanization and steep increase in domestic effluent load and other anthropogenic activities have caused heavy environmental stress in India, which is being felt all across in the form of air, water and soil pollution. Pollution varies in its complexity, nature and longevity depending upon the source and location specificity.

For complete environmental management plan in 88 polluted industrial clusters, the Central Pollution Control Board, Delhi studied their comprehensive environmental pollution index and classified them into four groups: Very Critically Polluted, 33 Critically Polluted (CEPI 70–80), 32 Seriously Polluted (CEPI 60–70) and, 10 Warning Zone (CEPI 50–60), besides 80 hazardous contaminated sites. Considering the above concern, Ministry of Environment & Forests, Government of India has prioritized remediation of these contaminated sites. The sites can be remediated by employing *in situ* and *ex situ* technologies like co-processing of waste in cement, in power plant, chemical oxidation/stabilization treatment and containment of waste *in situ* and capping, excavation and transferring to Treatment, Storage & Disposal Facilities (TSDF) and other technologies.

Most of the technologies of decontamination are energy consuming and lead to some chemical residues, which further contaminate the environment. On the other hand, bioremediation is core eco-friendly and cost-effective technology with no residues.

For bioremediation, the tremendous potential of physiological adaptation among microbes in general and bacteria in particular enables them to dwell in various habitats ranging from saline to acidic and thermophilic to mesophilic. These properties enable microorganisms for profound bioremediation under extreme conditions as well. Our country with large territory and diverse climatic conditions possesses a variety of environmental stresses where diverse groups of organisms thrive because they contain biomolecules, materials and metabolites which help in their establishment and consequently can reduce the pollution load in soil, air and water systems.

The book deals with the abatement of pollution caused by metals, chemicals and biomedical waste, and bioremediation of compounds such as cyanide, petroleum, hydrocarbons and others, providing sound solutions by employing biotechnology.

I am very hopeful that the readers would be highly benefited with the in-depth discussions on various biotechnological applications of microorganisms in the abatement of environmental pollution.

S.P. GAUTAM
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Preface

Microorganisms and their biosynthetic capabilities have solved several difficult problems in the welfare of mankind. The domains in which microorganisms have helped mankind include medical technology, human and animal health, food processing, food safety and quality, genetic engineering, environmental protection, agricultural biotechnology and more effective treatment of agriculture, and municipal and industrial waste treatment, which provide a most impressive record of achievements. Most of these technological advancements would not have been possible using conventional straightforward chemical and physical engineering methods, or if they were, they would not have been practically or economically feasible and might have caused environmental pollution.

In recent years, microbial technologies have been applied to several environmental problems with considerable success. These technologies have not, however, always been widely accepted by the scientific community because of the difficulties in consistently reproducing their beneficial effects. Microorganisms are effective only when they are presented with suitable and optimum conditions for metabolizing their substrates including available water, oxygen, pH and temperature. Meanwhile, various types of microbial cultures and inoculants available in the market today have rapidly increased because of the emerging technologies. Significant developments have been made in systems where technical guidance is coordinated with the marketing of microbial products. Since microorganisms are useful in solving problems associated with the use of chemical fertilizers and pesticides, they are now widely applied in nature farming and organic agriculture.

Environmental pollution caused by excessive soil erosion and the associated transport of sediment, chemical fertilizers and pesticides to the surface and groundwater, and improper treatment of human and animal wastes has caused serious environmental and social problems throughout the world. Often attempts have been made to solve these problems using the established chemical and physical methods. It has, however, been realized that such problems cannot be solved without using microbial and other biological methods and technologies.

The present book is an attempt towards understanding the diversity of microbes in natural as well as man-made normal and extreme environments and utilizing

microbes to solve a great variety of environmental issues. In order to review the progress made in solving environmental pollution and other problems, we have contacted scientists who have contributed significantly in understanding these aspects. We wish to thank all the contributors who have agreed to write and have submitted reviews. We thank Mr. Ankit Kumar for providing background support. Thanks are also due to Springer for publishing the book.

T. Satyanarayana
B.N. Johri
Anil Prakash
(Editors)

About the Editors

After completing M.Sc. and Ph.D. at the University of Saugar (India), **T. Satyanarayana** had post-doctoral stints at the University of Bhopal and France. In 1988, he joined the Department of Microbiology, University of Delhi South Campus as Associate Professor and became Professor in 1998. His research efforts have been focused on understanding the diversity of yeasts, and thermophilic fungi and bacteria, their enzymes and potential applications, heterotrophic carbon sequestration and meta-genomics, and cloning and expression of yeast and bacterial genes encoding industrial enzymes. He has published over 160 scientific papers and reviews, and edited three books. He is a fellow of the National Academy of Agricultural Sciences, Association of Microbiologists of India, Mycological Society of India and Biotech Research Society of India, and a recipient of Dr. G.B. Manjrekar award of the Association of Microbiologists of India in 2003 and Dr. V.S. Agnihotrudu Memorial award of MSI in 2009 for his distinguished contributions. He is one of the Editors for Indian Journal of Microbiology and a member in the editorial board of Bioresource Technology and Indian Journal of Biotechnology. He has over 37 years of research and teaching experience.

Bhavdish N Johri obtained his doctorate degree from the University of Alberta, Edmonton and carried out Post-Doctoral Research at the University of British Columbia, Vancouver (Canada). He has teaching experience of microbiology of over 35 years and research experience of nearly 40 yrs. He was Chairperson, Department of Microbiology at G.B. Pant University of Agriculture & Technology (Pantnagar) for considerable period, and was Dean, College of Basic Sciences & Humanities. During this long tenure of academics, he has won several accolades such as INSA Young Scientist Award (1974), UGC Carrer Award (1980), KN Katju Award (1985) and the prestigious Rafi Ahmed Kidwai Award of ICAR (2005). He is a Fellow of National Academy of Sciences (FNASc) and National Academy of Agricultural Sciences (FNAAS). He is the Past President, Association of Microbiologists of India. He has to his credit three edited volumes in the domain of microbiology and over 100 refereed and other publications. Currently he is Acharya PC Ray Silver Jubilee Fellow of the Madhya Pradesh Council of Science & Technology. His major research interests encompass rhizosphere biology and microbial ecology.

Dr. Anil Prakash obtained his B. Sc., M. Sc., M. Phil. and Ph. D. from Barkatullah University, Bhopal. Presently he is working as Professor at the Department of Biotechnology and Coordinator of Bioinformatics Centre. He is fellow of Academy of Science, Engineering and Technology. During 25 years of research and teaching career, he has guided more than twenty Ph. D. students. He has completed a number of projects funded by UGC, ICAR, DBT (Govt. of India), and others. Dr. Anil Prakash is member of several Indian scientific bodies. He has published several research papers and articles on PGPR and mycorrhiza in national and international books and Journals, and published two books.

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Chapter 1
Halophiles – Taxonomy, Diversity, Physiology and Applications

P.P. Kanekar, S.P. Kanekar, A.S. Kelkar, and P.K. Dhakephalkar

Abstract Halophiles are salt-loving organisms inhabiting environments with the capacity to balance the osmotic pressure of the environment and resist the denaturing effects of salts such as salt induced co-aggregation of proteins. They are diverse group of organisms that thrive extreme saline environments. Depending upon their requirement for salt concentration, they are classified as halotolerant, moderately halophile and extreme halophile.

Halophiles have been routinely isolated from marine salterns and hypersaline lakes with 3.5–4.5 M (20–30 g% NaCl). Extremely halophilic archaea were characterized from saline environment in different parts of Turkey, "Solar de Atacama", Chile, two salt lakes in Xin-Jiang, China. The family Halobacteriaceae contains 96 species classified in 27 genera.

Mechanism of salt tolerance and halophily has been studied to some extent. The organisms devise protection against high concentration of Na^+. They are reported to produce certain biochemicals like glycine betaine which acts as compatible solute for high concentration of NaCl. The cell envelope and outer membrane shows modification in composition based on ionic strength of outer environment.

Extremophiles are being looked upon as a treasure of novel biomolecules, biomaterials and metabolites. Haloarchaeal enzymes are unusually stable and adapted to extreme environments thus are suitable candidates for applications in industrial processes that are performed under harsh conditions of high ionic strength. Bacteriorhodopsin, a photochemical produced by halophiles has a potential in conversion of light energy into chemical energy and electricity. Halophiles have wide range of biotechnological potential in industry e.g. biosurfactant production,

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biopolymers in oil recovery, proteases and amylases in detergent industry, poly-beta hydroxyalkanoate as biodegradable plastic, exopolysaccharide and bioremediation of contaminated hypersaline brines etc.

Keywords Haloarchaea • Halobacteria • Halotolerant • Extreme halophile • Membrane lipids • Bacteriorhodopsin • Ectoine • Extremozymes • Bioremediation of contaminated hypersaline environments • Biopolymers

1.1 Introduction

The beauty of the mother earth lies in diversity of life borne by her. Whether it is human being, animals, plants or tiny microorganisms, variations in them are just amazing. Geological activities create a variety of habitats that add to the richness of the earth. Some of such habitats exhibit themselves as very extreme environments which are far remote from the normal environments. By virtue of their adaptability, microorganisms thrive in such harsh environments e.g. saline water bodies, thermal springs, soda lakes, acid mine waters, etc. These special groups of microorganisms, popularly known as extremophiles, existed in the nature since long although known to man only recently. Based upon the type of environment they inhabit, they are classified as thermophiles (occurring in thermal springs, oil wells and growing optimally above 55°C); alkaliphiles (from alkaline environments like soda lakes, optimally growing above pH 9.0); halophiles (from saline environments e.g. saltern pans and ponds, soda lakes, deserts, hypersaline soils, salted foods, optimally growing above 2–30% NaCl), acidophiles (from acid mine water, optimally growing at pH 1.8–2.0) and many others like psychrophiles (cold loving), barophiles (growing at high atmospheric pressure), etc. Compared to alkaliphiles or thermophiles, halophiles have been paid little attention and less studied.

Halophiles are salt-loving organisms that inhabit hypersaline environments. They include mainly prokaryotic and eukaryotic microorganisms with the capacity to balance the osmotic pressure of the environment and resist the denaturing effects of salts such as salt induced co-aggregation of proteins. They inhabit salt ponds, soda lakes and even rock, salt crystals as dormant cells or as biopolymers. These prokaryotic organisms were likely evolutionary adaptations of more conventional bacterial forms rather than a more fundamental group or branch on the evolutionary tree (Woese 1993). Examples of well-adapted and widely distributed extremely halophilic microorganisms include archaeal *Halobacterium* species, which belong to the order Halobacteriales of the family Halobacteriaceae (Grant et al. 2001).

Halophiles can survive and flourish in environments that limit the growth of most other organisms. Halophilic archaea represent a considerable fraction of the prokaryotic world in marine and terrestrial ecosystems, indicating that organisms from this domain might have a large impact on global energy cycles. Their unusual properties make them a potentially valuable resource in the development of novel biotechnological processes and industrial applications.

Today, the halophilic archaea have come to be recognized as a domain of ancient organisms that evolved under the conditions of the primitive earth such as high salinity (Kates 1992). Moderately halophilic bacteria constitute a heterogeneous physiological group of microorganisms which belong to different genera.

They are diverse group of organisms that thrive extreme saline environments. Depending upon their requirement for salt concentration, they are classified as halotolerant (0–5% NaCl), slightly halophiles (2–5% NaCl), moderately halophile (5–20% NaCl) and extremely halophile (20–30% NaCl). Halotolerant organisms can grow in high salinity as well in the absence of a high concentration of salt.

1.2 Taxonomy and Diversity

Halophilic microorganisms have been isolated from a range of environments including aquatic habitats of both high- and low salinity, marshy places (especially salty ones), saline lakes, the Dead Sea and a few other places (Javor 1989; Oren 2002). A lot of these sites may be considered as representative of ancient environments. Similarly, microorganisms isolated from these sites may also be considered as representatives of the ancient microbial populations. Hence, there is an increasing awareness and interest in the microbial communities associated with hypersaline environments.

Hypersaline waters contain more than 10–12% salt. Brines derived from sea water contain salt concentrations of up to 35%, when precipitation is just about to set in, e.g. thalassohaline brines (Rodríguez-Valera 1993). Athalassohaline hypersaline waters, on the other hand, contain salts from the dissolution of continental minerals (Rodríguez-Valera 1993). Halophilic microorganisms from ancient salt sediments had been frequently reported in the literature as reviewed by Grant et al. (1998) and McGenity et al. (2000). *Halococcus salifodinae* was the first strain isolated from ancient rock salt (Permian salt sediment) that was classified up to species level (Denner et al. 1994). Several such reports of haloarchaea isolated from ancient rock salt have subsequently appeared in the literature (Gruber et al. 2004; Stan-Lotter et al. 1999, 2002; Vreeland et al. 2002). The microbial diversity of hypersaline ecosystems from various geographical regions has been reported in the literature. Some of the hypersaline ecosystems investigated for the diversity of halophiles include the Great Salt Lake, Utah, USA, the Dead Sea, the extremely alkaline brines of the Wadi Natrun, Egypt and lake Magadi, Kenya (Oren 1994; Kamekura 1998). Majority of these hypersaline ecosystems are characterized by the low taxonomic diversity.

A large numbers of different 16S rRNA gene sequences or fragments have been detected in the rock salt upon application of advanced molecular methods (Fish et al. 2002; Radax et al. 2001). These results indicated the presence of a sizable microbial community in rock salt. However, a majority of these gene sequences have not yet been identified with any of the bacterial isolates. It may be attributed to the fact that culturing extremely halophilic Archaea for obtaining colony forming units (CFUs) is often very time consuming. Extremely halophilic archaea isolated

from rock salts, crystalline ponds, etc. often take months to produce visible colonies (Gruber et al. 2004; Burns et al. 2004). However, classical culturing techniques to obtain colony forming units of extreme halophiles are of utmost importance from the point of view of taxonomy of these valuable microorganisms.

Isolation and identification of extremophiles demands special enrichment techniques, biochemical and physiological characterization and genotyping. Several researchers across the globe have described various methods that they used for isolation of halophiles from different saline environments. Bykova et al. (2000) have used SDS gel electrophoresis to investigate total cell proteins of halophilic and halotolerant eubacteria isolated from marine sediments and highly mineralized formation waters of oil fields. The genera *Dietzia, Rhodococcus, Staphylococcus, Cytophaga, Brevibacterium and Archangium* were found to form clearly distinguishable clusters on the dendrogram derived from electrophoretic protein patterns. Arahal et al. (2000) carried out a taxonomic study including a phenotypic and genotypic characterization of extremely halophilic aerobic archaeal strains isolated from Dead sea water samples. The archaeal diversity was represented by genera Haloarcula, Haloferax and Halobacterium. Eder et al. (2001) investigated microbial diversity of the brine seawater interface of the Kebrit deep, red sea using 16S rRNA gene sequencing and cultivation methods. Novel halophiles were isolated under strictly anaerobic culture conditions, which represented the genus Haloanaerobium. Extreme halophilic archaea were isolated from the "solar de Atacama", Chile by Lizama et al. (2002), extensively characterized and subjected to numerical analysis. The studies revealed high diversity among the halophilic archaea isolated – including some new taxa. Biomarker technique was used to screen bacteria that produce polyunsaturated fatty acids (PUFA) by Nichols et al. (2002). Investigations of Antarctic sea ice have revealed a high diversity of novel bacterial taxa that produce PUFA and were represented by psychrophilic and halophilic bacteria. The phylogeny of PUFA production in gamma-Proteobacteria suggests the future use of PUFA genes for the assessment of microbial bacterial biodiversity. Litchfield and Gillevet (2002) have done preliminary assessment of microbial diversity and complexity in hypersaline environments. The microbial communities in solar salterns and a soda lake were characterized using BIOLOG to estimate the metabolic potential and amplicon length heterogeneity analysis to determine molecular diversity of these communities. The studies revealed population of halophilic bacteria and archaea in the Eilat, Israel saltern. The investigations indicated metabolic diversity and phylogenetic complexity of the microbial communities. The authors pose need to develop more versatile media for the cultivation of the diversity of bacteria in hypersaline environments. Prokaryotic genetic diversity throughout the salinity gradient of a coastal solar saltern (4–37% salt) was investigated by Benlloch et al. (2002) using microbial ecology techniques e.g. 16S rRNA gene sequencing from both denaturing gradient gel electrophoresis (DGGE) and clone libraries and culturing methods. An extremely halophilic bacterium, *Salinibacter ruber* occurred in 32% salt pond. As salinity increases, the number of different clusters decreased.

The diversity of archaea in hypersaline environments e.g. a crystallization pond of a solar saltern in Spain, an alkaline lake in Nevada USA and a small pond from a slag

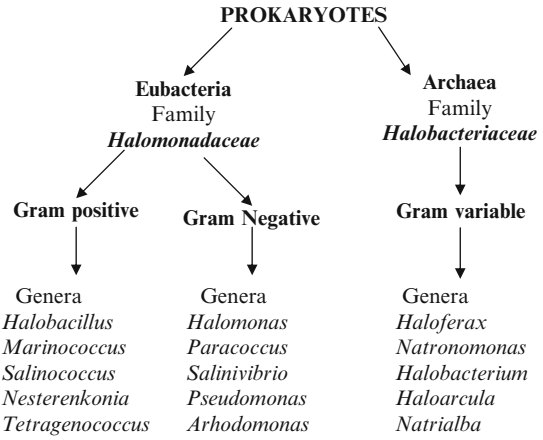

Fig. 1.1 Taxonomic grouping of halophiles

heap of a potassium mine in Germany, was investigated using (PCR) based molecular Phylogenetic techniques and cultivation methods by Ochsenreiter et al. (2002). It was found that halophilic archaea dominate the archaeal populations in these hypersaline environment. The organisms that were cultivated included *Natronococcus, Halorubrum, Natronomonas* and *Haloarcula*.

Mata et al. (2002) have made a detailed phenotypic characterization of the type strains of *Halomonas* species namely *H. eurihalina, H. halophila, H. maura* and *H. salina*. The authors used 234 morphological; physiological, biochemical, nutritional and antimicrobial susceptibility tests but suggest to minimize the tests required to arrive at reliable phenotypic characterization of *Halomonas* isolates and select most useful ones to differentiate *Halomonas* species from each other. Distribution of archaea in a black smoker chimney structures in microhabitats in a deep-sea hydrothermal vent from PACMANUS site in the Manus Basin near Papua New Guinea, was evaluated through the combined use of culture-independent molecular analyses and enrichment culture methods by Takai et al. (2001). The archaeal communities consisted of hyperthermophile members and extreme halophiles.

All these studies indicate growing interest of researchers in diversity of halophiles may be because they synthesize and accumulate biotechnologically important biomolecules and biomaterials.

The halophilic bacteria and archaea are classified according to the Bergey's Manual of Systematic Bacteriology, Second edition [Boone and Richard 2001 (Vol I) and Brenner et al. 2005 (Vol 2)] as presented in Fig. 1.1.

Some of the taxa are described below:

1.2.1 Genus Rhodospirillum

Rhodospirillum is classified under order Rhodospirillales and family Rhodospirillaceae. Members of the genus *Rhodospirillum* are Gram-negative, motile,

spiral-shaped bacteria. *Rhodospirillum* species are generally found in marine environments. Four halophilic species were classified together with several freshwater species of the genus *Rhodospirillum*. Optimum salt requirement for the three of these species namely *R. salinarum, R. sodomensis* and *R. mediosalina* is in the range of 8–12% while that for *R. trueperi* is 2%. The first three species have been isolated from saltern, salt lake and salty springs respectively whereas *R. trueperi* was isolated from marine sediments.

1.2.2 Genus Rhodovibrio

Rhodovibrio are spiral shape, Gram negative cells. They grow photoheterotrophically under anoxic conditions. Members of this genus usually inhabit anoxic zones of hypersaline environments such as salterns and salt lakes that are exposed to the light. *R. salinarum* is obligatory or moderately halophilic species that optimally grows at 42°C, pH 7.5–8 and 8–12% NaCl (range 3–24%). *R. sodomensis* members are obligately or moderately halophilic bacteria with optimum growth at 35–40°C, pH 7 and 12% NaCl (salt range 6–20% NaCl). *R. sodomensis* is extremely bromide tolerant, capable of growing well if 50–75% of NaCl is replaced by NaBr (1.5 M NaBr). Strains belonging to this species have been isolated from anoxic parts of sediments and water of the Dead Sea that are exposed to the light.

1.2.3 Genus Roseospira

Members of this genus are vibrioid to spiral shape, Gram negative and grow preferentially photoheterotrophically under anoxic conditions in the light. These are halophilic bacteria that require NaCl or sea salt for growth. Typical habitats for theses organisms include warm sulfur springs with elevated minerals salt concentrations. *R. mediosalina* is a halophilic species with optimum growth at 30–35°C, pH 7 and NaCl concentration of 4–7% (salt range: 0.5–15%). No growth is observed in absence of salt or temperature above 40°C. This species was first isolated from warm sulfur spring Astara (Azerbaijian) with elevated mineral salt concentration of 2%.

1.2.4 Genus Methylarcula

Members of this genus are Gram negative, aerobic and moderately halophilic strains that form white or pale pink colonies. NaCl is required for the growth of this organism. Ectoine is accumulated intra-cellularly as the main osmoprotectant in this organism. *M. marina* strain can tolerate up to 12% NaCl. These strains optimally grow at 29–35°C, pH 7.5–8.5, and 3–8% NaCl. The type strain of this species was

isolated from Azov sea estuary sea water. *M terricola* is able to grow at 10–4°C, pH 5.5–10, and in the presence of 0.05–14% NaCl (optimum 3–6% NaCl). The type strain of this species was isolated from costal salty soil of Black Sea.

1.2.5 Genus Rhodovulum

Cells are Gram negative, ovoid to rod shaped, photosynthetic that produce yellow – green to yellow – brown pigment when grown phototrophically and pink to red pigment under aerobic conditions. These are mesophilic marine bacteria that require sodium chloride for growth. They inhabit marine and hypersaline environments that are rich in organic matter, contain hydrogen sulfide and exposed to the light. Five of the six species of *Rhodovulum* namely, *R. sulfidophilum, R. adriaticum, R. euryhalinum, R. iodosum* and *R. robiginosum* grow optimally at NaCl concentrations of 5% or more. Only *R. strictum* optimally grow at 1% NaCl concentration. Typical habitats for the halophilic species of *Rhodovulum* include marine sediments, intertidal mud flats.

1.2.6 Genus Rhodothalassium

Members of this genus are Gram negative and vibrioid to spiral shape. These are obligatory halophilic bacteria that inhabit anoxic zones of hypersaline environments. *R. salexigens* is the only representative of this genus and is a moderately halophilic bacterium. It requires salt for growth and can tolerate up to 20% of the salt. Glycine betaine is accumulated as osmoticum and compatible solute by this organism in response to external elevated salt concentration.

1.2.7 Genus Dichotomicrobium

The genus *Dichotomicrobium* is classified under order *Rhizobiales* and family *Hyphomicrobiaceae*. These are aerobic, moderately thermophilic and halophilic microorganisms. They do not grow in anaerobic environment. They occur in saline ponds and lakes with temperature above 20°C. *D. thermohalophilum* grows optimally at 44–50°C, pH 8–8.5 and salinity of 8–14%. This organism can tolerate up to 22% NaCl. This organism is normally found in hypersaline, meriomictic and heliothermal solar lake.

1.2.8 Genus Rhodobium

Rhodobium is a member of family *Rhodobiaceae*. These are Gram negative ovoid to rod shaped bacteria. These are slightly halophilic bacteria that optimally grow in the

presence of 4–5% NaCl (range: 2–8%). No growth is observed in the absence of NaCl. The members of this genus are usually associated with tidal sea water pools and similar marine environments. Genus *Rhodobium* includes only two species namely, *R. orientis* and *R. marinum*, both species have similar salt requirements.

1.2.9 Genus Halothiobacillus

Halothiobacillus has been proposed as a new genus under gammaproteobacteria by Kelly and Wood (2000). They are now classified with purple sulfur bacteria. This genus is classified under order *Chromatiales* and family *Halothiobacillaceae.* All species of *Halothiobacillus* are obligate aerobic bacteria. They are halophilic and live in environments with high concentrations of salt.

1.2.10 Genus Nitrosomonas

Nitrosomonas has been classified under order *Nitrosomonadales* and family *Nitrosomonadaceae* which belong to class *Betaproteobacteria. Nitrosomonas* is a Gram negative spherical, ellipsoidal or rod shaped organism. Some species of *Nitrosomonas* are known to be halophilic or halotolerant in nature, e.g. *N. estuarii, N. marina, N. halophila* which are known to tolerate up to 8% NaCl. Common habitats for these species are brackish water environment, soda lakes and marine environment.

1.2.11 Genus Desulfovibrio

Cells of *Desulfovibrio* are Gram negative, curved or straight rods, sometimes sigmoid or spirilloid. *Type species* of *Desulfovibrio* is *D. desulfuricans* as described by Beijerinck. Strains of some species may show chemolithoheterotrophic growth, using H_2 as electron donor and assimilating acetate and CO_2. Common habitats of this genus include – anoxic mud of fresh and brackish water and marine environments; intestines of animals; manure and feces. Species of *Desulfovibrio* are known to tolerate 0.2–1% NaCl.

1.2.12 Genus Desulfohalobium

Species of genus *Desulfohalobium* are Gram negative, anaerobic and moderately halophilic. They can grow in presence of 6–24% NaCl and at 37–40°C. *Type strain of Desulfohalobium* is *D. retbaense* which can grow in the presence of 10% NaCl concentration. It is currently a member of the family *Desulfohalobiaceae* in the Class *Deltaproteobacteria.*

1.2.13 Genus Desulfonatronovibrio

Cells of *Desulfonatronovibrio* are Gram negative, vibrios, single or sigmoid in pairs and anaerobic. The genus currently includes only a single species i.e. *D. hydrogenovorans*. The species is *e*xtremely alkaliphilic, show no growth at pH 7 (optimal pH 9.0–9.7; maximum pH 10.2). The optimal NaCl concentration required is 3% (range 1–12%).

1.2.14 Genus Desulfonatronum

Cells of *Desulfonatronum* are vibrios, Gram negative, anaerobic and extremely alkaliphilic. No growth is observed at pH values below 8 (pH optimum, 9.5). The genus presently includes only a single species i.e. *D. lacustre* which can tolerate NaCl up to 10%, and is obligately dependent on sodium and carbonates.

1.2.15 Genus Desulfocella

Genus *Desulfocella* belongs to the family *Desulfobacteriaceae* within the class *Deltaproteobacteria*. Cells are Gram negative, vibrio-shaped and anaerobic. NaCl is required for growth of *Desulfocella*. The type species includes *D. halophila*, which is capable to tolerate up to 20% NaCl.

1.2.16 Archaea

Woese, Magrum, and Fox proposed "Archaebacteria" (Woese et al. 1978) as a group to include the methanogens, extreme halophiles, and thermoacidophiles on the basis of following common factors: (i) the possession of characteristic ribosomal RNAs and tRNAs; (ii) the absence of murein cell walls; and (iii) the presence of ether linked lipids in phytanyl chains.

1.2.17 Order Halobacteriales

Halophilic Archaea are found in hypersaline environments in which salt concentrations exceed 15–20 g%. Cells of the halophilic Archaea of the order *Halobacteriales* contain molar concentrations of ions, especially K^+ and Cl^- within the cells, required for unique adaptations of the enzymatic machinery to be able to function in the presence of high salt. These organisms contain retinal pigments, bacteriorhodopsin (outward proton pump) and halorhodopsin (inward chloride pump). Both proteins enable the direct use of light energy by the cells for energy transduction. The order *Halobacteriales*, at present contains single family, the *Halobacteriaceae* consisting 14 genera with 35 validly described species.

1.2.18 Genus Halobacterium

Members of this genus are Gram negative, rod shaped bacteria. They can tolerate up to 30% NaCl (optimum 23%). The type strain of genus *Halobacterium,* is *H. salinarum,* which has been isolated from salted cow hide. *Halobacterium* grows optimally at 35–50°C (optimum 44°C) and pH 7. It produces red/purple colored pigment.

1.2.19 Genus Halobaculum

Members of this genus are Gram negative, rod shaped bacteria. They can grow best at 14–15% NaCl concentration. The type strain of genus *Halobaculum,* is *H. gomorrense,* which has been isolated from Dead sea of Israel. It can grow at temperature of 40°C and pH 6–7 and produces red colored pigment.

1.2.20 Genus Halorubrum

Members of this genus are Gram negative, rod shaped bacteria. They can tolerate up to 30% NaCl (with optimum of 20% NaCl). The type strain of genus *Halorubrum,* is *H. saccharārovorum,* isolated from Saltern, California, grows optimally at 50°C and pH near neutrality. The member of this genus produces red/purple colored pigment.

1.2.21 Genus Haloarcula

Cells of member of this genus are Gram negative, flat pleomorphic rods or squared. They can grow in presence of 6–30% NaCl. The type strain of genus *Haloarcula,* is *H. vallismortis.* It can grow optimally at 40°C and at pH 7.4–7.5. The member of this genus produces red colored pigment.

1.2.22 Genus Natronomonas

Members of this genus are Gram negative, pleomorphic rods, aerobic, extremely haloalkaliphilic archaea. They are capable to grow optimally at 20% NaCl (Range: 10–30% NaCl). The type strain of genus *Natronomonas*, is *N. pharaonis.* Member of this genus grow optimally at 45°C and are alkaliphilic requiring pH around 9.5–10. The member of this genus produces red colored pigment.

1.2.23 Genus Halococcus

Member of this genus are coccoidal. Member of this genus are capable to grow optimally at 20% NaCl (Range: 8–30% NaCl). The type strain of genus *Halococcus,* is *H. morrhuae.* Halococcus found in environments with high salt levels, mainly inland bodies of salt water, but some may be located in highly salted soil or foods. Member of this genus grow optimally at 45°C and are alkaliphilic requiring pH around 9.5–10. The pigmented proteins in some species of *Halococcus* cause the reddish tint found in some areas of the Dead Sea and the Great Salt Lake, especially at the end of the growing season.

1.2.24 Genus Natrialba

Species of genus *Natrialba* are rod shaped and they thrive in alkaline hypersaline conditions. The members of this genus often tolerate high pH and high temperatures. They grow best at 40°C and pH around 6–9.5.

1.2.25 Genus Natronobacterium

Members of this genus are rod shaped. The type strain of this genus is *N. gregoryi* isolated from Lake Magadi, Kenya. It grows at around 37°C and at pH 9.5 requiring NaCl concentration around 10–30%. It produces red colored pigment.

1.2.26 Genus Halogeomatricum

Cells of *Halogeomatricum* are Gram negative Pleomorphic flat. They grow optimally at 25% NaCl concentration (range: 17–30% NaCl), requiring temperature of around 40°C at pH near neutrality. The member of this genus produces red colored pigment.

1.2.27 Genus Natronococcus

Members of this family are coccoidal, haloalkaliphilic, aerobic, archaea. The type strain of this genus is *N. occultus.* isolated from Lake Magadi, Kenya. The member of this genus can grow at temperature around 35–40°C and at pH 9.5 requiring NaCl concentration around 10–30%. It produces red colored pigment.

1.2.28 Genus Haloferax

The cells are Pleomorphic flat. The type strain of genus Haloferax is *H. volcanii* that has been isolated from high-saline environments. Members are commonly found in high-salinity aquatic environments, such as the Dead Sea the Great Salt Lake and oceanic environments with high NaCl concentrates. The member of this genus can grow at temperature around 40°C and at pH near neutrality requiring NaCl concentration around 6–30%. The members produce red to weakly pink colored pigment.

1.2.29 Genus Natrinema

The members of this genus are Gram-negative, non-motile, neutrophilic, pleomorphic and extremely halophilic. The type strain of this genus is *N. pellirubrum*. They are able to grow at 25–50°C, required at least 10% NaCl for growth (optimum at 18% NaCl) and grew over a pH range from 6.0 to 8.5 (optimum at pH 7.0).

1.2.30 Genus Halotarrigena

The members of this genus are coccoidal, neutrophilic, and extremely halophilic. The type strain of this genus is *H. turkmenica*. They require at least 15% NaCl for growth (optimum at 20% NaCl) are able to grow at 25–50°C, pH range from 6.0 to 9.

1.2.31 Genus Natronorubrum

These are pleomorphic flat and are extremely haloalkaliphilic archaea. The type strain of genus *Natronorubrum* is *N. bangense* that has been isolated from alkaline salt lake, Tibet. The member of this genus can grow at temperature around 44°C and at pH 8–9 requiring NaCl concentration around 20%. The members produce red to weakly pink colored pigment.

In addition to these genera, a number of halophilic and halotolerant microorganisms are reported as new genera/species or from new sources (Table 1.1)

Table 1.1 New reports of halophiles

Name of the organism	Source	Requirement/tolerance of salt	Reference
Salinisphaera shabanensis gen. nov., sp . nov.	Shaban Deep, Red Sea	1–28%	Antunes et al. (2003)
Halobiforma haloterrestris gen. nov., sp. nov.	Hypersaline soil close to Aswan (Egypt)	2.2 M NaCl	Hezayen et al. (2002)
Halomonas muralis sp. nov.	Samples of a wall and a mural painting, both heavily contaminated by microbial growth, inside the Saint-Catherine chapel of Castle Herberstein (Austria)	2.5–10% NaCl	Heyrman et al. (2002)
Lentibacillus salicampi gen. nov., sp. nov.	Salt field in Korea	4–8% NaCl	Yoon et al. (2002a)
Paraliobacillus ryukyuensis gen. nov., sp. nov.	Decomposing marine alga collected in Okinawa, Japan	0.75–3.0%	Ishikawa et al. (2002)
Haloferax lucentensis sp. nov;	Pond of a Spanish saltern Alicante	25%	Gutierrez et al. (2002)
Halosimplex carlsbadense gen. nov., sp. nov.	250-million-year-old Salado formation in southeastern New Mexico	20–30%	Vreeland et al. (2002)
Halococcus dombrowskii sp. nov.	Permian alpine salt deposit in Austrian salt mine	20–25%	Stan-Lotter et al. (2002)
Halomonas halocynthiae sp . nov.	Marine ascidian Halocynthia aurantium in coastal waters of the Sea of Japan	0.5–15% NaCl	Romanenko et al. (2002a)
Psychromonas marina sp. nov.	Monbetsu coast of the Okhotsk Sea in Hokkaido, Japan	3–5%	Kawasaki et al. (2002)
Psychrobacter submarinus sp. nov. and *Psychrobacter marincola* sp. nov.	Sea water and the internal tissues of an ascidian Polysyncraton sp.	12–15%	Romanenko et al. (2002b)
Salinicoccus alkaliphilus sp. nov.	Baer Soda Lake in Inner Mongolia Autonomous Region, China	10%	Zhang et al. (2002)
Saccharomonospora halophila sp. nov.	Marsh soil in Kuwait	10–30%	Al-Zarban et al. (2002)
Vibrio calviensis sp . nov.	Sea water (Western Mediterranean Sea, Bay of Calvi, Corsica, France)	2.5–3.5%	Denner et al. (2002)
Salinibacter ruber gen. nov., sp . nov.	Saltern crystallizer ponds, Alicante Mallorca, Spain	20–30%	Anton et al. (2002)

(continued)

Table 1.1 (continued)

Name of the organism	Source	Requirement/tolerance of salt	Reference
Halorubrum tebenquichense sp. nov.	Atacama Saltern, Chile	Saturated concentrations of NaCl	Lizama et al. (2002)
Halomonas alimentaria sp. nov.	Jeotgal, a traditional Korean fermented seafood	1–23%	Yoon et al. (2002b)
Alcalilimnicola halodurans gen. nov., sp. nov.	Sediments of soda-depositing Lake Natron, East Africa Rift Valley	3–8%	Yakimov et al. (2001)
Methanohalophilus zhilinae sp. nov.	Bosa Lake of the Wadi el Natrun in Egypt	0.2–2.1 M	Mathrani et al. (1988)
Haloarcula marismortui (Volcani) sp. nov.	Dead Sea	1.7–5.1 M	Oren et al. (1990)
Chromohalobacter salexigens sp. nov.	Saltern	7.5–10%	Arahal et al. (2001)
Thalassomonas viridans gen. nov. sp. nov.	Mediterranean coast at Valencia (Spain)	2–4%	Macian et al. (2001)
Natronorubrum bangense gen. nov., sp. nov. and *Natronorubrum tibetense* gen. nov. sp. nov.	Soda lake in Tibet	12%	Yi et al. (1999)
Haloferax prahovense sp. nov.	Telega Lake, a hypersaline environment in Prahova county, Romania	3.5 M	Enache et al. (2007)
Natrialba hulunbeirensis sp. nov. and *Natrialba chahannaoensis* sp. nov.	Soda lakes in Inner Mongolia Autonomous Region, China	12–30%	Yi et al. (2001); Kamekura and Dyall-Smith (1995)
Alkalispirillum mobile gen. nov. spe. nov.	Cultures of the anoxygenic phototroph *Halorhodospira halophila* SL-1	2%	Rijkenberg et al. (2001)
Halomonas marisflavae sp. nov.	Yellow Sea in Korea	0.5–12%	Yoon et al. (2001)

Selenihalanaerobacter shriftii gen. nov., sp. nov.	Dead Sea sediments that respires selenate	21%	Blum et al. (2001)
Halonatronum saccharophilum gen. nov. sp. nov.	Lake Magadi, Kenya	7–12%	Zhilina et al. (2001)
Filobacillus milensis gen. nov. sp. nov.	Near to a shallow water hydrothermal vent area of Palaeochori Bay beach, Milos, Greece.	2–23%	Schlesner et al. (2001)
Nocardiopsis kunsanensis sp. nov.	Kunsan, Republic of Korea	3–20%	Chun et al. (2000)
Methylarcula marina gen. nov. sp. nov. and *Methylarcula terricola* sp. nov.	Coastal saline environments;	0.5–1 M	Doronina et al. (2000)
Halomonas campisalis, Alkalibacillus haloalkaliphilus	Alkaline soda lake of Lonar, India.	2%	Joshi et al. (2008)

1.3 Physiology

1.3.1 Requirement for Salt

Halophily is demonstrated by the requirement of a high salt concentration for optimum growth however halotolerance describes the ability to grow at a salt concentration higher than optimum (Imhoff and Thiemann 1991; Ollivier et al. 1994). The degree of salt tolerance of bacteria depends upon the composition of the growth medium and growth temperature. (Kushner 1993) Osmotic stress is one such restrictive environmental factor, which is caused by large concentrations of either salts or non-ionic solutes (sugary plant saps, honey etc.) in the surrounding medium with the resulting deficit of water. Osmotic adaptation has largely focused on halophilic and halotolerant organisms (Kushner 1978; Larsen 1986; Oren 1994; Galinski 1995). Osmoadaptation has the broadest meaning, covering both physiological and genetic manifestation of adaptation to a low water environment. Various aspects of the subject from different angles are reviewed by Yancey et al. (1982), Imhoff (1986), Reed and Stewart (1988), Brown (1990), Csonka and Hanson (1991) and Kinne (1993).

Considerable diversity exists in the mechanisms of halophilic and halotolerant microorganisms that withstand the large osmotic pressure exerted by their highly saline surrounding medium. As biological membranes are permeable to water, all microorganisms have to keep their cytoplasm at least isosmotic with their environment. When a turgor pressure is to be maintained, the cytoplasm should even be slightly hyperosmotic. (Oren 2002). Halophiles which require more than 0.5 M NaCl for optimal growth have developed two different basic mechanisms to cope with osmotic stress.

1.3.1.1 Salt-in-Cytoplasm Strategy

Organisms following this strategy adapt the interior protein chemistry of the cell to high salt concentrations. The thermodynamic adjustment of the cell can be achieved by raising the salt concentration in the cytoplasm. This mechanism, first discovered in Halobacteria, is considered the typical archaeal strategy of osmoadaptation. The salt -in strategy seems to be restricted to

(i) the extremely halophilic Archaea of family Halobacteriaceae, which includes the extreme halophiles of genera such as *Halobacterium*, *Haloarcula, Haloquadratum, Halorhabdus, Natronobacterium* and *Natronococcus,* (ii) the halophilic bacteria of the order Haloanaerobiales, and (iii) the bacterium *Salinibacter ruber* (Oren 2008).

Sulfate reducers *Desulfovibrio halophilus* and *Deslfohalobium retbaense* are now known to employ this strategy (Zhilina and Zavarzin 1990; Gaumette et al. 1991).

Despite the abundance of NaCl in the typical haloarchaeal environment, halophilic Archaea keep the cytoplasm relatively free of sodium. Instead, potassium

accumulates in the cell and together with its counter ion Cl^-, K^+ can be found in molar concentration in the cytoplasm. Because the K^+ concentration inside the cell is 100 times higher than in the surrounding environment, a part of the proton motive force must be used to maintain the ion gradient. In case of halophilic anaerobic bacteria, *Haloanaerobium pravalens*, K^+ is the dominant cation but that Na^+ levels are also relatively high (Oren et al. 1997; Mermestein and Zeikus 1998).

While much understanding has been achieved on the intracellular sodium and potassium concentrations of halophilic organisms and their regulation, the metabolism of anions is becoming interestingly clear. Chloride has specific functions in haloadaptation in different groups of halophilic microorganisms (Müller and Oren 2003). In *Halobacillus halophilus* certain functions such as growth, endospores germination, motility and glycine betaine transport are chloride dependant. A very high requirement for chloride was demonstrated in two groups of bacteria that accumulate inorganic salts intracellularly rather than using organic osmotic solutes, the anaerobic Haloanaerobiales and the aerobic extremely halophilic *Salinibacter ruber*. A specific requirement for chloride for growth of these organisms has been demonstrated and in some cases chloride is required for activation of certain enzymes. It is becoming increasingly clear that chloride has specific functions in haloadaptation in different groups of halophilic organisms.

1.3.1.2 Organic Osmolytes Mechanism

The organic osmolytes mechanism is widespread among bacteria, Eukarya and also present in some methanogenic Archaea (Robertson et al. 1992; Roberts et al. 1992). In response to an osmotic stress, these organisms mainly accumulate organic compounds like sugars, polyols, amino acids and/or amino acid derivatives either by de novo synthesis or by uptake from the surrounding environment. These non-ionic highly water soluble compounds do not disturb the metabolism even in high cytoplasmic concentrations and are thus aptly named ‘compatible solutes’ (Brown 1976). Organic osmolytes fall into three general chemical categories.

(1) Zwitterionic solutes, (2) Noncharged solutes and (3) Anionic solutes.

The range of solutes, their diverse biosynthetic pathways and physical properties of the solutes that effect molecular stability are reviewed by Roberts (2005).There must also be a mechanism for efficiently regulating the concentration of the osmolyte in response to external NaCl. Osmolytes also have other roles in cells – most notably they aid in stabilizing macromolecule structures (Bolen and Baskakov 2001).

Until the advent of high-resolution NMR spectroscopy, little was known of the range of solutes used for osmotic balance in cells. However, in 25 years the library of organic osmolytes has increased significantly in large part due to diverse NMR experiments. Three categories of compatible solutes are given below:

1. **Zwitterionic solutes**: Free polar amino acids in cells might be expected to play role in osmotic balance. However, neutral amino acids are not accumulated to high concentrations, presumably because they are intermediates in protein

Table 1.2 Zwitterionic solutes accumulated in halotolerant and halophilic microorganisms

Type of organic solute	Halotolerant and halophilic microorganisms accumulating organic solutes	References
Zwitterionic solutes		
1. Betaine	*Thioalkalivibrio versutus*; *Actinopolyspora halophila*; *Halorhodospira halochloris, Methanohalophilus portulcalensis* FDF1; *Methanosarcina thermophila*; *Synechococcus* sp. DUN 52	Imhoff and Rodriguez-Valera (1984), Robertson et al. (1990a), Nyyssölä et al. (2000, 2001), Canovas et al. (1998), and Roberts et al. (1992).
2. Ectoine	*Sporosarcina pasteurii*.; *Brevibacterium epidermidis*; *Thioalkalimicrobium aerophilum*; *Vibrio cholerae* and *V. costiocola*, *Chromohalobacter israelensis*; *Chromohalobacter salexigens*; *Halorhodospira halochloris*; *Halomonas elonga*ta, *H. variabilis*; *Methylarcula marina* and *M. terricola*; *Methylophaga alcalica* and *Methylophaga natronic;* aerobic, halophilic isolates from Mono Lake	Galinski (1995), Ciulla et al. (1997), Doronina et al. (2000, 2003), Regev et al. (1990), Onraedt et al. (2004), and Pflughoeft et al. (2003)
3. Hydroxyectoine	*Halomonas elongata; Nocardiopsis halophila*	Canovas et al. (1999)
4. N-γ-acetyldiamino-butyrate	*Halomonas elongata CHR63*	Canovas et al. (1999)
5. N-ε-acetyl-β-lysine	*Methanosarcina thermophila*; *Methanothermococcus thermolithotrophicus*; *Methanosarcina mazei* Gö1, *Methanohalophilus portucalensis* FDF1; *Methanohalophilus* Z7302	Sowers et al. (1990)
6. β Glutamine	*Methanohalophilus portucalensis* FDF1	Lai et al. (1991)

synthesis. Many bacterial and archaeal cells synthesize and accumulate a few zwitterionic molecules derived from amino acids as compatible solutes. Occurrence of these solutes in halophilic and halotolerent microorganisms is presented in Table 1.2.

2. **Non-charged solutes**: The few polar molecules lacking any formal charges have been identified as osmolytes in halophilic bacteria, archaea and eukaryotes. They include several carbohydrates and amino acid/dipeptide as detailed in Table 1.3.
3. **Organic anions**: Anionic solutes in halotolerant and halophilic microorganisms are the most diverse group of osmolytes. Negatively charged solutes can balance high intracellular K^+ as well as counteract osmotic pressure. The negative charge on the solute is provided by three functional groups: carboxylate, phosphate or sulfate. They are presented in Tables 1.4 and 1.5.

Table 1.3 Noncharged solutes accumulated in halotolerant and halophilic microorganisms

Type of organic solute	Halotolerant and halophilic microorganisms accumulating organic solutes	References
Noncharged solutes		
1. α-Glucosylglycerol	Marine & freshwater cyanobacteria: *Synechocystis* sp. *Microcystis firma;* phototrophic eubacteria: *Rhodovulum sulfidophilum; Pseudomonas mendocina, P. pseudoalcaligenes*; *Stenotrophomonas*	Borowitzka and Brown (1974) and Roder et al. (2005)
2. α-Mannosylglyceramide	*Rhodothermus marinus (R.obamensis)*	Silva et al. (1999)
3. Trehalose	*Actinopolyspora halophila; Chromohalobacter israelensis; Desulfovibrio halophilus; Rhodothermus obamensis; Natrialba magadii.*	Welsh et al. (1996) and Nyyssölä and Leisola (2001)
4. Sucrose	Diverse cyanobacteria (*Synechocystis* sp. strain PCC 6803, Anabaena spp.) and proteobacteria	Reed et al. (1986) and Deplats et al. (2005).
5. N-α-carbamoyl-L-glutamine 1-amide	*Ectothiorhodospira mobilis (marismortui)*	Galinski and Oren (1991).
6. N-acetylglutaminyl-glutamine amide	*Sinorhizobium meliloti*; *Rhizobium leguminosarum; Pseudomonas aeruginosa* PAO1, Purple sulfur bacteria.	Smith and Smith (1989) and D'Souza-Ault et al. (1993).

From the studies of different halotolerant and halophilic organisms, it is clear that most cells use combination of solutes, not a single one, for osmotic balance. The diversity and distribution of known classes of compatible solutes found in prokaryotes as well as the increasing knowledge of the genes and pathways involved in their synthesis have been discussed in review (Empadinhas et al. 2008).

1.3.2 Halophilic Adaptation of Enzymes

The entire intracellular machinery of halophilic organisms has to be functional in the presence of molar concentration of salt. Halophilic proteins employ different adaptation mechanisms. The proteins of *Halobacterium* and other halophiles using "salt-in" strategy show unusual properties when compared with their nonhalophilic counterparts. They generally show a high excess of acidic amino acids (glutamate and aspartate) over basic amino acids (lysine and arginine). In addition, their content of hydrophobic amino acids is relatively low. Such proteins not only are soluble and functional at high salt, they even require molar concentration of salt for activity and stability (Dennis and Shimmin 1997; Lanyi 1974; Maverech et al. 2000).

Table 1.4 Anionic solutes (carboxylates) accumulated in halotolerant and halophilic microorganisms

Type of Organic solute	Halotolerant and halophilic microorganisms accumulating organic solutes	References
Anionic solutes (carboxylates)		
1. L-α-glutamate	*Halomonas elongata; Methanohalophilus portucalensis*-FDF1; *Halobacterium* sp. NRC-1 and *H. salinarum*	Roberts et al. (1990), Martin et al. (2001), and Robertson et al. (1990b).
2. β-Glutamate	*Methanothermococcus thermolithotrophicus*; *Methanocaldococcus jannaschii; Methanotorris igneus, Nocardiopsis halophila*	DasSarma and Arora (2002).
3. Hydroxybutyrate	*Photobacterium profundum*	Martin et al. (2002)
4. Poly-β-hydroxy butyrate	*Photobacterium profundum Methylarcula marina* and *M. terricola.*	Doronina et al. (2000)
5. α-Glucosylglycerate	*Agmenellum quadruplicatum; Stenotrophomonas maltophilia, Methanohalophilus portucalensis* FDF1	Robertson et al. (1992)
6. α-Mannosylglycerate	*Methanothermus fervidus; Pyrococcus Efuriosus;Rhodothermus marinus* (*R. obamensis*)	Silva et al. (1999).

Table 1.5 Anionic solutes (phosphate, sulfate) accumulated in halotolerant and halophilic microorganisms

Type of organic solute	Halotolerant and halophilic microorganisms accumulating organic solutes	References
Anionic solutes (phosphate, sulfate)		
1. α-Diglycerol phosphate	*Archaeoglobus fulgidus*	Lamosa et al. (2000).
2. Di-*myo*-inositol-1,1′-phosphate	*Archaeoglobus fulgidus*; *Methanotorris igneus*; *Pyrococcus furiosus* and *P. woesei*; *Pyrodictium occultum*; *Thermotoga maritima.* *Methanobacterium thermoautotrophicum* H and Marburg.	Lamosa et al. (2000), Martins et al. (1996) and Ciulla et al. (1994)
3. Mannosyl-di-myo-inositol-1, 1′-phosphate	*Thermotoga maritima* and *T. neapolitana*	Martins et al. (1996)
4. Cyclic - 2, 3-diphosphoglycerate	*Methanothermobacter thermoautotrophicus; Methanopyrus kandleri; Methanothermus fervidus.*	Kanodia and Roberts (1983), Seeley and Farney (1983), Gorkovenko and Roberts (1993),
5. Sulfotrehalose	*Natronococcus occultus; Natronobacterium* sp.	Desmarais et al. (1997)

The definition and composition of halophilic enzymes, the effects of salt on their activity, solubility and stability are reviewed by Madern et al. (2000).Enzymatic activities of moderately halophilic bacteria and their relation to salt have been discussed by Ventosa et al. (1998).Three distinct categories of enzyme activities such as cytoplasmic enzymes, stress proteins, membrane bound enzymes are discussed in depth. The modification in cell morphology and structure of bacteria under saline conditions is discussed by Zahran (1997).The current proteome study of halophilic archaea and efficient procedures for screening and confirming the function of novel halophilic enzymes is illustrated in review by Joo and Kim (2005).

1.3.3 Cell Envelopes of Halophilic Microorganisms

The cytoplasmic membrane of halophilic microorganisms forms the barrier between the cytoplasm and the environment with its high and possibly fluctuating salinity. The potential to adjust the membrane according to the external salinity seems to be prerequisite for halophilic microorganisms. Alterations of membrane composition have been extensively studied in a range of halophilic eubacteria (Vreeland et al. 1984; Adams et al. 1989; Thiemann and Imhoff 1991; Adams and Russell 1992; Ritter and Yopp 1993. Salt dependant changes in membrane lipid composition of halotolerant moderately halophilic and extremely halophilic bacteria are described in review by Kates (1986).

Many members of the *Halobacteriaceae* are characterized by the presence of beautiful red orange pigments, called bacterioruberins, in cytoplasmic membrane, which help to screen out UV radiation and protect the cells from the harmful effects of sunlight.

Archaeal membrane lipids are unique in containing diphytanylglycerol diether lipids. Lipids of Halophilic Archaea are characterized and their extraction, separation and analysis are described by Corcelli and Lobasso (2006). Most of the phospholipids and glycolipids of extreme halophiles are anionic so that they impart a high negative charge density on halophile membranes. Glycolipids have become useful taxonomic markers in classification of different genera of halophilic Archaea. The study of lipid composition of extreme halophilic archaea is of particular interest because of its relevance for taxonomy and presence of a large variety of unusual structures of potential biotechnology applications.

1.4 Applications

Physiological features of the halophiles contribute to their resourcefulness. They are being looked upon as a treasure of biomolecules, biomaterials and metabolites. To protect themselves against high concentration of Na^+/NaCl, they synthesize certain biochemicals like glycine betaine which acts as a compatible solute. A number of such compatible organic solutes have been reported from halophiles which become technologically important products. The cell envelope and outer membrane shows

modification in composition based upon ionic strength of outer environment (Reed 1986). The membrane lipids of halophilic archaea are diether lipids and thus become source of novel lipids. Recently Oren (2010) has reviewed applications of halophilic microorganisms.

1.4.1 Enzymes of Halophiles

Halophilic enzymes are unusually stable than their normal counterparts. It has been proven that many halophilic enzymes are polyextremophilic. These enzymes not only remain active and stable in high salt environments but are also thermotolerant and alkaliphilic (Moreno et al. 2009). These properties made halophilic enzymes attractive for various biotechnological applications. Gomes and Steiner (2004) have reviewed potential of enzymes of extremophiles including halophiles. Recently Setati (2010) has revisited applications of enzymes from halophilic and halotolerant microorganisms which produce a wide array of enzymes useful in food industry, laundry detergents and textile industries. Some of the enzymes of halophiles are summarized in Table 1.6.

1.4.2 Production of β-Carotene by Dunaliella

Cultivation of the green algae *Dunaliella salina* and *D. bardawil* for the production of β-carotene is the major successful application of halophilic algae (Ben-Amotz et al. 1989; Borowitzka 1999; Raj et al. 2007). The first pilot plant for mass culture of *Dunaliella* was set up in the mid-1960s in the Ukraine.

The pigment β-carotene is in high demand as an antioxidant, as a source of pro-vitamin A (retinol) and as a food colouring agent. Its antioxidant activities make it popular for use in health food. *D. salina* and *D. bardawil* produce large amounts of *β*-carotene when grown under suitable conditions. The pigment is found concentrated in small globules between the thylakoids of the cell's single chloroplast. The main environmental conditions that stimulate accumulation of the pigment are high light intensities, high salinity and nutrient limitation; the slower the cells grow in the presence of high irradiation levels, the more pigment is formed.

1.4.3 Ectoine – A Multi-purpose Osmotic Solute

As discussed earlier, most halophilic and halotolerant microorganisms produce or accumulate small organic compounds intracellularly to provide osmotic balance with their hypersaline environment. One of the most common osmotic solutes in the domain Bacteria is ectoine (1, 4, 5, 6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid). It was first discovered in the haloalkaliphilic photosynthetic sulfur bacterium

Table 1.6 Stability to NaCl of enzyme produced by halophiles

Sr. No.	Name of the organism	Enzyme	Stability to NaCl	Reference
1	*Halobacillus* sp.	Amylase	5%	Amoozegar et al. (2003)
2	*Haloferax mediterranei*	α-Amylase	2–4 M	Perez-Pomares et al. (2003)
3	Halophilic bacterium, CL8	Xylanase	4 M	Wejse et al. (2003)
4	*Halorhabdus utahensis*	β-Xylanase, β-xylosidase	5–15%, 5%	Waino and Ingvorsen (2003)
5	*Pseudoalteromonas* sp. strain CP76	Protease CPI	7.5%	Sánchez-Porro et al. (2003)
6	*Halothermothrix orenii*	α-Amylase	5%	Mijts and Patel (2002)
7	*Halomonas* sp. # 593	Nucleoside diphosphatekinase	NA	Yonezawa et al. (2003)
8	*Halomonas salina* strain AS11	Aldehyde dehydrogenase	5%	Sripo et al. (2002)
9	*Haloferax mediterranei*	2-Hydroxy acid dehydrogenase	4 M	Bonete et al. (2000)
10	*Nesterenkonia* sp.	Endo-1,4-β-Xylanase	2.5%	Govender et al. (2009)
11	*Glaciecola mesophila*	Endo-β-Xylanase	2.5 M	Guo et al. (2009)
12	*Chromohalobacter* sp.	Xylanase	20%	Prakash et al. (2009)

NA Not available

Ectothiorhodospira halochloris, but later a great variety of halophilic and halotolerant bacteria were found to produce this compound, often together with its 5-hydroxy derivative. Ectoine can protect many unstable enzymes and also nucleic acids against the detrimental action of high salinity, thermal denaturation, desiccation and freezing, thereby increasing shelf life and activity of enzyme preparations (Lippert and Galinski 1992; Galinski et al. 1989, 1991; Kolp et al. 2006). For example, ectoine inhibits spontaneous conversion of trypsinogen to trypsin and of trypsin-catalyzed conversion of chymotrypsinogen to chymotrypsin, and it also stabilizes the activity of trypsin and chymotrypsin.

In recent years additional properties of interest were found for ectoine. It is claimed that it counteracts effects of ultraviolet UV-A-induced and accelerated skin ageing, and therefore the cosmetics industry started to add ectoine to dermatological cosmetic preparations as moisturizers in cosmetics for the care of aged, dry or irritated skin. Ectoine is commercially produced by extracting the compound from halophilic bacteria. Industrial processes for mass production of ectoine and hydroxyectoine were developed using *Halomonas elongata* and *Marinococcus* M52, respectively.

1.4.4 Bacteriorhodopsin

Bacteriorhodopsin is a photochemical material produced by halophiles. It is a highly unusual protein within the proteome of *Halobacterium*. Its function was discovered in early 1970s within the cell membrane of *Halobacterium salinarum*. It serves as a light-driven proton pump. After excitation by light of a suitable wavelength, a complex photocycle is initiated in which the Schiff base is deprotonated and reprotonated. The protonation/deprotonation reactions are so arranged that the proton is taken up from the cytoplasmic side and released to the outside of the cell so that light absorption results in the generation of a proton gradient that can be used for generation of adenosine triphosphate (ATP). Proteins of *Halobacterium* require high salt concentrations for structural stability and activity. However, bacteriorhodopsin is stable in absence of salts, retains the photochemical properties over long periods, functions between 0°C and 45°C in the pH range 1–11. It tolerates temperatures over 80°C when in aqueous solution while when dry, it tolerates temperature up to 140°C. It is reported to be stable to sunlight for years and resists digestion by most proteases. Bacteriorhodopsin is manufactured in form of purple membrane patches from *Halobacterium salinarum*, however, its commercial application is yet to start. Probable applications include conversion of light energy into chemical energy and electricity and desalination of sea water.

1.4.5 Exopolysaccharides

Microorganisms produce a number of compounds to protect themselves from harsh and adverse conditions. Exopolysaccharides (EPS) are one of them. These are polysaccharides produced extracellularly by microorganisms and compose of simple

sugars. Microbial polysaccharides are water soluble polymers and may be ionic or non-ionic in nature. The repeating units of these polysaccharides are very regular, branched or unbranched and interconnected by glycosidic linkages. Microbial polysaccharides find a wide range of applications in food, pharmaceutical, petroleum and other industries (Sutherland 1990, 1998; Tombs and Harding 1998). They have a crucial role in metal biosorption process (Brown and Lester 1979) and thus could be used in heavy metal bioremediation. EPS have a number of applications e.g. as gelling agents and emulsifiers. They are also used in microbially enhanced oil recovery. Among the halophilic bacteria, *Halomonas* has been reported for production of EPS. *H. maura, H. eurihalina* produce large amounts of extracellular polyanionic polysaccharide having application as emulsifier (Davis 1974). EPS 'mauran' produced by *H. maura* has been reported to have application as pseudoplastic (Arias et al. 2003) and as an immunomodulator (Bejar et al. 1998; Arias et al. 2003). Among archaea, *Haloferax mediterranei* secretes anionic exopolysaccharide. The sulfated acidic heteropolysaccharide of *Haloferax* sp. has a high viscosity at low concentrations, exhibits excellent rheological properties and is resistant to extremes of pH and temperature. It has a potential application in enhanced oil recovery.

1.4.6 Biodegradable Plastic – Poly-hydroxyalkanoate (PHA)

Synthetic plastic has been a major environmental concern in past decades because of its resistance to degradation by air, water or any biological entity including plants and animals. Polyhydroxyalkanoate (PHA) has been explored as biodegradable plastic, an alternative to the synthetic plastic. PHA is one of the major reserve materials produced by eubacteria as well as archaea. To date, around 300 microbial species including halophiles are reported for production of biodegradable plastic in form of Poly-hydroxyalkanoate (PHA). These are produced under unbalanced nutritional conditions and limiting condition of oxygen or potassium or some other elements. They are microbial polyesters stored in form of granules inside the cells. Among the bacteria, *Alkaligenes* sp., *Halomonas* sp. etc. have been most extensively investigated for the production of PHA. However, commercial production of PHA is yet to become economically feasible. Extraction of PHA involves use of organic solvents like chloroform, methanol etc. thus adding extra cost to the production of PHA. Thus, a cost effective production of biodegradable plastic is a major objective from industrial point of view.

A few species of *Halomonas*, viz. *H. maura,* isolated from hypersaline soil and *H. ventosa* isolated from saline soils in Jaen (South eastern Spain) have been reported to accumulate PHB granules intracellularly (Bouchotroch et al. 2001; Martinez-Canovas et al. 2004). Quillaguaman et al. (2005) for the first time reported production of PHB by moderately halophilic *Halomonas boliviensis* LC1 isolated from the shores of a hypersaline lake located in the Andean region of Bolivia. Another species, *Halomonas profoundees*, sp. nov., isolated from a deep sea hydrothermal vent shrimp was reported to produce PHB and PHB-co-PHV using carbon source like glucose and glucose + valeric acid respectively (Simon-Colin et al. 2008).

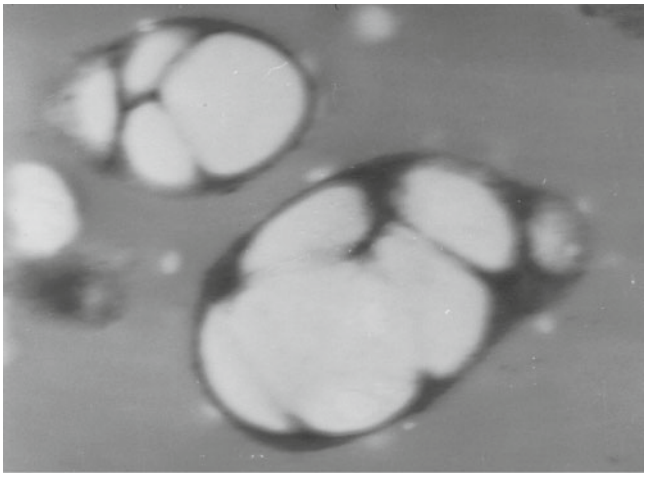

Fig. 1.2 Transmission electron micrograph of *Halomonas campisalis* showing presence of PHA granules, grown in the PHA production medium of pH 9 for 24 h

Mothes et al. (2008) reported a moderately halophilic *H. elongata*, which was found to synthesize PHA and ectoine simultaneously. Recently, Biswas et al. (2009) studied the production of PHB in another moderately halophile *H. marina* HMA103, isolated from a solar saltern in Orissa, India. Quillaguaman et al. (2006, 2007, 2008) have further improved the production of PHA by *H. boliviensis.* Joshi et al. (2007) have reported accumulation of PHA granules in *Halomonas campisalis* isolated from alkaline soda lake of Lonar, India, when grown in medium containing maltose (Fig. 1.2).

Recently Kulkarni et al. (2010) have reported production of copolymer, PHB-co-PHV by moderately haloalkalitolerant bacterium *Halomonas campisalis* isolated from alkaline soda lake of Lonar, India.

Halophilic archaea (haloarchaea) could be explored for more efficient production of PHA. Haloarchaea inhabit the hypersaline environments containing salt concentrations in excess of seawater. They are distributed throughout the world, requiring high salinity (20–30 g% NaCl) for their growth (DasSarma et al. 2001; Tindall 1991). Literature survey of haloarchaea reveals that species from genus *Haloferax* have been reported to produce PHA (Fernandez-Castillo et al. 1986; Huang et al. 2006). Lillo and Rodriguez (1990) reported that *H. mediterranei* grows optimally with 25% (w/v) salts and glucose or starch or extruded rice bran as sole carbon source in the medium and accumulates 60–65% PHB intracellularly. This organism also accumulates a co-polymer of PHB-co-PHV using an enzymatic extruded starch without addition of any precursor (Chen et al. 2006).

One of the advantages of using haloarchaea for the production of PHA is the reduced cost of extraction. Haloarchaea could be lysed by simply suspending in distilled water. PHA can be extracted in distilled water and can be recovered by evaporation. The PHA pellet can be redissolved in a small volume of chloroform and

a PHA film can be casted. Thus, the cost of solvent extraction would be significantly reduced on an industrial scale. Further, chances of microbial contamination during fermentation of PHA could be minimized since the haloarchaea outgrow contaminating bacteria in the presence of high salinity. Moreover, the organism can grow on simple carbon source like glucose and low amount of yeast extract (0.1 g%) to produce PHA which also reduces the cost of production (Asker and Ohta 2002).

1.4.7 Bioremediation of Saline Waste Waters

There are a number of industrial processes which generate saline waste waters e.g. food industries making pickles and brines, leather making in tanneries, manufacturing of basic dyes, etc. Moderately halophilic *Halomonas* sp. has been reported for biological treatment of saline phenolic waste water (Hinteregger and Streichsbier 1997). Likewise Kubo et al. (2001) have described treatment of hypersaline waste water with salt tolerant microorganisms. Anaerobic biodegradation has been proposed to remove nitrate from brines in a membrane reactor using denitrification process of *Haloferax denitrificans* (Cyplik et al. 2007). *Haloferax mediterranei* also shows potential in bioremediation of nitrate and nitrite in saline groundwater in coastal areas (Martinez-Espinosa et al. 2007). Riffat and Krongthamchat (2007) have described anaerobic treatment of high saline waste water using halophilic methanogens. The halophilic anaerobic bacterium *Haloanaerobium lacusrosei* has been reported to be useful in an anaerobic packed bed reactor for remediation of chemical waste waters containing salt concentration up to 10% (Kapdan and Erten 2007; Kapdan and Boylan 2009). Veena Gayatri and Vasudevan (2010) have reported biodegradation of phenol by moderately halophilic organisms under saline conditions. Since many industrial waste waters contain chlorides, nitrates etc., halophiles would be the best candidates for the bioremediation.

1.5 Future Perspectives

Many researchers have stated that what we know about Extremophiles is far less than what exists in the nature. Among all the extremophiles, halophiles have been paid little attention. With new approaches derived from genomics and proteomics, it is likely that the knowledge would be generated on biodiversity of halophiles. A number of saline environments on the earth are yet to be explored for isolation of halophiles.

It is hypothesized that studying Extremophiles on earth may provide helpful metrics in our search for life elsewhere in the universe. Species that thrive in the hypersaline environments are among the most fascinating models for studying mechanisms of survival away from our planet. Extremely halophilic microorganisms thrive in these extreme environments, turning the Great Salt Lake into a deep red

colour that is visible from space. Likewise, deep red colour of Mars observed from the space indicates the possibility of presence and survival of extremely halophilic archaea in harsh and saline environments of Mars. They might also be capable of surviving travel between planets for instance, encased in salt crystals and thus protected from damaging radiation (Das Sarma 2006). This hypothesis opens up a new horizon to microbiologists interested in biodiversity of halophiles.

Although a number of compounds have been identified from halophiles, only a few e.g. ß-carotene and ectoine are produced. Many ideas are yet to be exploited e.g. production of liposomes for the cosmetic industry. Bacteriorhodopsin is the novel compound produced by halophiles and has wide range of applications. As suggested by Oren (2010) the molecule could be exploited for construction of bioelectronic elements of computer memories and information processing units. A high density of information storage, both permanent optical image storage, data storage and transient optical image storage (data processing) by bacteriorhodopsin are possible. The molecule could also be explored for construction of artificial retinas, nanotechnology applications such as the construction of molecular transistors, molecular motors and molecular sensors.

With isolation of new halobacteria and haloarchea, novel molecules could be identified for application in environment management, healthcare and agriculture.

1.6 Conclusions

Halophiles are comparatively a less studied group of extremophilic organisms. To date, a very few ecosystems have been explored for isolation of halophiles. Depending upon their requirement of salt, halophiles are classified as slightly, moderately and extremely halophilic organisms. Halotolerant organisms grow both at high concentration of salt and in absence of salt. Among the halophilic bacteria, *Halomonas* is a well studied genus particularly because of its potential in production of a number of compounds like PHA, exopolysaccharide and in bioremediation of saline waste waters. The halophilic archaeon, namely *Haloferax* has been found to be an interesting organism having application in production of bacteriorhodopsin, PHA, exopolysaccharide, etc. Addition of new taxa of halophiles will widen the horizon of biotechnological applications for environment management, betterment of human health and agriculture.

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Chapter 2
Diversity of Enteropathogens in River Narmada and Their Environmental and Health Implications

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Abstract The global diversity is changing at an unprecedented rate as complex response to several human induced perturbations in the environment. Though many of the microorganisms, the earliest forms of life on earth, have been commercially exploited, our knowledge of their diversity and key roles in sustaining global life supporting systems is limited. The microbial population in a body of natural water, to a large extent is determined by the physical and chemical condition, which prevails in that habitat. The discharge of domestic sewage and industrial effluents are the main sources of river water pollution that propagates the pathogens especially the enteropathogenic microbial populations. River Narmada holds the life line of central India. The most prevalent mechanism of dispersion being the oral-fecal route. The emergence and persistence of multidrug resistant pathogens in the aquatic gene pool of river Narmada posses a catastrophic situation for all forms of life and humans in special. The study of genetic diversity of pathogens helps in better implementation of public health intervention strategies and provides a better insight for understanding evolution, taxonomy, and pathogenecity. Such an enterprise is useful for rational development of diagnostics, therapeutics, and vaccines.

Keywords River Narmada • Taxonomy • Diversity • Central India • Enteropathogens • Multidrug resistance • Health implications

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2.1 Introduction

> "What makes a river so restful to people is that it doesn't have any doubt - it is sure to get where it is going, and it doesn't want to go anywhere else."
>
> Hal Boyle
> Pulitzer prize-winning columnist

Enteropathogen means 'An organism that is capable of producing intestinal disease' (Janda and Abbott 2006).

Rivers are the major source of fresh water available for many purposes e.g. consumption, industry, and agriculture. There is an increasing awareness of the necessity for conserving fresh water sources. Microbial communities' play an important role in the biodegradation of pollutants derived from human activities, and contributes to natural self purification (Takehiko et al. 2001). Among many other factors related to human health, water is implicated in the transmission of infections. Worldwide, inadequate treatment of gastroenteritis kills 5,000–8,000 people per year, (Kasper et al. 2005) and is a leading cause of death among infants and children under the age of 5 (King et al. 2003).

Biodiversity has become a popular buzzword among the politicians, economists and the public in general. The values and benefits of studying biodiversity are multifold and include:

- Expanding the frontiers of knowledge about the strategies and limits of life.
- Using diversity pattern of microorganisms for monitoring and predicting environmental changes.
- Discovering the untapped diversity of microorganisms as a resource for new genes and organisms of value to biotechnology
- Importance of microorganisms to the sustainability of life on earth.
- Developing microbial community as models for understanding biological interaction as evolutionary history and health quandary (Hunter-Cevera 1998).

Attempts to investigate the diversity of prokaryotic species in an environmental sample have been performed for decades by plating dilution onto certain standard growth media and on the recovery of certain physiological and morphological distinct groups. Affiliation of strains to species were done originally on the basis of superficial phenotypic tools, followed later by investigation of chemo taxonomic properties and more recently of molecular properties. The development of molecular phylogenetics has recently enabled characterization of naturally occurring microbial biota, without cultivation. Free from biases of the culture based studies, molecular phylogenetic surveys have revealed a vast array of new microbial groups. Many of these new microbes are widespread and abundant among contemporary macrobiota and fall within novel divisions that branch deep within the tree of life. The breadth and extent of microbial diversity has become much clearer (Delong and Pace 2001). The ability to quantify the number and kinds of microorganisms within a community is fundamental to the understanding of the structure and function of an ecosystem. Molecular approaches based on 16S ribosomal RNA (rRNA) sequence analysis allows direct investigation of the community structure, diversity, and phylogeny of microorganisms in almost any environment (Theron et al. 2001).

2.1.1 Narmada Basin

The basin has five well defined physiographic regions. They are: (1) The upper hilly areas covering the districts of Shahdol, Mandla, Durg, Balaghat and Seoni, (2) The upper plains covering the districts of Jabalpur, Narsinghpur, Sagar, Damoh, Chhindwara, Hoshangabad, Betul, Raisen and Sehore, (3) The middle plains covering the districts of East Nimar, part of west Nimar, Dewas, Indore and Dhar, (4) The lower hilly areas covering part of the west Nimar, Jhabua, Dhulia, Narmada and parts of Vadodara, and (5) the lower plains covering mainly the districts of Narmada Bharuch, and parts of Vadodara. The hilly regions are well forested. The upper, middle and lower plains are broad and fertile areas, well suited for cultivation. The Narmada basin mainly consists of black soils. The coastal plains in Gujarat are composed of alluvial clays with a layer of black soils on the surface (Narmada valley Development Authority 2002) (Fig. 2.1).

2.1.2 The River Environment

River Narmada (21°23′–24°46′ N Latitude, 72°32′–81°46′E Longitude) is the largest west flowing river in peninsular India. The total length of the river from the head to its outfall into the sea is 1,312 km. The first 1,077 km is in M.P., the next 35 km forms boundary between the states of Madhya Pradesh and Maharashtra, a further 39 km forms the boundary between Maharashtra and Gujarat and the rest of the 161 km lies in Gujarat. The basin has an elongated shape almost like a thin ribbon with a maximum length of 953 km east to west and a maximum width of 234 km north to south (Narmada valley Development Authority 2002). Originating from Amarkantak at 1,151 m altitude (Latitude 22°40′ N and Longitude 81°46′ E) in Shahdol district from the Maikal ranges, the river flows through different cities, which are surrounded with dense human population, agricultural farms, and large and small industries. The catchment area of river Narmada sustains's various agriculture and industrial communities (Sharma et al. 2008a) (Table 2.1).

This water system also serves as a main source of fresh water; used for drinking, washing, bathing, aesthetic, recreational and agricultural purposes to the large population, which resides in this region (Sharma and Rajput 1996; Sharma and Khokale 2005, 2006; Sharma and Chaturvedi 2007; Sharma et al. 2009a). Similar to other resources, this river is also under environmental stress due to human encroachment, high macrophytic population and sewage inputs from various sources.

2.2 Diversity of Enteropathogens in Fresh Water Riverine System

The multifarious uses of water for drinking, bathing, washing and cooking are well known. Water meant for human consumption should be free from pollution, safe and acceptable. Indeed, the microbial quality of water sources should not exceed the

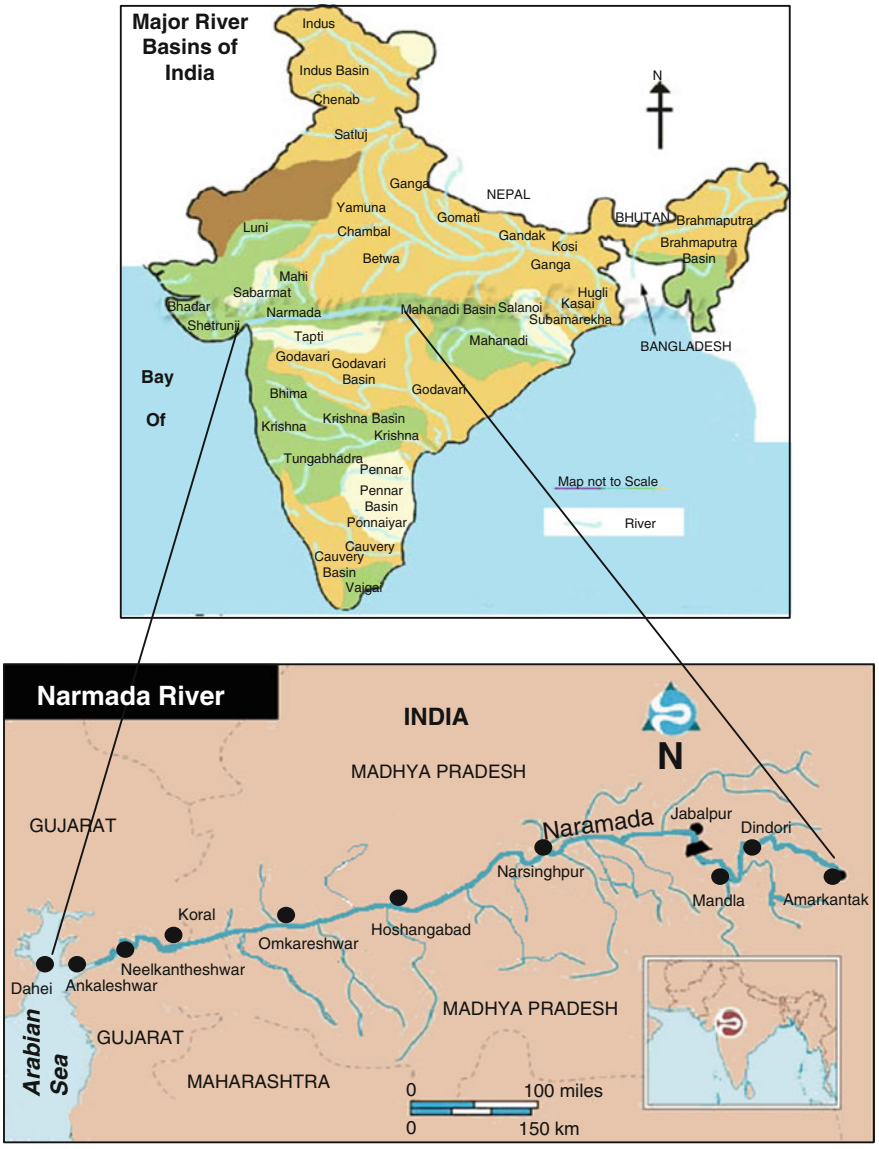

Fig. 2.1 Map showing different sampling stations of river Narmada

maximum limits specified in water quality guidelines. Disease-causing organisms (pathogens) transmitted via drinking water are predominantly of fecal origin (and therefore known as enteric pathogens) (Ashbolt 2004; Hunter et al. 2002). Pathogenic microorganisms found in non-treated wastewater have the ability to

Table 2.1 Locations of the sampling stations on river Narmada and their characteristics

Sampling stations	Name	Distance from sources (km)	Altitude (M)	Latitude	Longitude	Source of pollution
1	Amarkantak	0	1,151	22°-40′ N	81°-46′ E	Domestic
2	Dindori	91	665	22°-57′ N	81°-14′ E	Domestic
3	Mandla	212	438	22°-43′ N	80°-35′ E	Domestic and sewage
4	Jabalpur	309–326	475	23°-10′ N	79°-59′ E	Domestic, sewage and industrial
5	Narsinghpur	437	359.8	22°-57′ N	79°-15′ E	Domestic and sewage
6	Hoshangabad	623	331	22°-46′ N	77°-45′ E	Domestic and sewage
7	Omkareshwar	966	321	22°-46′ N	77°-37′ E	Domestic and sewage
8	Koral	1,113	Sea level	21°-41′ N	73°-01′ E	Domestic and sewage
9	Neelkantheshwar	1,163	”	21°-41′ N	73°-01′ E	Domestic and sewage
10	Ankaleshwar	1,259	”	21°-38′ N	73°-02′ E	Domestic, sewage and industrial
11	Dahej	1,312	”	21°-42′ N	72°-38′ E	Domestic and industrial

reproduce easily due to the large amount of available nutrients, thereby affecting the environment and presenting a great risk to health (Pusch et al. 2005; Gilbride et al. 2006). In India, mostly the house hold waste water in tribal regions is discharged in rivers. This could be the cause of contamination of many entrtic-bacteria. While the normal microbiota has been implicated as a critical defense against invading pathogens, the impact of enteropathogenic infection and host inflammation on intestinal microbial communities to significantly reduce the total numbers of resident colonic bacteria.

The World Health Organization (WHO) estimates that about 1.1 billion people globally drink unsafe water (Kindhauser 2003) and the vast majority of diarrheal disease in the world (88%) is attributable to unsafe water, sanitation and hygiene (WHO 2006). Fresh water and the related microbial population in the distribution system constitute one of the most extensively studied oligotrophic systems (LeChevallier et al. 1982; Pedersen 1990). Gastrointestinal infections are the most common diseases caused by enteric bacteria. Some examples are salmonellosis (*Salmonella* sp.), cholera (*Vibrio cholerae*), dysentery (*Shigella* sp.) and other infections caused by *Campylobacter jejuni, Yersinia* sp. and *Escherichia coli* O157:H7 and many other strains. *E. coli* O157:H7 successfully causes infections because of its low infectious dose (ID), which can be as few as ten cells (Rosen 2000).

Most frequent disease associated with enteropathogens is diarrhea. According to the World Health Organization, each year there are an estimated four billion cases of diarrhea. In 2007, 1.8 million people died worldwide from diarrheal diseases (including cholera), with 88% of these diarrheas being attributable to unsafe water supply, inadequate sanitation and hygiene. They were the second leading cause of disability-adjusted life years (DALY) lost and were the third leading cause of death (4% of all deaths) (WHO 2006). Shigellosis is caused by *Shigella*, in case of *Salmonella* symptoms are associated with enteric fever, also called typhoid, whereas *Klebsiella* is responsible for nosocomial pneumonia, septicemia and also the common cause of Urinary tract infection (Podschun and Ullmann 1994). The most prominent disease linked to members of the genus *Citrobacter* involves infection of the central nervous system (CNS), in 1960, it was recognized that *Citrobacter* had the capacity to cause serious life-threatening infections such as meningitis in neonates.

Aeromonas is a common component of the bacterial population of drinking water in distribution systems but comprises of only a small fraction of the heterotrophic population (Leclerc and Buttiaux 1962; Schubert 1976; van der Kooij 1977; LeChevallier et al. 1982; Havelaar et al. 1990). During national survey in the Netherlands, no evidence was obtained that the aeromonads present in drinking-water were enteric pathogens (Havelaar et al. 1992). Still, in drinking-water legislation in the Netherlands, a maximum value for *Aeromonas* of 1,000 CFU/100 ml is included, aiming at limiting the exposure of the consumer to this organism (VROM 2001). *Proteus* is important agent of human disease causing urinary tract infection and also in a number of miscellaneous illness including wound, bone, and joint infections and disease of the respiratory tract. Identification of such pathogens is of utmost importance mainly in rivers, which are the reservoirs of fresh water source used for drinking purposes in many of the countries including India.

2.2.1 Genetic Diversity of Enteropathogens in River Narmada

Freshwater biodiversity is an important source of food, income, and livelihood, particularly to rural communities in developing countries. Studies have estimated the economic value of river basins in the billions of dollars (Schuyt 2005). River Narmada is no exception, being an important source of fresh water in the states of Madhya Pradesh and Gujarat. The numerous taxonomic, molecular and genetic advances made over the past decade have helped in identification of pathogenic microbes unraveling across the diverse members of family Enterobacteriaceae, Aeromonadaceae, and Vibrionaceae, thus ringing alarm bells concerning the level of pollution the in riverine systems ascertained to the industrial malpractices, meager sanitary and unhygienic conditions along with poor socio-economic conditions. Since the pathogenic organisms studied are autochthonous inhabitants of the aquatic environments, it is important to study their diversity in the fresh water ecosystem. Most of the pathogenic organisms are not able to grow in the laboratory conditions, so to study their diversity in the aquatic environment molecular analysis will be the most important tool for analyzing the biodiversity of river Narmada in Madhya Pradesh.

Traditionally, identification of pathogenic bacteria has been based on phenotypic characteristics and mainly involves analysis of differential metabolic properties and reactions of specific antibodies. Recently, molecular analysis of phylogenetic markers has been recognized as a very useful tool for identification of bacterial genera, species, or subspecies (Goscon et al. 1998; Carroll et al. 2000). Among these markers, 16S rRNA gene is particularly useful because these molecules are present in every living cell and their function is highly conserved. However, an approach based on utilization of universal primer PCR (UPPCR) for conserved regions, such as 16S rRNA genes, can be used to study almost all bacteria (Marchesi et al. 1998). The bacteria have to be characterized further by subsequent steps including restriction fragment length polymorphism analysis single-strand conformation polymorphism analysis, or sequencing analysis (Goscon et al. 1998; Osorio et al. 1999; Peng et al. 2000).

Vibrios are Gram-negative bacteria, which are often associated with cholera, gasteroenteritis and other enteric diseases in humans. *V. cholera* (Finkelstein 1973) and *V. mimicus* (Davis et al. 1981) have been associated with excessive watery diarrhea, while *V. parahaemolyticus* (Paramasivam et al. 2004) and *V. vulnificus* (Chakraborty et al. 1997) have been determined as causal agents of acute gasteroenteritis and septicimea, respectively. Cholera, caused by infection with the toxigenic bacteria *V. cholerae* O1 or O139, continues to cause severe outbreaks of dehydrating diarrhea in much of the developing world. Occurrence of vibrios in River Narmada at Jabalpur dates back as early as 1995 (Sharma and Rajput 1995). During the course of study (Feb. 1991–Feb. 1992), a total number of 96 isolates were identified based on biochemical parameters. *V. vulnificus*, *V. parahaemolyticus*, *V. mimicus,* and *V. fluvialis* accounted for the different species of vibrios identified during the study period.

A total of 28 *Vibrio* isolates represented by *V. furnissi, V. vulnificus,* and *V. metschnikovii* were identified in river Narmada (August 1996–August 1998) at Jabalpur (Sharma et al. 2004). The population of *Vibrio* sp. in the river Narmada at Jabalpur during the period January 2002–December 2003 indicated the presence of 115 strains of *Vibrio* sp. isolated from four different sampling points. The identification of *V. cholera* non-O1, *V. fischeri*, *V. hollisae*, *V. mimicus*, *V. parahaemolyticus*, *V. proteolyticus*, and *V. vulnificus* was carried out on the basis of ARDRA. Statistical analysis of isolation rate with various physico-chemical parameters revealed positive correlation with temperature, alkalinity, chloride, COD, phosphate, total coliforms, fecal streptococci, heterotrophic bacterial count, DO and negative correlation with pH, BOD, nitrate, fecal coliforms. Significant correlation was observed between water temperature and *Vibrio* densities, whereas alkalinity was not strongly correlated with distribution of *Vibrio* sp. (Sharma and Chaturvedi 2007). Considerable variation was associated with occurrence of *Vibrio* sp. at different sampling stations, along with regular seasonal variation. The isolation rate was much higher during the summer season, but substantial decrease in *Vibrio* count was observed during the winter season. An overall reduction in count was observed across all the sampling stations during winter season that could be accounted by the VBNC (viable but non culturable) state of the organism, a survival strategy use to counter temperature stress (Oliver et al. 1995). Repeated failure to isolate the organism during the winter season summarized the trivial count of vibrios during winter or their ability to undergo and reside in the VNBC state (Sharma and Chaturvedi 2007). Both ARDRA and RAPD-PCR showed high degree of heterogeneity amongst *Vibrio* isolates. Unlike ARDRA, where the differentiation was species specific, in RAPD the isolates were found to be distributed in all the clusters, depending on the banding pattern. This observation establishes the pre-eminence of ARDRA over RAPD in establishing the genetic delineation among the fresh water isolates of the river Narmada at Jabalpur (MP) (Chaturvedi 2006).

Isolation of *Vibrio* sp. from river Narmada at Amarkantak and Hosangabad was reported during the year 2006–2007 (Sharma et al. 2009a). A total of 32 *Vibrio* strains were isolated from the different sampling sites of Amarkantak while 27 strains were isolated from the four sites of Hoshangabad. Identification on the basis of biochemical characteristics and ARDRA distinguished the 59 isolates into six different species (*V. cholera non-O1, V. parahaemolyticus, V. vulnificus, V. fischeri, V. proteolyticus,* and *V. mimicus*). UPGMA cluster analysis of RAPD profile designated two major clusters (A & B) for the six identified species. *V. vulnificus* was the sole representative of major cluster A, while cluster B was divided into three subdivisions; B1 (*V. fischeri*), B2 (*V. proteolyticus* and *V. mimicus*) and B3 (*V. cholera*, and *V. parahaemolyticus*). However UPGMA cluster analysis of ARDRA profile delineated the six species into distinct clusters, thus establishing the superiority of ARDRA over RAPD. Both these characterization tools have been used efficiently for molecular characterization and epidemiological studies for a number of pathogens including *Salmonella enterica* (Olsen et al. 1992), *Staphylococcus aureus* (Blumberg et al. 1992), *Enterobacter* sp. (Dour 2006) and *Shigella* sp. (Sharma et al. 2009b).

For many years only *V. cholera* qualified as human pathogen, occasionally contaminating aquatic environments leading to human diseases. While there were cases of suggested *Aeromonas* caused diseases in humans in the 1930 (Aiken et al. 1936), a case reported by Hill et al. (1961) is considered to be the first verified instance of human disease caused by *Aeromonas*. The enteropathogenecity of the organism was first established by Caselitz in 1955. Since then, many studies on the relationship of *Aeromonas* sp. and diarrheal disease have been subsequently reported and vary from single case reports to multiple cases (Austin and Adams 1996).

The occurrence of Aeromonads in river Narmada was first documented during 1992 (Sharma and Khokale 2005). The presumptive identification based on biochemical characters demarcated the genera into six species (*A. hydrophila*, *A. salmonicida*, *A. liquifaciens*, *A. punctata*, *A. veronii*, and *A. formicans*). The incidence of isolation was found to be higher during the month of June at all sampling sites when water temperature was near to 29.2°C. Lowest *Aeromonas* count was observed during the month of November, when water temperature was recorded around 20°C. The occurrence of Aeromonads in river Narmada was further substantiated by another study (Aug 1993–1994), where five species of *Aeromonas* (*A. caviae*, *A. salm*, *A. salmonicida*, *A. sobria*, and *A. hydrophila*) were identified from the water samples across the different sampling stations of Jabalpur (Sharma et al. 1995; Sharma and Rajput 1996). Aeromonads were isolated from four major sampling stations of river Narmada at Jabalpur, followed by identification and species differentiation following the scheme proposed by Abbott et al. (2003) and Altwegg et al. (1990). The intra and interspecies differentiation of 110 isolates using UPGMA cluster analysis resulted in identification of eight genemospecies (*A. hydrophila*, *A. schubertii*, *A. sobria*, *A. eucrenophila*, *A. HG* 11, *A. veronii* bt *veronii*, *A. veronii* bt *sobria*, and *A. caviae*.). Occurrence of high percentage of *A. hydrophila* at most of the stations indicated low level of fecal pollution. The predominance of *A. veronii* bt *sobria*, *A. veronii* bt *veronii*, and *A. schubertii* in environmental samples are significant as well as of immediate concern as these strains are frequently present in clinical samples (Sharma et al. 2005).

Analyses of water samples from river Narmada at Jabalpur during 2002, resulted in isolation of 30 isolates and were identified accordingly as: *A. hydrophila*, *A. caviae*, *A. sobria*, *A. veronii* bt *veronii*, *A. jandei*, and *A. eucrenophila* (Sharma et al. 2005). Genetic differentiation on the basis of RAPD analysis is considered as a reliable tool for differentiation between strains within a species (Austin and Adams 1996). Different RAPD profiles were recorded for different species of *Aeromonas* although UPGMA analysis revealed that the collection of *Aeromonas* under study comprises a closely related population. UPGMA cluster analysis on the basis of biochemical characteristics as well as RAPD patterns of *Aeromonas* demarcated all the isolates into two major clusters, suggesting that RAPD pattern is species specific (Sharma et al. 2005).

The diversity of *Serratia* in river Narmada was first studied during the period 2001–2003. *Serratia* as a virulent organism has been established as causative agent of endocarditis, osteomyletis and arthritis (Ania 2002). It has also been isolated from patients suffering from gall bladder emphysema (Anahory et al. 1998)

and septicemia (Darbas et al. 1994). ARDRA and RISA differentiated the 27 strains of *Serratia* isolated during the study into six species (*S. marcescens*, *S. fonticola*, *S. plymuthica*, *S. rubidaea*, *S. marinorubra*, and *S. odorifera*). The UPGMA cluster analysis places all the isolates into two major divisions. The species-specific patterns suggested a close phylogenetic relationship among the different species of *Serratia*. These patterns thus could be used for designing of specific markers through modern molecular methods for determination of diversity and variation within the same genus (Tiwari 2004).

The diversity of *Enterobacter* in river Narmada was studied during the period 2002–2003. Among 57 isolates of *Enterobacter*, 22 were identified as *E. cloacae*, 11 isolates were identified as *E. taylorae*, 9 were identified as *E. aerogens*, 4 were identified as *E. sakazakii*, 6 were identified as *E. agglomerans*, 4 were identified as *E. gergoviae* and 1 was identified as *E. asburiae*. UPGMA cluster analysis revealed that, all the isolates were grouped in three clusters and the different species of *Enterobacter* were phenotypically closely related. UPGMA cluster analysis based on ARDRA analysis successfully demarcated the *Enterobacter* strains into four distinct clusters. *E. cloacae*, *E. asburiae and E. aerogenes* were grouped under cluster I, while *E. agglomerans* and *E. taylorae* were grouped under cluster II and III respectively, whereas *E. gergoviae* and *E. sakazakii* were placed under cluster IV (Dour 2006).

Bacillary dysentery was first differentiated from amoebic dysentery with respect to an etiologic agent (Shiga 1898). *Dysenteriae* type 1, the first *Shigella* species isolated was discovered by Kiyoshi Shiga in 1896 (Shiga 1898). *Shigella* infections are usually self-limiting, but bacillary dysentery can be life threatening in infants as a result of dehydration or chronic malnutrition. Diarrheal disease is most often associated with *S. sonnei* infection, whereas *S. flexneri* and *S. dysenteriae* may have a prodrome of diarrhea but or usually characterized by dysentery (Hale 1991; Niyogi 2005). Biochemical characterization of the *Shigella* isolates resulted in the differentiation of 40 isolates into 3 species (*S. dysenteriae*, *S. flexneri*, and *S. sonnei*) during 2005–2006. Both ARDRA and RAPD-PCR showed that the *Shigella* isolates examined were very heterogeneous. Unlike the ARDRA, where the differentiation was species specific, in RAPD the isolates were found to be distributed in all the clusters, depending on the banding pattern. This observation establishes the pre-eminence of ARDRA over RAPD in establishing the genetic delineation among the fresh water isolates of the river Narmada (Sharma et al. 2009b).

Klebsiella spp. is mostly confined to environmental niche, however clinically important species include *K. oxytoca* and *K. pneumoniae*. A high degree of genetic heterogeneity is associated with these two species demonstrated by capsular typing, protein profiling, ribotyping and RAPD analysis (Wong et al. 1993). A total of 40 isolates of *Klebsiella* spp. were identified based on biochemical and molecular identification during the study on river Narmada (2005–2006). Biotyping and ARDRA analysis indicated the presence of three species (*K. pneumoniae, K. terrigena, and K. oxytoca*). Variation in distribution of the isolates was observed throughout the different sampling points. A higher load of *K. pneumoniae* in environment source raised concern over the pollution index of the river as it is the most important source of drinking water supply and other recreational activities in this region (Sharma et al. 2007).

The presence of diverge species of enteropathogenic bacteria in river Narmada and their persistence raises questions over the pollution status of the river and also makes a compulsory mandate for regular surveying, sampling, identification and characterization of these bacteria so as to provide important information related to public welfare and health safety regulations. Molecular typing methods have played an important role in both identification and characterization of strains and establishing their phylogenetic relationship.

Molecular phylogenetic analysis plays a very important role in the study of microbial diversity for both cultivated and uncultivated microbes, the comparative sequence analysis of 16S rRNA gene investigation of phylogenetic relationships among microorganisms in a manner that was not feasible through traditional microbiological methods. As a result, there are drastic revisions in our understanding of microbiology (Tian et al. 2003). The phylogenetic diversity based on the comparative sequence analysis of 16S rRNA gene produced new insights into biodiversity conservation. It provides a method for biodiversity judgment and guidelines for biodiversity. Also, it produces the aim of conservation, which is to sustain the phylogenetic information as much as possible.

In principle, all genotypic, phenotypic and phylogenetic information may be incorporated in polyphasic taxonomy. Genotypic information is derived from proteins, their functional different chemotaxonomic markers and a wide range of other expressed features (Vandomme et al. 1996). The first attempt to characterize environmental samples by studying rRNA began about two decade ago. In these studies, 5S rRNA gene molecules were directly extracted from mixed samples, the molecules belonging to different community members were electrophoretically separated and a comparative sequence analysis yielded phylogenetic placements (Amann et al. 1995).

2.2.2 Important Factors Related to Microbial Virulence and Pathogenecity

Important enteric pathogens are becoming increasingly resistant to the major antibiotics that are needed for optimal treatment of patients. The bacterial pathogens are very different from one another. They cause quite different clinical syndromes; their ecology, epidemiology, and modes of transmission are distinct; and they are widely separated genetically. The fact that such different organisms are becoming increasingly antibiotic-resistant underlines the pervasiveness of the pressures that lead to the emergence and spread of resistance (Sack et al. 2001).

Apart from chemical pollution caused by antibiotics themselves, the use of antibiotics may also accelerate the development of antibiotic resistance genes (ARGs) and bacteria, which shade health risks to humans and animals (Kemper 2008). These bacteria might be transmitted from environment to human via direct or indirect contact (Iversen et al. 2004; Kim et al. 2005; Rodríguez et al. 2006). Considering the growing evidences that clinical resistance is intimately associated

with environmental ARGs and bacteria (Tatavarthy et al. 2006; Prabhu et al. 2007; Abriouel et al. 2008), it is quite clear that the research activities need to be extended to include nonpathogenic or environmental microorganisms. Applications of antibiotics in human, veterinary medicine, and agriculture for nearly 60 years have exerted a major impact on bacterial communities, resulting in various resistances to the antibiotics, which is genetically controlled by ARGs. The use of antibiotics results in hundreds of ARGs being detected in various water environments.

2.2.3 Mechanism and Route of Microbial Resistance

The emergence and maintenance of drug resistance in cholera is governed by a complex series of biological, environmental, and behavioural factors. Transposons, plasmids, mobile gene cassettes, and integrons mediate the rapid and broad dissemination of genetic information across species lines. Although antimicrobial resistance can result from the accumulation of chromosomal point mutations, the vast majority of clinically relevant resistance in *V. cholerae* is due to exchange of genetic information among bacterial strains via plasmids and transposons. Laboratory experiments have shown that such exchange of bacterial genetic material can take place by conjugation, transduction, or transformation (Ogg et al. 1978).

In clinical settings, plasmid-mediated transfer has accounted for the emergence and dissemination of resistance genes in cholera. Most plasmids isolated from *V. cholerae* O1 are cryptic, but some encode antibiotic resistance determinants (R-factors). These R-plasmids are large (110–170 kb), self-transmissible, and usually of the "C" incompatibility group. In the 1970s it was reported that, in the laboratory, R-plasmids were unstable in *V. cholerae* and were easily eliminated in drug-free conditions (Yokoto et al. 1972).

A similar observation had been made earlier by Kuwahara et al. (1963) after *in vitro* transmission of plasmids from *Shigella* spp. to *V. cholerae*. However, stability of certain R-plasmids was reported some years later (Rahal et al. 1978). R-plasmids have been found that carry genes encoding resistance to ampicillin, chloramphenicol, gentamicin, kanamycin, spectinomycin, streptomycin, sulfonamides, tetracycline, and TMP, with up to seven resistance determinants on a single plasmid (Threlfall et al. 1980).

Most of these genes are prevalent among the members of *Enterobacteriaceae*. Plasmids of the "C" incompatibility group are found in a wide variety of bacterial genera, including *Pseudomonas*, *Proteus*, *Klebsiella*, and *Serratia*. Bacteria may acquire resistance genes from other species of the normal intestinal flora under the selective pressure of antimicrobial use. *V. cholerae* R-plasmids have been shown to carry resistance determinants (e.g. for ampicillin and TMP) that are common in enteric bacteria (Young and Amyes 1986). When Rahal et al. (1978) examined the transferability and maintenance of plasmids, they found that although plasmids of most incompatibility groups could be transferred from *E. coli* to *V. cholerae*, only those of groups "C" and "J" were maintained.

2.3 Multidrug Resistance, Virulence Genes and ARGs Circulating in the Enteropathogen Gene Pool of River Narmada

The percentage multiple resistance exhibited by the isolates of *V. cholerae* non-O1, *V. fischeri*, *V. parahaemolyticus*, *V. vulnificus*, *V. mimicus*, *V. hollisae*, and *V. proteolyticus* from river Narmada was determined to be 78.56%, 30%, 42.85%, 67.87%, 76.87%, 42.85%, and 37.16% respectively. Cholera toxin gene (*ctxA*) and the gene for toxin co-regulated pili (*tcpA* of ElTor variants) were present in 14 isolates (~58.53% *V. cholerae* non-O1). The identity of *Vibrio cholerae* was confirmed by screening with the *OmpW* gene, which showed 100% specificity for all *V. cholerae* strains under study. The nucleotide sequence of the *tcpA* and *ctxA* gene has revealed an amplicon of 758 and 564 base pair. The sequences were submitted in NCBI Gen Bank with the accession number **DQ 132784** and **DQ 132785.** The virulence of vibrios is regulated by the *ctxAB* and *tcpA* genes. These genes are alleged to be exclusively associated with clinical strains of O1 and O139 serogroups. Cholera toxin gene (*ctxA*) and the gene for toxin coregulated pili (*tcpA* of Classical variants) were present in 14 non-O1 *V. cholerae* isolates (~13%) (Sharma et al. 2009a; Chaturvedi A 2006; Sharma and Chaturvedi 2006). In the period of 2009, multidrug resistance increased in the environmental *Vibrio* isolates, out of 59 isolates more than 50% showed resistance against five commonly used antibiotics; ampicillin, ceftazidime, erythromycin, chloramphenicol, and cefuroxime. Plasmids of 6 kb were detected in 11 resistant isolates and class 1 intergron was detected in 16 resistant isolates. Plasmid analysis showed that plasmid of 6 kb size was present in 11 *Vibrio* isolates, that can be responsible for β-lactamases and broad spectrum cephalosporin resistance, in addition to the aminoglycoside and co-trimoxazole resistance corresponding to their dissemination through mobile genetic elements. Plasmids and class 1 integrons have contributed to the circulation of multidrug resistance determinants in *Vibrio* species (Sharma et al. 2009a).

The strains of *Aeromonas* showed highly active hydrolytic enzymes like protease, amylase, lipase and DNase. Isolates were also found to produce beta-hemolysin, *hly* A gene coding for hemolysin activity was detected in the environmental strains of *Aeromonas*. Multiple drug resistance associated with such strains may aggravate the infection with this pathogen and the marked resistance of the strains against beta-lactam antibiotics increases the risk of infection and the dissemination of antibiotic resistance genes among the closely related members in the riverine system (Sharma et al. 2005).

Out of the 57 isolates of *Enterobacter*, 42 (73.68%) were found to be ESBL producers. Among these 42 ESBL producers, 23 (40.35%) were found to be ESBL producers with derepressed mutants while the remaining 19 (33.33%) were plain ESBL producer. Seven (12.28%) isolates of *Enterobacter* were only derepressed and 2 (9.09%) isolates were AmpC producers. A multiplex PCR was designed to detect bla_{TEM}, bla_{SHV} and *Amp*C genes in *Enterobacter* strains. Each PCR amplification

reaction generated a unique DNA fragment of the expected size bla_{TEM} (1,079 bp), bla_{SHV} (868 bp) and *Amp*C (750–800 bp). PCR amplification products for *Amp*C were obtained for all the *Enterobacter* strains tested. *E. taylorae* and *E. agglomerans* have been reported for the first time as β-lactamases positive species. These strains of *Enterobacter* are likely to express chromosomal AmpC β-lactamases. The study elaborates the knowledge about the β-lactamases of the six *Enterobacter* species that are likely to be human pathogens present in riverine environment (Sharma et al. 2008b). Within the family of Enterobacteriaceae, natural resistance to majority of the most β-lactam antibiotics is attributed predominantly to the action of chromosomally expressed AmpC or class A β-lactamases. With few exceptions, each species within the Enterobacteriaceae is thought to express its own β-lactamase. The *Enterobacter* isolates were found to be highly resistant against 1st, 2nd, and 3rd generation cephalosporin. All isolates were resistant to ampicillin; 45.61% to cotrimoxazole, 64.91% to amikacin, 57.89% to ceftazidime, 66.66% to gentamicin, 22.80% to chloramphenicol, 38.59% to cefotaxime and 45.61% to nalidixic acid. Plasmid analysis indicated that the 30 kb gene fragment is responsible for the epidemics that occurs throughout the world. In 10.5% isolates, class 1 integron were detected, indicating the role of integrons in the development of multidrug resistance in the pathogen. It is significant that the environmental gene pool holds the key to transfer the drug resistant genes in clinical sources (Sharma et al. 2009c).

The antibiogram analysis of *Shigella* spp. showed that out of 7 isolates of *S. dysenteriae,* 71.4% of the strains were resistant to amoxicillin, 57% to gentamycin, 42.8% to each amikacin and nalidixic acid, 28.5% to each ampicillin, streptomycin and tetracycline, 14.3% to ceftazidime, ciprofloxacin and norfloxacin (Sharma et al. 2009b). The protein product of *IpaH* gene is necessary for the invasion of colonic epithelial cells and also for the detection of *Shigella* in the environment. The invasive gene *ipaH* (427 bp) was present in all the 40 isolates confirming the presence of *Shigella* in river water. The *ipaBCD* gene (500 bp) was present in all the 23 isolates of *S. flexneri*, while it was absent in all the isolates of *S. dysenteriae* and *S. sonnei*. The shiga toxin (*stx1*) gene was present in all the 7 isolates of *S. dysenteriae* and absent in all the isolates of *S. flexneri* and *S. sonnei* (Sharma et al. 2009b).

Analysis of plasmid DNA revealed that most of the *Shigella* isolates contained multiple plasmids (1–4 plasmid bands) ranging from 18 to 220 kb and formed a number of unique banding patterns. Twelve distinct plasmid profiles were identified. The presence of *Shigella* in the aquatic environment and dispersion of different virulence genes among the isolates, which appears to constitute an environmental reservoir of *Shigella* specific virulence genes. Since critical virulence genes in *Shigella* are carried by plasmids or mobile genetic elements, the environmental gene pool may contribute to an optimum combination of genes, causing the emergence of virulent *Shigella* strains, which is facilitated in particular by close contact of the population with surface water (Sharma et al. 2010).

2.3.1 ARGs in Environmental Gene Pool (Table 2.2)

2.3.1.1 ARGs Related to Tetracycline

Tetracycline-resistant bacteria were found to emerge in the environments with the introduction of tetracycline (Dancer et al. 1997). There have been at least 38 different tetracycline resistance (*tet*) genes and three oxytetracycline resistance (*otr*) genes characterized to date (Roberts 2005; Thompson et al. 2007). These genes include 23 genes, which code for efflux proteins (efflux pump mechanism), 11 genes for ribosomal protection proteins (target modification mechanism), and three genes for an inactivating enzyme and one gene with unknown resistance mechanism (Levy et al. 1999; Roberts 2005).

Among them, more than 22 *tet* or *otr* genes have been found in bacterial isolates from water environments. Most environmental *tet* genes code for transport proteins, which pump the antibiotics out of the bacterial cell and keep the intercellular concentrations low to make ribosomes function normally (Roberts 2002). The efflux genes of *tet*A, B, C, D, and E frequently appeared in various environmental compartments including activated sludge of sewage treatment plants (STPs) (Guillaume et al. 2000), fish farming ponds (Schmidt et al. 2001; Dang et al. 2007), surface water (Poppe et al. 2006), and swine lagoon (Macauley et al. 2007).

2.3.1.2 ARGs Related to Aminoglycoside

Different from tetracycline resistance mechanisms mentioned above, the most major mechanism of aminoglycoside resistance is direct deactivation of this type of antibiotics by enzymatic modification (Shakil et al. 2008). More than 50 modification enzymes have been found so far (Vakulenko and Mobashery 2003; Ramon-Garcia et al. 2006).

These enzymes are divided into three groups based upon their biochemical actions on the aminoglycoside substrates, including acetyltransferases, phosphotransferases, and nucleotidyltransferases (adenylyltransferases), encoded by three types of genes, namely, *aac, aph,* and *ant* (*aad*), respectively. Different aminoglycoside-modifying enzymes have been reported in a broad range of bacteria isolated from patients or clinical environments (Filipova et al. 2006; Kelmani Chandrakanth et al. 2008).

The *aac, aph*, and *ant* genes are widely distributed in various genera including *Aeromonas*, *Escherichia*, *Vibrio, Salmonella*, and *Listeria* spp. isolated from polluted or natural water environments (Tennstedt et al. 2003; Henriques et al. 2006a; Mukherjee and Chakraborty 2006; Moura et al. 2007).

Table 2.2 Antibiotic resistance genes present in the different Enteropathogens inhabiting water environment

Gene	Biological source	Reference
Tetracycline resistance genes		
*tet*A	*Aeromonas, Alcaligenes, Arthrobacter, Comamonas, Escherichia, Listeria, Pseudomonas, Salmonella,* and *Vibrio*	Szczepanowski et al. (2004), Agersø and Sandvang (2005), Srinivasan et al. (2005), and Tennstedt et al. (2005)
*tet*D	*Aeromonas, Escherichia*; microbial community	Agersø and Sandvang (2005), Akinbowale et al. (2007a), and Macauley et al. (2007)
*tet*E	*Aeromonas, Pseudoalteromonas,* and *Vibrio*	Schmidt et al. (2001), Dang et al. (2006), and Agersø and Petersen (2007)
*tet*M	*Aeromonas, Bacillus, Escherichia, Pseudoalteromonas, Lactococcus* and *Vibrio*; microbial community	Chee-Sanford et al. (2001), Pei et al. (2006): Mackie et al. (2006), Akinbowale et al. (2007b), Auerbach et al. (2007), Dang et al. (2007), Kim et al. (2007), Nonaka et al. (2007), Hu et al. (2008), Rahman et al. (2008), and Suzuki et al. (2008)
Aminoglycoside resistance genes		
*aad*A1	*Aeromonas, Escherichia* and *Vibrio*	Dalsgaard et al. (2000), Tennstedt et al. (2003), Heuer et al. (2004), and Taviani et al. (2008)
*aad*A5	*Escherichia* and *Vibrio*	Park et al. (2003), Tennstedt et al. (2003), and Mohapatra et al. (2008)
*sat*1 and *sat*2	*Aeromonas* and *Escherichia*	Henriques et al. (2006a) and Moura et al. (2007)
Macrolide, chloramphenicol, and vancomycin resistance genes		
*erm*C, *erm*E, *erm*F (Macrolide resistance genes)	Microbial community	Chen et al. (2007) and Patterson et al. (2007)
flo_R (Chloramphenicol resistance genes)	*Listeria, Pseudoalteromonas, Salmonella* and *Vibrio*	Srinivasan et al. (2005), Poppe et al. (2006), and Dang et al. (2007)
*cat*B3 and *cat*II	*Aeromonas* and *Vibrio*	Jacobs and Chenia (2007) and Dang et al. (2007)
Sulphonamide and trimethoprim resistance genes		
*dfr*A1, *dfr*A12, *dfr*A15, *dfr*A18 (Dihydrofolate reductase encoding genes)	*Aeromonas, Escherichia*, and *Salmonella*	Henriques et al. (2006a), Mukherjee and Chakraborty (2006), and Moura et al. (2007)
sulI (Dihydropteroate synthase encoding genes)	*Aeromonas*, *Escherichia*, and *Listeria;*	Heuer et al. (2004), Lin and Biyela (2005), and Schlüter et al. (2005)
sulII	*Acinetobacter, Escherichia, Salmonella*, and *Vibrio*	Pei et al. (2006), Agersø and Petersen (2007), Cernat et al. (2007), Hu et al. (2008), and Mohapatra et al. (2008)

2.3.1.3 ARGs Related to Macrolide–Lincosamide–Streptogramin, Chloramphenicol, and Vancomycin

Although structurally unrelated to each other, the three antibiotics, macrolides, lincosamide, and streptogramin, are often investigated simultaneously for microbial resistance, since some macrolide resistance genes (*erm*) encode resistance to two or all three of these compounds (Roberts et al. 1999).

Totally, more than 60 different genes confer resistance to one or more of the macrolide–lincosamide–streptogramin (MLS) antibiotics have been identified (Roberts 2008), including the genes associated with ribosomal RNA (rRNA) methylation, efflux, and inactivation. MLS resistance is mostly mediated by rRNA methylases (encoded by *erm* genes), which methylate the adenine residues to prevent the three antimicrobials from binding to ribosomal protein (Roberts 2002; Cetin et al. 2008).

2.3.1.4 ARGs Related to Sulfonamides and Trimethoprim

Sulfonamides are the first antibiotic developed for large scale introduction into clinical use, which target dihydropteroate synthase (DHPS). Trimethoprim competitively inhibits dihydrofolate reductase (DHFR), which is responsible for the reduction of dihydrofolate to tetrahydrofolate (Alekshun and Levy 2007).

The most widespread trimethoprim resistance mechanism is the replacement of a trimethoprim-sensitive DHFR by a plasmid-, transposon-, or cassette-borne trimethoprim-resistant DHFR (Skold 2001; Blahna et al. 2006). *dfr*A1 is one of the static resistance genes located on class 2 integrons (Blahna et al. 2006), and *dfr* gene cassettes are frequently found in the variable regions of integrons and are often the only gene cassettes present in environmental isolates (Antunes et al. 2006; Mukherjee and Chakraborty 2006).

2.3.1.5 ARGs Related to β-Lactam

β-Lactams are the most widely used antibiotics, and resistance to these antibiotics is a severe threat because they have low toxicity and are used to treat a broad range of infections (Livermore 1996). *bla* genes often coexist with other antimicrobial resistance determinants and can also be associated with mobile genetic elements, increasing the possibility of multidrug resistance and environmental dissemination (Tennstedt et al. 2003; Weldhagen 2004; Schluter et al. 2007).

The plasmids containing *bla* gene obtained from a wastewater treatment plant are frequently associated with transposons and integrons and often simultaneously carry other resistance determinants including *aad* (or *aac*) encoding aminoglycoside nucleotidyltransferase (or acetyltransferase), *cml* encoding chloramphenicol efflux protein, and *cat* encoding chloramphenicol acetyltransferase (Tennstedt et al. 2003).

2.4 Molecular Techniques for the Detection and Characterization of Environmental ARGs

Considering that ARGs are widespread in aquatic environments mentioned above, there is a need for the development and application of molecular methods to investigate the occurrence, transport, and fate of the environmental ARGs. So far, the methods used for detection, typing, and characterization of ARGs have covered, but not been limited to, specific and multiplex polymerase chain reaction (PCR), real-time PCR, DNA sequencing, and hybridization-based techniques including microarray (Zhang et al. 2009).

2.4.1 Molecular Hybridization

Molecular hybridization has been used to detect presence/absence of specific ARGs for nearly 30 years (Mendez et al. 1980). Many improvements have been made on molecular hybridization, especially in probe design and synthesis, so that the technique, especially Southern blot, is still often applied to distinguish different ARGs in one group (i.e., *tet* genes) from each other (Levy et al. 1999) or to identify presence of specific genes in certain environment (Agersø and Petersen 2007; Malik et al. 2008). Southern hybridization and filter-mating experiments demonstrated that tet and class 1 integrons can be co-transferred from soil isolates to *E. coli* and/or *Pseudomonas putida* (Agersø and Sandvang 2005). Using Southern blot or dot blot coupled with PCR method, Malik et al. (2008) found that *amp*C was frequently present in soil samples irrigated with wastewater. PCR-Southern blot assays showed that *tet*39 and *sul*II were common resistance genes in *Acinetobacter* spp. isolates from water and sediments of fish farms. With a number of non radiolabeled systems becoming commercially available, radioactive labelling is no longer an option to label probes. As an important non radiolabeled method, fluorescence in situ hybridization (FISH) has been established and implemented successfully for clinical detection of microbial resistances. The use of FISH technique has been described for the rapid identification of macrolide resistances caused by ribosomal mutations (Russmann et al. 2001).

2.4.2 PCR (Simple and Multiplex PCR)

PCR assays have been widely used in both pure cultures and mixed environmental samples for detection of specific ARGs encoding resistances to aminoglycoside (Mohapatra et al. 2008), chloramphenicol (Dang et al. 2008), β-lactam (Taviani et al. 2008), macrolide (Chen et al. 2007; Patterson et al. 2007), penicillin (Srinivasan et al. 2005), sulphonamide (Agersø and Petersen 2007), tetracycline (Jacobs and Chenia 2007), trimethoprim (Moura et al. 2007), and vancomycin (Caplin et al.

2008). Environmental target DNA or RNA at low concentrations can be amplified and detected by PCR-based methods. However, a false-positive result is often given in the PCR assay. Southern hybridization of PCR products labelled and used as DNA probes to plasmid or chromosome DNA samples from strains harboring target genes can avoid the false-positive PCR results (Akinbowale et al. 2007b). In addition to DNA hybridization, DNA sequencing is another common method used to verify the PCR products of certain ARGs (Thompson et al. 2007).

In order to save time and effort, multiplex PCR methods have been developed and often used for simultaneous detection of more than one environmental ARG, including the genes encoding resistances to vancomycin (Bell et al. 1998), macrolide (Jensen et al. 2002), tetracycline (Agerso et al. 2007), sulfamethoxazole, and trimethoprim (Ramachandran et al. 2007). With various primer pairs in the same PCR reaction system, multiplex PCR can amplify the DNA fragments of several ARGs at the same time (Gilbride et al. 2006). The method saves considerable time and cost when different target regions are investigated simultaneously, but as a result of all the reactions taking place at the same conditions, some DNA amplifications can be inhibited and false-negative results are probably obtained. Another disadvantage of multiple PCR is that the dimer formation between primer pairs can disturb experimental results and lead to poor sensitivity (Markoulatos et al. 2002). Despite of the drawbacks mentioned above, multiplex PCR is still considered as a rapid and convenient method for the detection of multiple ARGs in isolated bacteria or environmental DNA (Agerso et al. 2007).

2.4.3 Quantitative PCR

The quantitative real-time PCR (qRT-PCR) is usually used to quantify target DNA on the basis of the principle that initial concentration can be estimated according to the change of PCR product concentration with amplification cycles (Zhang and Fang 2006). Among the several fluorescent reagents developed for qRT-PCR, SYBR Green is the most common method used to quantify ARGs in bacterial isolates of clinical origin, including *tet* (Morsczeck et al. 2004), *mef*, and *erm* genes (Reinert et al. 2004). Recently, the technique has been frequently used to quantify ARGs in environmental samples, including *tet* genes in beef cattle farms (Yu et al. 2005), groundwater (Mackie et al. 2006), river sediments (Pei et al. 2006), and STPs (Auerbach et al. 2007), as well as *sul* genes in river sediments (Pei et al. 2006) and *npt* genes in river water (Zhu 2007).

2.4.4 DNA Microarray

Compared with other molecular methods, DNA microarray technique is a genomic analysis technique with highthroughput, high-speed and high-delicacy. For detection of antibiotic resistances, DNA microarray can provide detailed, clinically relevant

information on the isolates by detecting the presence or absence of a large number of ARGs simultaneously in a single assay (Gilbride et al. 2006).

Microarray allows detection of antibiotic resistance determinants within several hours and can be used as a time-saving and convenient tool supporting conventional resistance detection assays (Antwerpen et al. 2007). Microarray has been widely used to clinically detect antibiotic resistance of human pathogens *E. coli* (Zhu et al. 2007a), *H. pylori* (Chen et al. 2008a), *Salmonella enterica* (Guard-Bouldin et al. 2007), and *S. aureus* (Zhu et al. 2007b; Spence et al. 2008). The technique can also be applied to analyze genotypic resistance mechanisms of certain antibiotics (Chen et al. 2008b).

2.5 Health Implications of Virulent Enteropathogens in River Narmada

Understanding the ecology of *Shigella* had been limited mainly due to the lack of suitable techniques to detect the presence of *Shigella* in environment samples (Faruque et al. 2002). In developing countries, where sanitation is poor, fresh contamination of surface water by fecal material of dysentery patients may result in the presence of *Shigella* in surface water. Several previous studies have also detected *Shigella* in surface waters or sewage samples (Faruque et al. 2002 and Obi et al. 2004) and have indicated that *Shigella* strains can be transported by surface waters (Mathan et al. 1984). However, the absence or deletion of crucial virulence genes in environmental *Shigella* isolates suggests that the presence of these strains in river water may not be due to fresh fecal contamination of river water, but instead, these strains have survived and persisted in the aquatic environment (Sharma et al. 2009b). It appears that since in the environment the invasive genes do not have any known function, the bacteria apparently lost part of the plasmid-encoded genes. However, a chromosomally located multicopy virulence genes, *ipaH*, which is known to have a role in producing invasive characteristics (Faruque et al. 2002) was found to be more stable and was present in all the environmental strains analyzed. Therefore, PCR screening of these strains for *ipaH* genes proves to be a better indicator for the presence of *Shigella* than screening for the plasmid caring *ipa*BCD genes (Sharma et al. 2010).

Since vibrios are autochthonous to the aquatic environments, monitoring this bacterium is imperative for the control of cholera and other gastrointestinal infections (Choopun et al. 2002). Until recently, all recorded pandemic and epidemic cases of cholera were associated with strains carrying the type O1 antigen. The remaining non-O1 strains were considered of minuscule epidemic significance. However, an epidemic, which began in India, late in 1992 and spread, to several neighbouring countries was caused by an O139 strain (Nair et al. 1994). Intrigued by the surfacing of O139 strain; investigators have focused on the genetic diversity and population structure of *V. cholerae* non-O1 and other *Vibrio* species (Beltran et al. 1999; Dalsgaard et al. 1995).

Enterobacter spp. has been an important source of transferable antibiotic resistance. ESBL production is coded by genes that are prevalently located on large conjugative plasmid of 80–160 kb in size. Since these plasmids are easily transmitted among different member of Enterobacteriaceae, accumulation of resistance genes results in strain that contain multidrug resistant plasmid. For this reason, ESBL producing isolates are resistant to a variety of classes of antibiotics. Moreover the emergence of these multiple resistant *Enterobacter* strains is unfortunately accompanied by a relatively high stability of the plasmid encoding ESBLs (Velasco et al. 2007). Pioneering work has led to the discovery of class 1 integrons in riverine system from central India. The eneteropathogens in river Narmada seem to harbor class 1 integron and also the different antibiotic gene cassettes associated with this class of integron which may act as a reservoir as well as a medium for the dispersion of bacterial antibiotic resistance genes in the environment (Sharma et al. 2009a, c).

2.5.1 Future Prospectives

Predicament linked with the Drug resistant pathogens of River Narmada to the human life can be proposed by studying the Diversity of Enteropathogens. We can correlate information about the phylogenetic identity with the physical and chemical condition of the River water prevailing in the habitat. Ecological modifications of natural environment, climate changes, and the increasing number of susceptible people around the water source are only a few examples of favorable conditions for bacterial proliferation and dissemination. Within this context there are two important matters that are closely connected with emerging infections. The first is the need to ensure the safety of the consumer's health. The second regards the exact identification of the pathogens for the proper cure against them. The presence and persistence of endemic pathogens and their ARG's in the environmental gene pool prescribes the development of cheaper but novel Diagnostic tools for their detection, as an endeavor to improve public health and related safety concerns.

Good vaccine candidates can be developed against the enteropathogens for long lasting memory response and better protection against the enteric diseases in an endemic region.

2.5.2 Conclusions

The river Narmada is a very significant source of fresh water, sustaining millions of populace, and also used for recreational purposes, the occurrence of toxigenic bacterial enteropathogens raises a question regarding potential risk of human exposure; hence it's indispensable to monitor the river water recurrently to check the possibility of any epidemic. Moreover, many studies have been carried out almost exclusively with clinical strains; while there have been few studies on the pathogenic aspects of environmental strains. Since the aquatic environment is

implicated as the reservoir for these microorganisms, and consequently responsible for their transmission in humans, it is obvious that detail studies on the pathogenic potential of the environmental bacterial strains will certainly contribute to understanding the virulence properties of these bacteria and to establish the importance of these significant pathogens of aquatic systems.

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Chapter 3
Insights into the Genetic Relationships Between Environmental and Clinical Strains of *Yersinia enterocolitica* Biovar 1A

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Abstract *Yersinia enterocolitica*, an important food- and water-borne gastrointestinal agent is currently regarded as an emerging pathogen worldwide. *Y. enterocolitica* is represented by six biovars (1A, 1B, 2, 3, 4 and 5) and more than 70 serotypes. Biovar 1A strains have generally been regarded as nonpathogenic as they lack pYV (plasmid for *Yersinia* virulence) plasmid and major chromosomal virulence genes. Despite the lack of known virulence determinants, some biovar 1A strains are associated with the disease indistinguishable from that produced by known pathogenic biovars (1B, 2–5). Genotyping of *Yersinia enterocolitica* biovar 1A revealed two clonal groups (A and B). Multilocus variable number tandem repeat analysis (MLVA) not only supported the genotyping data but also revealed that wastewater isolates represent the ancestral group while the clinical isolates originated from them. Further, multilocus enzyme electrophoresis (MLEE) revealed the genetic relationships among strains of *Y. enterocolitica* biovar 1A, and clustered these into four groups. Multilocus restriction typing (MLRT) data also clustered biovar 1A strains into two clonal groups (A and B). The analysis of MLRT data by BURST (Based Upon Related Sequence Types) programme revealed that wastewater isolates represent the ancestral strains while the clinical isolates occupied radial position as single locus variants. All these studies suggested that the clinical strains probably originated from the wastewater strains, by host adaptation and genetic change. Proteomic analysis using whole cell protein profiling displayed sufficient diversity among strains of *Y. enterocolitica* biovar 1A, and the phylogenetic relationships obtained were in good agreement as established by genotyping techniques. Some pathogenic determinants have also been identified by suppression subtractive hybridization between wastewater and clinical strains. In future, further studies on

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pathogenic markers of *Y. enterocolitica* biovar 1A need to be undertaken. So, this would help us in understanding the pathogenic potential and true public health significance of *Y. enterocolitica* biovar 1A.

Keywords Biovar 1A • REP-ERIC PCR • Multilocus variable number tandem repeat analysis (MLVA) • Multilocus enzyme electrophoresis (MLEE) • Multilocus restriction typing (MLRT) • Suppression subtractive hybridization (SSH) • Based Upon Related Sequence Types (BURST) • India

3.1 Introduction

Yersinia enterocolitica is facultatively anaerobic, gram-negative, asporogenous rod shaped bacterium with a tendency toward pleomorphism ranging from small coccobacilli to more elongated bacilli. Optimal growth occurs at 27–30°C (Bottone 1999). It is an important food- and water-borne gastrointestinal agent and is regarded as an emerging pathogen (Ostroff 1995; Sharma et al. 2003). It causes a variety of gastrointestinal symptoms ranging from mild gastroenteritis to more invasive diseases like terminal ileitis, mesenteric lymphadenitis mimicking appendicitis, and blood transfusion related septicemia (Bottone 1999). High mortality has been reported due to Blood transfusion related septicemia (Leclercq et al. 2005). *Y. enterocolitica* infection is typically initiated by ingestion of contaminated food or water especially through eating raw pork or pork products. *Y. enterocolitica* initially travels to the small intestine and invades the intestinal barrier *via* M cells, a specialized group of follicle-associated epithelial cells that function in antigen uptake (Bottone 1999; Pujol and Bliska 2005). After invasion of the intestinal epithelium, the bacteria replicate within lymphoid follicles or the Peyer's patches. *Y. enterocolitica* survive and multiply in lymphoid tissues due to presence of an approximately 70 kb virulence plasmid, termed pYV (plasmid for *Yersinia* virulence). The bacteria can spread from lymphoid follicles to mesenteric lymph nodes, resulting in mesenteric lymphadenitis. *Y. enterocolitica* is quite heterogeneous species (Fredriksson-Ahomaa and Korkeala 2003) and well characterized biochemically, serologically and pathogenically, and has been classified into six biovars (1A, 1B, 2, 3, 4 and 5) and more than 50 serotypes. Concerning its pathogenicity, *Y. enterocolitica* can be broadly divided into three groups *viz.* highly pathogenic strains (biovar 1B), strains with moderate pathogenicity (biovars 2–5) and the non-pathogenic biovar 1A strains (Cornelis et al. 1987). Pathogenicity of biovars 1B and 2–5 is due to the presence of virulence genes which are present on both pYV (plasmid for *Yersinia* virulence) plasmid as well as on chromosomal DNA. Plasmid-encoded virulence factors including *Yersinia* adhesin A (YadA) and Ysc-Yop type III secretion system, which are responsible for injection of Yop effector proteins into the host cells (Cornelis and Wolf-Watz 1997). Chromosomally borne virulence genes include *ail* (attachment and invasion locus), *inv* (invasin), *yst* (*Yersinia* stable toxin), *myf*A (mucoid *Yersinia* factor) (Revell and Miller 2001) and genes related to iron utilization (fepA, fepD & fes)

(Schubert et al. 1999). Biovar 1A strains have generally been regarded as avirulent or non-pathogenic (Bottone 1999), because these strains lack both pYV plasmid and major chromosomal virulence genes (Tennant et al. 2003). But, there is growing epidemiological, clinical, and experimental evidence, however, to suggest that some biovar 1A strains are virulent and can cause gastrointestinal diseases (Burnens et al. 1996; Morris et al. 1991). Biovar 1A strains have also been implicated in nosocomial (Ratnam et al. 1982) and food-borne outbreaks (Butt et al. 1991; Greenwood and Hooper 1990), and were isolated from extra-intestinal infections (Bissett et al. 1990). The biovar 1A strains are highly heterogeneous encompassing a plethora of serotypes and non-agglutinable (NAG) forms, and have been isolated from diverse sources (Sinha et al. 2000; Tennant et al. 2003). However more studies are in progress with the other biovars of *Yersinia enterocolitica* including genotyping, molecular and biochemical characterization of strains, identification of virulent genes, which play an important role in their pathogenicity. However analysis of biovar 1A strains by REP-PCR, ERIC-PCR genomic fingerprinting (Sachdeva and Virdi 2004), ribotyping, PCR-ribotyping and *gyr*B gene polymorphism (Gulati and Virdi 2007) generally revealed limited genetic heterogeneity. In this chapter we would highlight our findings on the genetic insights between clinical and environmental strains of biovar 1A strains of *Y. enterocolitica*. The majority of these strains have been isolated from India.

3.2 Studies from India

In India, *Y. enterocolitica* has been isolated from stools of both children and adults suffering from gastroenteritis. First isolation of *Y. enterocolitica* from humans was reported in 1980 (Pramanik et al. 1980).

The first *Y. enterocolitica*-related outbreak of food poisoning in India was reported from North Arcot district of Tamil Nadu (Abraham et al. 1997). *Y. enterocolitica* has been isolated from wastewater (Sinha et al. 2000), river and ground water (Singh et al. 2003), well water (Abraham et al. 1997), pork (Singh and Virdi 1999), fresh buffalo milk (Toora et al. 1989), ice-cream and dairy products (Ramesh et al. 2002) cane juice (Ram et al. 1987), pig throat, intestinal content and rectal swabs (Pramanik et al. 1980; Singh et al. 2003), blood cultures of septicemic patients and stools of the diarrheic human subjects (Pramanik et al. 1980; Ram et al. 1987; Singh et al. 2003).

Although biotyping data of all the strains isolated from India is not available, it was apparent that most of the strains belonged to biovar 1A (Singh and Virdi 1999; Sinha et al. 2000; Singh et al. 2003) except for two biovar 4 strains which were implicated in the food-borne outbreak in Southern India (Abraham et al. 1997) and septicemia (De et al. 1993). *Y. enterocolitica* serotypes and biotypes that have been isolated from India are summarized in Table 3.1. *Y. enterocolitica* isolates belonging to biovar 1B have not been isolated from India as yet. Although most of the Indian strains of *Y. enterocolitica* belong to biovar 1A but encompasses diverse serotypes

Table 3.1 Serovars and biovars of *Y. enterocolitica* isolated from India

Source	Serovar(s)	Biovar	Reference(s)
Humans			
Diarrheic human stools	O:6,30; O:6,30-6,31; NAG	1A	Singh et al. (2003)
	O:3	4	Abraham et al. (1997)
	O:4	ND	Varghese et al. (1984)
	O:9	–	Lal et al. (2003)
	O:1,2a,3	–	Ram et al. (1987)
	O:9,16	–	Pramanik et al. (1980)
Septicemic patients	O:3	4	De et al. (1993)
Animals			
Healthy pigs	ND	1A	Singh et al. (2003)
	O:9,16	–	Pramanik et al. (1980)
	O:3; O:9; O:12,25	4	Verma and Mishra (1984)
Food			
Pork	O:7,8-8-8,19	1A	Singh and Virdi (1999)
Raw milk and milk products	ND	1A	Toora et al. (1989), Vaishnavi et al. (2001), and Ramesh et al. (2002)
	O:5	1A	Ram et al. (1987)
Sugarcane juice	O:22	1A	Ram et al. (1987)
Aquatic			
	O:6,31; O:6,30-6,31; O:10–34; O:15, O:41,42;	1A	Sinha et al. (2000) and Singh et al. (2003)
	O:41,43; NAG	4	
	O:3		Abraham et al. (1997)

NAG non-agglutinable; *ND* not determined

(Table 3.1). *Y. enterocolitica* serotypes O:6,31, O:6,30-6,31, O:15, O:10–34, O:41,42 and O:41,43 belonging to biovar 1A have been recovered from sewage effluents in Delhi (Sinha et al. 2000). As regards isolation from food, 50 isolates of *Y. enterocolitica* have been recovered from fresh buffalo milk in Chandigarh (Toora et al. 1989). All of these isolates were of biovar 1A; however, none of these were serotyped.

In an another study, two isolates of biovar 1A were isolated from sugarcane juice (serotype O:5) and cow milk (serotype O:22). *Y. enterocolitica* strains of serotype O:7,8-8-8,19 and some non-agglutinable strains were also isolated from pork samples collected from a slaughterhouse in Delhi (Singh and Virdi 1999). Besides it, pathogenic serotypes have also been reported from various sources. Isolates of serotype O:9 have been isolated from diarrheic stools (Lal et al. 2003) and apparently healthy pigs (Verma and Mishra 1984). In another study the presence of *Y. enterocolitica* serotype O:9 in swine was evidenced in Bangalore (Krishnappa et al. 1980). Serotype O:3 was isolated from diarrheic human subjects (Abraham et al. 1997), septicemic patients (De et al. 1993) and apparently healthy pigs (Verma and Mishra 1984) in India. Other serotypes which have been isolated from human subjects include O:1,2a,3 (Ram et al. 1987), O:4 (Varghese et al. 1984) and O:9,16 (Pramanik et al. 1980).

3.3 Genotyping of *Yersinia enterocolitica*

Genotyping has been used increasingly to supplement the biotyping and serotyping methods in order to further subtype strains belonging to the same bioserovars. For the genotyping of *Y. enterocolitica* diverse methods *viz.* Microarray (Howard et al. 2006), Repetitive sequence-PCR (REP-ERIC PCR) based genotyping (Kim et al. 2003; Sachdeva and Virdi 2004), 16S-23S intergenic spacer region typing (Lobato et al. 1998), Multilocus sequence typing (MLST) (Kotetishvili et al. 2005), Pulsed-field gel electrophoresis (PFGE) (Fredriksson-Ahomaa et al. 1999, 2004; Capilla et al. 2003), Randomly amplified polymorphic DNA (RAPD), PCR-ribotyping (Wojciech et al. 2004), Variable Number of Tandem Repeats (VNTR) (Gierczyński et al. 2007), Amplified fragment length polymorphism (AFLP) (Fearnley et al. 2005), which have been used for the genotyping of *Y. enterocolitica* vary in their discriminatory power and also in typeability, reproducibility, use and ease in interpretation (Sachdeva and Virdi 2004; Fredriksson-Ahomaa et al. 2006). Most of these studies pertained to pathogenic strains belonging to biovars 1B, 2–4 and serotypes O:3, O:5,27, O:8 and O:9. The genetic diversity of biovar 1A strains belonging to diverse serotypes has been reported only recently (Sachdeva and Virdi 2004; Gulati and Virdi 2007). As compared to pathogenic biovars (1B and 2–4), *Y. enterocolitica* strains belonging to biovar 1A are more diverse. PFGE typing is considered to be the gold-standard technique in discriminating *Y. enterocolitica* strains of same bioserovars and thus it is highly effective in molecular epidemiological studies of *Y. enterocolitica* (Buchrieser et al. 1994).

3.3.1 Repetitive Sequence-PCR (REP-ERIC PCR) Based Genotyping

Two families of repetitive elements, the repetitive extragenic palindrome (REP) and enterobacterial repetitive intergenic consensus (ERIC) sequence have been found in many members of the family *Enterobacteriaceae* and used extensively in molecular typing studies of a wide range of gram-negative bacteria (Versalovic et al. 1991). All these motifs are genetically stable and differ only in their numbers and chromosomal locations between the strains. Repetitive element sequence-based PCR (rep-PCR) genomic fingerprinting has been reported to be reproducible, rapid and sensitive for discrimination of strains of a wide range of bacterial species (Kim et al. 2003; Sachdeva and Virdi 2004).

Odinot et al. 1995 studied *Y. enterocolitica* strains by ERIC-PCR, but did not provide any clear information about the results obtained. In our study, when a collection of 81 strains of *Y. enterocolitica* biovar 1A isolated from India, Europe and USA studied by REP- and ERIC-PCR fingerprinting and then 19 and 23 genotypes were reported respectively (Sachdeva and Virdi 2004). The strains having different serotypes were found to produce identical rep-profiles, and all the isolates clustered into two major groups. Each group included strains from widely separated geographical regions and exhibited similarities ranging from 85% to more than 95%

indicating existence of limited number of clonal groups among biovar 1A strains. These authors also showed that within one group, members of serogroup O:6,30-6,31 isolated from aquatic sources clustered more tightly while those of clinical origin formed a yet another discrete cluster as shown in dendrogram (Fig. 3.1) constructed using UPGMA method (Sachdeva and Virdi 2004).

Another study using rep-PCR showed limited heterogeneity in *Y. enterocolitica* isolated from humans, pigs and foxes. It was observed that human isolates shared common genotypes with pig isolates which further supports epidemiological evidence that swine is source of infection and major reservoir of *Y. enterocolitica* (Wojciech et al. 2004). More studies using rep-PCR based genotyping of strains of *Y. enterocolitica* belonging to serogroups O:3, O:9, O:8, O:5,27 of diverse geographical origin are however warranted in future.

3.3.2 16S-23S Intergenic Spacer Region Typing

16S-23S intergenic spacer region typing, also called as PCR-ribotyping or SR (spacer region) ribotyping, differentiates closely related strains based on variations in the number of copies and sizes of the 16S-23S spacer region of *rrn* operon. Only two studies have reported application of this technique to unravel heterogeneity of clinical strains of *Y. enterocolitica* (Wojciech et al. 2004). Using this method, a group of 84 strains of *Y. enterocolitica* were differentiated into 13 SR ribotypes. It was however observed that same PCR-ribopatterns were assigned to strains belonging to different serotypes (Lobato et al. 1998). A recent study discerned nine different types based on internally transcribed sequence (ITS)-typing of 35 strains of *Y. enterocolitica* isolated from humans, pigs and foxes (Wojciech et al. 2004).

Gulati and Virdi (2007) used 81 strains of *Y. enterocolitica* biovar 1A and analyzed their ribopattern. Ribotyping with *Bgl*I, *Nci*I and *Eco*RV distinguished 81 strains into 4, 3 and 2 ribotypes respectively. *Bgl*I-*Nci*I combination gave the highest Simpson's diversity index (DI = 0.43). Strains with identical ribotypes were further differentiated by PCR-ribotyping. This suggested that combination of the two might be used for molecular epidemiological studies of *Y. enterocolitica* biotype 1A. This study also provides the evidence for the presence of two groups among the strains of *Y. enterocolitica* biovar 1A despite their diverse geographical origins. These data also grouped clinical and non-clinical strains of serotype O:6,30-6,31 into discrete subgroups (Gulati and Virdi 2007).

3.3.3 PCR-Restriction Fragment Length Polymorphism (RFLP) of gyrB Gene

It is becoming increasingly clear that phylogeny based on analysis of a single locus may be flawed (Fukushima et al. 1993). PCR-RFLP of single locus may provide phylogenetic relationships among bacterial strains (Wojciech et al. 2004). Currently

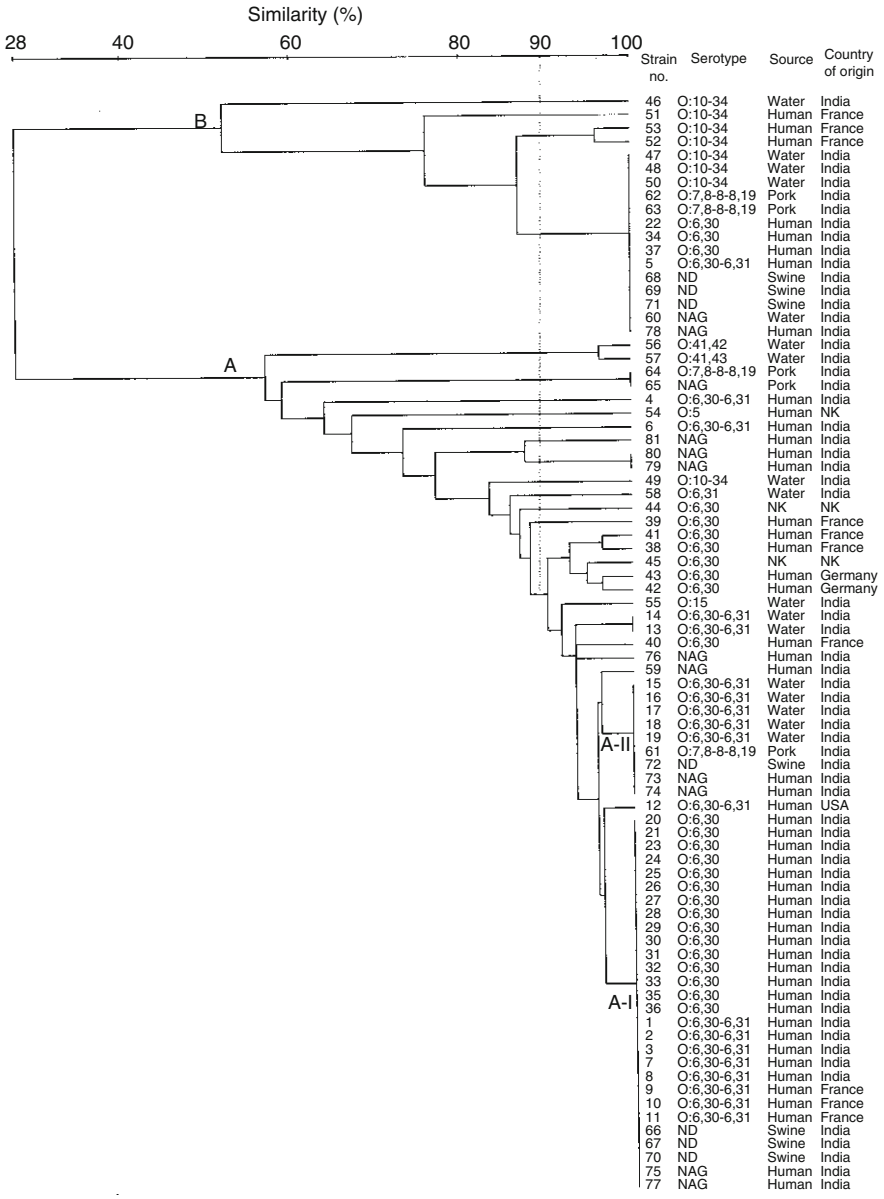

Fig. 3.1 Concatenated dendrogram of REP- and ERIC-PCR fingerprints of *Y. enterocolitica* biotype 1A. The similarities between the fingerprints were calculated by Jaccard coefficient and clustering was performed by UPGMA. *NAG* Non-agglutinable, *ND* Not determined, *NK* Not known (Reproduced from Sachdeva and Virdi 2004)

*gyr*B gene (encodes the B subunit of DNA gyrase) is frequently being used to understand phylogenetic relationships among strains of several organisms apart from the ribosomal genes (Yamamoto and Harayama 1996; Wojciech et al. 2004).

It has been reported that *gyr*B RFLP efficiently detected genomic variability in strains of *Y. enterocolitica* biovar 1A (Gulati and Virdi 2007). *gyr*B RFLP with *Alu*I, *Msp*I and *Hinf*I distinguished 81 strains into four types respectively. PCR-RFLP of *gyr*B gene confirmed the existence of two clonal groups in strains of *Y. enterocolitica* isolated from India. *gyr*B RFLP data also supported the REP-ERIC PCR data which provided the unequivocal evidence for the presence of two clonal groups amongst strains of *Y. enterocolitica* biovar 1A despite their diverse geographical origins (Gulati and Virdi 2007).

3.3.4 PCR-Restriction Fragment Length Polymorphism (RFLP) of bla Genes

Strains belonging to different bioserovar combinations shows different susceptibilities to β-lactams (Pham et al. 2000; Stock et al. 2000), which may be due to the expression of two chromosomal beta lactamases (Bla-A & Bla-B). Bla-A is a class-A constitutive broad spectrum penicillinase whereas Bla-B is a class C inducible cephalosporinase (AmpC). The distribution and expression of the two lactamases in different biovars of *Y. enterocolitica* has been well studied (Pham et al. 1999; Stock et al. 2000; Sharma et al. 2004). Both *bla*A and *bla*B genes were detected in biovar 1A strains of *Y. enterocolitica* by PCR amplification (Sharma et al. 2006). PCR-RFLP revealed heterogeneity in *bla*A, which was represented by three restriction profiles. But, *bla*B failed to reveal any such genetic heterogeneity. The cluster analysis of the restriction profiles of *bla*A provided information broadly similar to that inferred previously from rep (REP/ERIC)- PCR fingerprinting of the isolates (Sachdeva and Virdi 2004). Serogroup O:6,30-6,31 isolates of clinical origin clustered into one group, whereas those of non-clinical origin clustered into another group (shown in Fig. 3.2).

A correlation between REP/ERIC types and restriction profile of *bla*A was also discerned. For example, REP type 1 (R1) was predominantly associated with *bla*A group A; whereas ERIC type 4 (E4) was exclusively associated with *bla*A group C. Another study reported that restriction profiles of β-lactamase genes (blaOXY-1, blaOXY-2) of *Klebsiella oxytoca* correlated very well with ERIC-PCR profiles (Granier et al. 2003). These observations suggest that restriction analysis of β-lactamase genes may be used to study epidemiology, or discern phylogenetic relationships.

3.4 Genetic Relationship Between Environmental and Clinical Strains

Analysis of data based upon genotyping of *Yersinia enterocolitica* biovar 1A strains using different methods *viz* Repetitive sequence-PCR (REP-ERIC PCR) based genotyping (Kim et al. 2003; Sachdeva and Virdi 2004), 16S-23S intergenic spacer region typing (Lobato et al. 1998), Multilocus sequence typing (MLST)

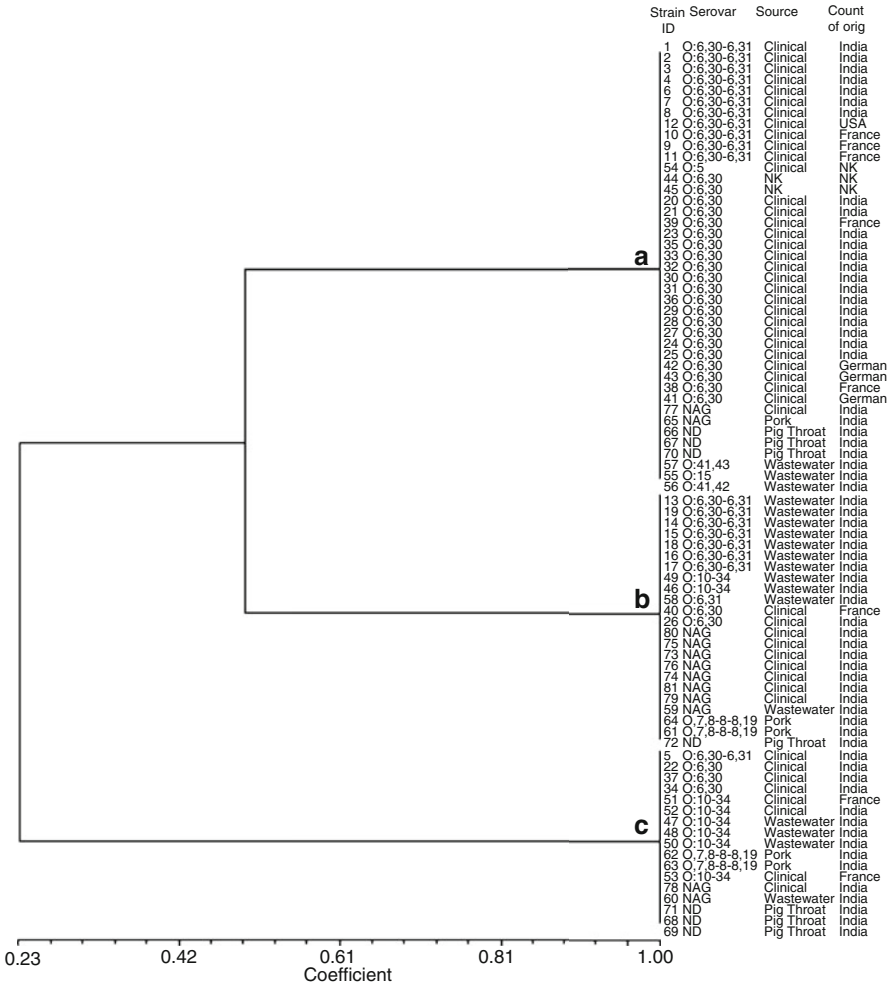

Fig. 3.2 Phylogenetic analysis of blaA gene of *Yersinia enterocolitica* biovar 1A based on restriction profiling. (*ND* not determined; *NK* not known; *NAG* nonagglutinable (Reproduced from Sharma et al. 2006)

(Kotetishvili et al. 2005), Pulsed-field gel electrophoresis (PFGE) (Fredriksson-Ahomaa et al. 1999, 2004; Capilla et al. 2003), PCR-ribotyping (Wojciech et al. 2004), Variable Number of Tandem Repeats (VNTR) (Gierczyński et al. 2007), Amplified fragment length polymorphism (AFLP) (Fearnley et al. 2005), *gyr*B gene polymorphism (Gulati and Virdi 2007) generally revealed limited genetic heterogeneity. Genotypic methods could reveal the clonal groups of clinical and environmental strains of *Y. enterocolitica* biovar 1A strains (Sachdeva and Virdi 2004; Gulati and Virdi 2007), but these studies failed to reveal any genetic association between serotypes and the sources of isolation of these strains. However

genotyping methods did not provide any information regarding the evolution of these strains. Whether the clinical strains originated first or environmental strains served as the ancestral types.

Multilocus variable number tandem repeat analysis (MLVA) has shown to be a promising method for genotyping several pathogenic bacteria (Lindstedt 2005; Sperry et al. 2008). The genetic relationship among 88 strains of *Y. enterocolitica* using polymorphism at four variable number of tandem repeats (VNTR) loci were examined by cluster analysis and a dendrogram was constructed. MLVA clustered *Y. enterocolitica* biovar 1A strains clearly into two major groups - A and B. Group-A included most of the isolates of *Y. enterocolitica* biovar 1A, which was further divided into subgroups namely A-I, A-II and A-III (Gulati et al. 2009). Subgroup A-I exclusively comprised of all the human serotype O:6,30-6,31 and O:6,30 isolates along with the three pig and one pork isolates. On the other hand, all wastewater serotype O:6,30-6,31 isolates generated identical MLVA genotype and clustered in subgroup A-II. All the reference strains (biovar 1B, 2 & 4) except one grouped in subgroup A-III. Genotypically group-B was found to be very homogeneous. All Indian isolates in this group were identical in their MLVA profiles.

Population modelling using the minimum spanning tree (MST) supported the two groups – A and B among strains of *Y. enterocolitica* biovar 1A. Each of the two groups was represented by a predominant MLVA genotype. The other MLVA genotypes radiated from these two dominant ancestral profiles. MST also revealed that in group-A, wastewater O:6,30–6,31 isolates represent the ancestral strains while the clinical O:6,30–6,31 isolates occupy a radial positions (Fig. 3.3) (Gulati et al. 2009).

Genetic relationships among 81 strains of *Y. enterocolitica* biovar 1A isolated from clinical and non-clinical sources were also discerned by multilocus enzyme electrophoresis (MLEE) and multilocus restriction typing (MLRT) (Mallik and Virdi 2010a).

Multilocus enzyme electrophoresis (MLEE) has been used to study genetic relationships where allelic variations in housekeeping genes are indexed using electrophoretic mobilities of corresponding enzymes (Selander et al. 1986; Caugant et al. 1989). The technique has been used to study epidemiology of several pathogenic bacteria (Farfán et al. 2000; Scortichini et al. 2003). Activities of six enzymes (malate dehydrogenase, adenylate cyclase, glutamine synthetase, glucose-6-phosphate dehydrogenase, isocitrate dehydrogenas and glutamate dehydrogenase) were detected using MLEE in 81 strains of *Y. enterocolitica* biovar 1A and including one reference strain *Y. enterocolitica* 8081. MLEE revealed the genetic relationships among strains of *Y. enterocolitica* biovar 1A, and clustered into four groups (Mallik and Virdi 2010a). In MLEE dendrogram, two ETs showed some pork and pig strains to be identical to the strains isolated from diarrheic human subjects suggesting that like pathogenic biovars, pigs may be the source of biovar 1A strains isolated from human patients. No such grouping of human and pork/pig isolates was evident as from earlier studies (Gulati and Virdi 2007; Sachdeva and Virdi 2004). However, this study needs to be explored globally, by making use of a larger number of pig/pork isolates belonging to biovar 1A. A close analysis of data presented by Dolina and Peduzzi (1993), who studied human, animal and aquatic strains of *Y. enteroco-*

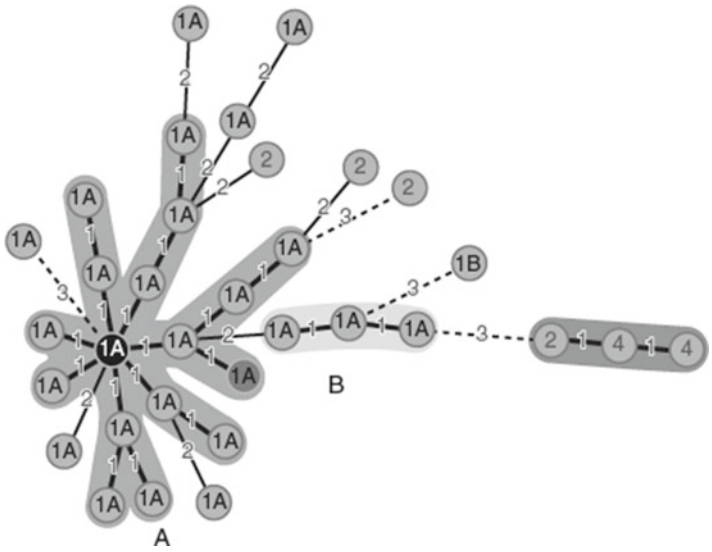

Fig. 3.3 Population modelling by minimum spanning tree (MST) using MLVA data. Each *circle* in the tree represents a different MLVA type; *letter* in the circle denotes biovar of the isolate. The MLVA data separates the isolates into two distinct MST tree branches – A and B. Biovars *2* and *4* form a separate group. The *thickness* and the *dotting* of the lines indicate the distance between the circles. A *thicker line* denotes closer distance than a *thin line*, and a *thin line* denotes closer distance than a *dotted line*. The *number* on each line denotes the number of loci that differ between the MLVA types. The *black circle* indicates wastewater serotype O:6,30–6,31 isolates, while the *grey circle* indicates clinical serotype O:6,30–6,31 isolates (Reproduced from Gulati et al. 2009)

litica isolated from Switzerland by MLEE, revealed that 51 biovar 1A strains clustered into two major groups, although minor clusters having one and six isolates each were also observed.

Multilocus restriction typing (MLRT), a recently developed tool analyses restriction fragment length polymorphism of several housekeeping genes (Coenye and Lipuma 2002; Bennett and cafferkey 2003). Mallik and Virdi (2010a) used six loci for MLRT and PCR amplicons of genes were obtained for the 81 strains of *Y. enterocolitica* biovar 1A. Collating the PCR-RFLP data for six loci in 81 strains, 12 restriction types (RTs) were identified. MLRT also clustered biovar 1A strains into two clonal groups (A and B) (Mallik and Virdi 2010a). The analysis of MLRT data by BURST program identified two clonal complexes (Fig. 3.4) corresponding to the clonal groups as identified above. The clonal complex A comprising 9 RTs (64 strains) revealed that wastewater serotype O:6,30-6,31 isolates represented by RT2 were present in the innermost circle as ancestral strains. The clinical serotype O:6,30-6,31 strains represented by RT1 and RT12 were present in the outer circle as single locus variants (Fig. 3.4a) The double locus variants (RT5 and RT9) and the satellite RTs (RT6 and RT10) were represented by serotypes which are relatively not

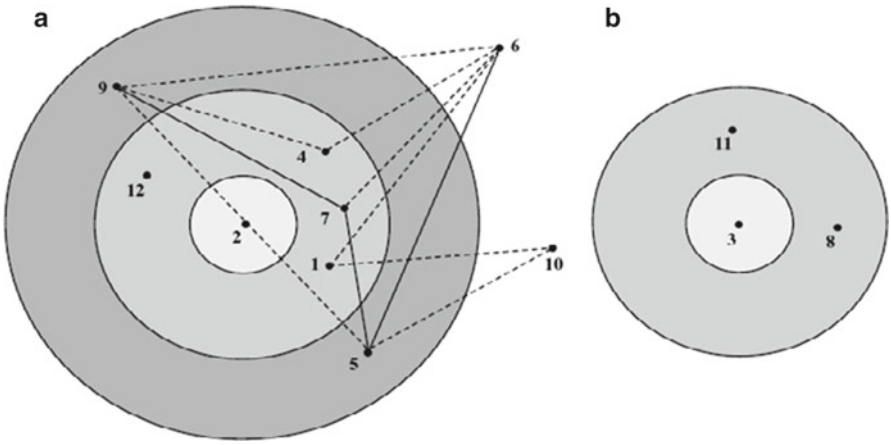

Fig. 3.4 Clonal complexes identified among 81 strains of *Y. enterocolitica* biovar 1A by BURST analysis of MLRT data. (**a**) Clonal complex A, (**b**) Clonal complex B. Each *number* denotes a restriction type. Radial distribution shows divergent RTs. *Ancestral RT* is shown in the innermost circle (Reproduced from Mallik and Virdi 2010a)

common. However, not much information could be inferred from clonal complex B (Fig. 3.4b).

The approach used in the BURST (Based Upon Related Sequence Types) analysis specifically examines the relationships between closely related genotypes in the clonal complexes (Jolley et al. 2001). This analysis revealed that in the primary clonal complex, wastewater serotype O:6,30-6,31 isolates represented the ancestral strains while, clinical serotype O:6,30-6,31 strains occupied radial position as single locus variants. This observation corroborates the recent findings obtained from the study of VNTR loci which also suggested that the clinical serotype O:6,30-6,31 strains probably originated from the wastewater strains, by host adaptation and genetic change (Gulati et al. 2009).

3.5 Genomic Comparison by Suppression Subtractive Hybridization

Comparisons of clinical and environmental strains are of great biological and clinical interest today. There are many general procedures for genomic comparison, and to detect virulence genes such as microarray (Salama et al. 2000), differential display polymerase chain reaction (DD PCR) (Rivera-Marrero et al. 1998), and suppression subtractive hybridization (SSH) (Diatchenko et al. 1996). For the identification of genetic differences between clinical and environmental (virulent and avirulent) strains SSH is powerful and efficient method. This method provides valuable information for presumptive virulence genes of pathogenic bacteria.

SSH is an efficient PCR-based subtractive hybridization method for genome comparison, which is used to selectively amplify the target sequences and simultaneously suppress non-target DNA amplification (Diatchenko et al. 1996). In principle, SSH is based on suppression PCR, which means using the long inverted terminal repeats (LITR) to link to the ends of the tester sample to form stable panhandle-like loop structures in each denaturation–annealing cycle. This resulting "panhandle- like" structure can prevent amplification of non-targeted sequences in PCR reaction, which is the so-called suppression PCR effect. Consequently, no exponential amplification occurs in non-target sequences.

This method is generally divided into six steps, including; (1) synthesis of tester or driver DNA (2) digestion of tester or driver DNA by restriction enzyme (3) adapter ligations to tester DNA (4) two successive subtractive hybridization (5) PCR amplification of target sequences; and (6) construction of the subtracted library. The schematic representation of SSH is shown in Fig. 3.5.

For the comparison and identification of potential virulence genes in pathogenic strains of *Y. enterocolitica* biovar 1A, Tennant et al. (2005) used genomic subtractive hybridization to determine genetic differences between two biovar 1A strains: a clinical isolate, *Y. enterocolitica* T83 (Biovar 1A, O:5, clinical isolate, New Zealand) as the tester, and an environmental isolate, *Y. enterocolitica* IP2222 (Biovar 1A, O:36, water isolate, Japan) as the driver strain. After SSH three genes (*tcb*A, *tca*C, and *tcc*C) specific to *Y. enterocolitica* T83 were identified, that showed homology to the insecticidal toxin complex (TC) genes first discovered in *Photorhabdus luminescens*. In *P. luminescens*, the TC genes encode high-molecular weight toxins capable of killing insects (Bowen et al. 1998). The results supported by TC genes inactivation in *Y. enterocolitica* T83, resulted in mutants, which were attenuated in the ability to colonize the gastrointestinal tracts of perorally infected mice. These results indicate that products of the TC gene complex contribute to the virulence of some strains of *Y. enterocolitica* biovar 1A, possibly by facilitating their persistence *in vivo*. This approach led to the identification of novel virulence-associated genes of *Y. enterocolitica* biovar 1A, related to the insecticidal toxin complex (TC) genes of other bacterial species.

In our study, Suppression subtractive hybridization was carried out between a clinical (serotype O: 6,30-6,31, Human stools, tester) and environmental strain (serotype O: 6,30-6,31, waste water, driver) strain of *Yersinia enterocolitica* to identify the tester-specific sequences that are exclusively present in tester strain. After SSH, The genomic fragments which were not found in *Yersinia enterocolitica* (Serotype O: 6,30-6,31, Driver strain) were sequenced. These sequences were then analyzed at both nucleotide and protein level using BLAST homology search. The results showed that these sequences having homology to proteins involved in iron acquisition system, hemin storage system, outer membrane protein PgaA, flagellar hook proteins, secretion system, transport systems and others of unknown function. These are shown in Table 3.2. All these sequences showed high homology to *Yersinia enterocolitica* 8081 (Biovar 1B). However these sequences are also showing homology to *Y. pseudotuebculosis*, *Salmonella enterica*, *Aeromonas* spp., *Serratia* spp. and others.

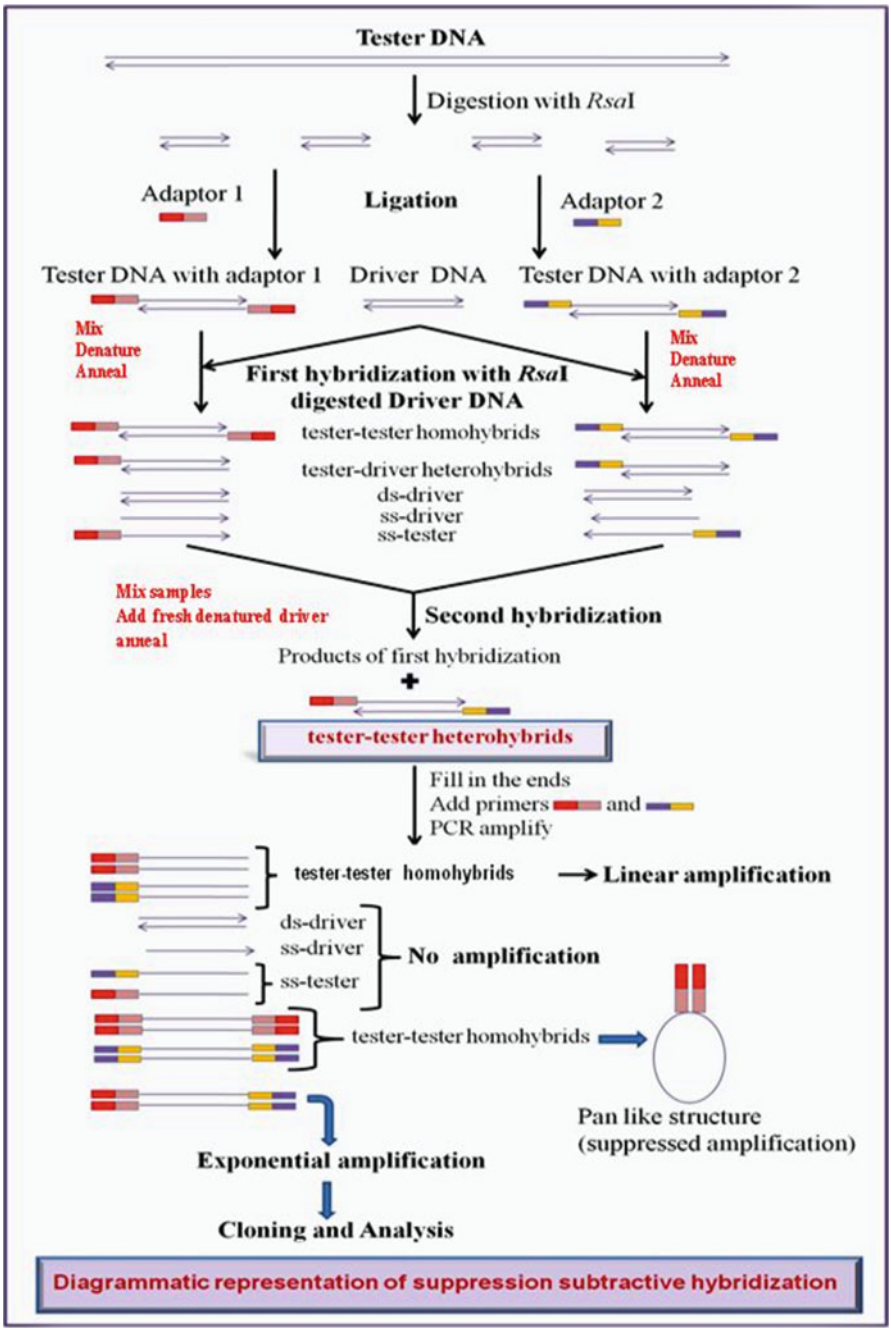

Fig. 3.5 Suppression subtractive hybridization

Table 3.2 Genes identified by SSH in tester strain

Clone no.	Insert size	Homologies to predicted encoded protein	E-value	Similarity (% amino acid)	Genbank accession no.
Clone no 8	276	Conserved hypothetical protein	2e-39	81/84 (96%)	GU253386
Clone no 10	236	Hemophore A (HasA)	2e-14	42/52 (80%)	GU253387
Clone no 18	265	Type 1 secretion protein	2e-48	110/123 (89%)	GU253388
Clone no 51	643	Haemin storage system (HMS protein)	8e-104	212/218 (97%)	GU253389
Clone no 57	682	Flagellar hook protein	1e-71	206/216 (95%)	GU253390
Clone no 66	402	Putative 5′ nucleotidase	5e-94	110/113 (97%)	–
Clone no 70	521	Restriction modification system	7e-90	127/140 (96%)	GU253391
Clone no 87	492	Putative exported protein	2e-34	133/166 (80%)	GU253392
Clone no 124	430	ABC type multidrug transport system	2e-68	122/139 (87%)	GU253393

The application of the SSH to a prokaryotic genome study was originally performed with the gram-negative gastric pathogen *Helicobacter pylori* (Akopyants et al. 1998). SSH has been used in different bacterial species (Winstanley 2002) to identify genomic differences between virulent and avirulent strains (Zhang et al. 2000; Janke et al. 2001). SSH has also been applied to the different species of *Yersinia viz. Yersinia pestis* (Erhei et al. 2005), *Yersinia pseudotubercolosis* (Xiaoyi et al. 2006) and other biotypes of *Yersinia enterocolitica* (Iwobi et al. 2003).

3.6 Proteomic Analysis

Using different genotyping methods, we could collect significant information regarding the clonal groups and association between clinical and environmental strains of *Y. enterocolitica* biovar 1A. Other than the genotyping methods, whole cell proteins analysis has been shown to be useful for discriminating bacteria at species levels (Veriśsimo et al. 1996; Vandamme et al. 1998; Sánchez et al. 2003). Utility of whole cell protein profiling in studying strain diversity and a correlation between whole cell protein profiles and some genotyping methods has also been demonstrated (Duim et al. 2004; Konecka et al. 2007).

SDS-PAGE whole cell protein profiling was performed with 81 strains *Y. enterocolitica* biovar 1A isolated from clinical and non-clinical sources (Mallik and Virdi 2010b). As a result of, 21 distinct protein profile types were obtained among these strains. Whole cell protein profiling also clustered the strains into two distinct clonal groups. The clinical and the aquatic serotype O:6,30-6,31 strains were clustered into two separate subgroups. This method displayed sufficient diversity among strains of *Y. enterocolitica* biovar 1A and provide another platform to obtain

Table 3.3 Discriminatory indices and 95% confidence interval of various typing techniques for *Y. enterocolitica* biotype 1A

Genotyping method	Simpson's index of diversity (DI)	Confidence interval (95% CI)	Reference
PAGE	0.80	0.76–0.83	Mallik and Virdi (2010b)
MLVA[a]	0.87	0.85–0.90	Gulati et al. (2009)
Rep-PCR[b]	0.84	0.81–0.88	Sachdeva and Virdi (2004)
*gyr*B-RFLP	0.75	0.72–0.77	Gulati and Virdi (2007)
Ribotyping	0.72	0.69–0.74	Gulati and Virdi (2007)

[a] Multilocus VNTR analysis
[b]Repetitive elements PCR

phylogenetic relationships as inferred earlier by genotyping techniques. The discriminatory index (DI) for whole cell protein profiling was (0.80) compared to DI of genotyping data obtained with rep-PCR, MLVA, 16 S–23 S IGS and *gyr*B shown in Table 3.3.

It was observed that DI of protein profiling was lower than the DI of rep-PCR and MLVA but higher than that of 16S–23S IGS or gyrB. Nevertheless, whole cell protein profiling has the potentiality to be used as an adjunct tool to study epidemiology of *Y. enterocolitica*.

3.7 Future Perspectives

Pathogenic markers or virulence-associated factors that are known to play role in other biovars but have not been investigated for their role in biovar 1A strains such as insecticidal toxin complex, superoxide dismutase, urease, lipopolysaccharide, type two secretion system (T2SS), iron acquisition system, and siderophores production. Homologues of insecticidal toxin complex (*tc*) namely *tcbA*, *tcaC* and *tccC* were identified in biovar 1A while elucidating genomic differences between clinical and environmental strain, and shown to play role in virulence (Tennant et al. 2005). An isogenic mutant for *tc* complex genes exhibited decreased ability to colonize ilea, ceca and colons of perorally inoculated mice. These studies suggested the probable role of insecticidal toxin in persistence of bacterium in the gastrointestinal tract (Tennant et al. 2005). However this toxin gene complex has not been elucidated in Indian biovar 1A strains. Likewise, urease is very well recognized virulence determinant in gastroduodenal pathogen like *Helicobacter pylori*. The role of gastric acid as an innate defense against *Y. enterocolitica* was demonstrated recently (Tennant et al. 2008). *Y. enterocolitica* biovar 1A also produce urease and it is conceivable that it might contribute to their survival in the gut (Bhagat and Virdi 2009). Further work using an isogenic urease mutant of *Y. enterocolitica* biovar 1A would elucidate the role of urease in the acidic pH.

Similarly, *Hre*P of a family of subtilisin-kexin like proteases is one of the host responsive elements (*hre*) which is expressed *in vivo* early during infection but

not *in vitro* and was identified using *in vivo* expression technology (IVET) (Heusipp et al. 2001). The distribution of *hreP* gene in biovar 1A strains correlates with the clonal group A strains which also carry *ystB* (Bhagat and Virdi 2007). So this could be an important virulence factor in biovar 1A strains, however, needs to be investigated.

The role of lipopolysaccharide (LPS) in virulence of *Y. enterocolitica* biovar 1B has been shown by signature-tagged transposon mutagenesis (Darwin and Miller 1999; Gort and Miller 2000), hence, the role of O-antigen in virulence of biovar 1A strains needs to be investigated. Similarly, two types of type II secretion systems (T2SS) have been identified in *Y. enterocolitica*: *yts*1 and *yts*2 (Iwobi et al. 2003). Among these, *yts*1 is present in highly pathogenic strains (biovar 1B) and plays role in virulence while *yts*2 is common to all biovars (Iwobi et al. 2003). It would be remarkable to explore the role of *yts*2 in strains of *Y. enterocolitica* biovar 1A.

It has also been reported that *Y. enterocolitica* biovar 1A produce water-soluble siderophores, which have not been biochemically or molecularly characterized as yet (Chambers and Sokol 1994). The biovar 1A strains also contain iron acquisition genes, which may utilize iron by making use of the exogenous siderophores (Chambers and Sokol 1994). Genes related to iron acquisition and their role in virulence of biovar 1A needs to be further investigated.

3.8 Conclusions

The biovar 1A strains of *Y. enterocolitica* lack both pYV plasmid and major chromosomal virulence genes, but are considered as non-pathogenic. However, strains of biovar 1A analyzed using diverse genotyping methods *viz*. REP-PCR, ERIC-PCR genomic fingerprinting (Sachdeva and Virdi 2004), ribotyping, PCR-ribotyping and *gyr*B gene polymorphism (Gulati and Virdi 2007) generally revealed limited genetic heterogeneity. Genotypic methods not only provide the information of clonal groups of *Y. enterocolitica* biovar 1A strains but also revealed that clinical and wastewater O:6,30-6,31 (biovar 1A) isolates clustering into two separate groups (Sachdeva and Virdi 2004; Gulati and Virdi 2007). These studies failed to reveal any genetic association between serotypes and the sources of isolation of these strains. Nevertheless more diversity was observed among clinical and non-clinical strains of *Y. enterocolitica* biovar 1A when MLEE was used. Sixty-two electrophoretic types were identified using MLEE, which clustered 81 strains into four distinct groups. While 12 restriction types were identified by MLRT, which was distinctly less discriminatory, clustering the strains into two groups. The BURST analysis of the MLRT data nevertheless provided newer insights into the probable evolution of clinical strains from those present in the aquatic environments (Mallik and Virdi 2010a). In future, detailed studies on pathogenic markers like insecticidal toxins; urease, host responsive elements, flagella, siderophore production and the host immune response to these clonal groups need to be undertaken. This would help in fully understanding the pathogenic potential and true public health significance of *Y. enterocolitica* biovar 1A.

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Chapter 4
Role of Microorganisms in Remediation of Contaminated Soil

Manab Das and Alok Adholeya

Abstract Starting from the mining of raw materials to production, transportation, use by end users, disposal or accidental spills of chemicals often contaminate soil to the extent that threaten the health of human life, livestock, wildlife and indeed whole ecosystems. Traditional methods to cleanup or decontaminate the soil are expensive, labour intensive, do not always ensure that pollutants are completely removed or destroyed and often result in abrupt changes to the physical, chemical, and biological characteristics of the treated soil. Use of microorganism have shown promises in remediation of soil contaminated with heavy metals and radionuclide, organic compounds including chlorinated solvents like TCE; explosives such as TNT, RDX; petroleum hydrocarbons including PAHs; PCBs and pesticides such as atrazine and organophosphates. Aesthetically pleasing cleaning methodology, minimal disruption and preservation of top soil, usefulness in treating broad range of environmental contaminants and low cost (60–80% or even less costly than conventional methods) are the advantages associated with microorganisms mediated soil remediation technology and so it has gained increasing attention over the past 15 years. The review focuses on an overview of various physico-chemical methods used earlier for soil remediation purposes, what are the bioremediation techniques used nowadays and how it works; finally future perspectives of bioremediation techniques and conclusion.

Keywords Bioremediation • Heavy metal • Pesticides • Polycyclic aromatic hydrocarbons • Microorganism

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4.1 Introduction

Worldwide, contamination of soil due to various anthropogenic activities is a severe problem. Intensification of agriculture and expansion of industries has resulted in increased release of a wide range of xenobiotics. These contaminants render harm to humans, livestock, wildlife, crops, or native plants causing ecological problems leading to imbalance in nature. The scientists all over the world are trying to solve it through several means such as physical, chemical and thermal processes resembling excavation and transportation of contaminated soil. Unfortunately, those methods are expensive, labour intensive, do not always ensure the pollutants are completely removed or destroyed and often result in abrupt changes to the physical, chemical and biological characteristics of the treated soil. The search for alternative methods for traditional methods to clean polluted sites resulted in evolution of bioremediation techniques. Bioremediation approaches have gained considerable interest and a plenty of research have been carried out and published on the application of various microorganisms for decontamination of different type of pollutants in soil. However, still more and more research is required for complete understanding of available techniques followed by suitable modification of the same to gain maximum output from that and at the same time exploration of new possibilities from day by day experiences.

4.2 Common Source of Pollutants in Soil

Soil pollution is defined as the build-up in soils of persistent toxic compounds, chemicals, salts, radioactive materials, or disease causing agents, which have adverse effects on plant growth and animal health. There are many different ways that soil can become polluted, such as (1) seepage from a landfill (2) discharge of industrial waste into the soil (3) percolation of contaminated water into the soil (4) rupture of underground storage tanks (5) excess application of pesticides, herbicides or fertilizer (6) solid waste seepage (7) improper installations, detonation, dismantlement of munitions. The most known chemicals involved in causing soil pollution are presented in Table 4.1.

Table 4.1 List of well-known soil pollutants

Type of pollutants	Examples
Heavy metal/trace element	As, Cd, Cr, Co, Cu, Hg, Ni, Pb, Zn, Se, Sb, F, Be, Mn
Radionuclide	^{137}Cs, ^{90}Sr, ^{40}K, ^{232}Th
Munition wastes	TNT, HMX, RDX
BTEX	Benzene, toluene, ethyl benzene, xylene
Polycyclic aromatic hydrocarbons	Napthalene, anthracene, fluoranthene, pyrene, Benzo(a) pyrene
Pesticides	DDT, BHC, organophosphates, aldrin, malathion, dieldrin, Lindane, Sevin, Zectrion, Atrazine, Bentazone
Others	Pentachlorophenol, trichloroethylene, 4-chlorobiphenyl, 2,4-dichlorobiphenyl

The environmentalists around the world are trying to overcome such huge load of pollutants in soil by several means.

4.3 Different Physico-chemical Soil Remediation Technologies

Physical methods of soil reclamation are those that do not change the physico-chemical properties of the pollutants accumulated in the soil to be cleaned. Physical methods of soil reclamation could be divided into Ex-situ methods and In-situ methods.

4.3.1 Ex-situ Methods

It requires the transportation of the polluted soil to the place of cleaning and comprises of two techniques namely mechanical separation and extraction and storage.

Mechanical separation: The mechanical separation of soil is a physico-chemical process in which the contaminated parts of soil are separated. This process leads to a decrease in volume of contaminated soil. The techniques most often used for separation are: gravitational separation (based on differences in density between fractions) or in cyclones (based on the Coriolis Effect), sieve analysis (different grain size of elements) or magnetic separation (based on magnetic induction). Although significant amount of volume reduction can be achieved through this mechanical separation but it is not applicable for pollutants homogenously distributed in soil. Besides, the separated fraction containing the pollutants must be cleaned up or neutralized in another process.

Extraction and storage: This method is the simple extraction of contaminated soil cover, using a digger or bulldozer, and its storage in an appropriate place for further clean-up using another method. The storage site has to be sheltered to prevent wind and water erosion. Short excavation time, requirement of simple equipment make this method popular but excavation is possible only upto a depth of 3 m and is not suitable for small sites because of high expenses.

4.3.2 In-situ Methods

It could be applied on-site, without the removal of the soil from the polluted site. Common techniques available for in-situ method include electroremediation, cofferdam system and soil covering.

Electroremediation: This method is based on the phenomenon of pollutant migration in an electric field. Migrating particles have to have a permanent electric charge or have to be polarized, so the technique is used to remove heavy metals or polar compounds. Electrodes are inserted into the ground on opposite sites of the contaminated area. Contaminants under the influence of an electromagnetic field migrate through the soil within the cathode or anode area, where they are removed in a few possible ways: chemical precipitation, adhesion to the electrodes' surfaces, removing and processing the contamination beyond the remediate site. However, such treatment results acidification of soil and often produce unwanted chemicals due to oxidation-reduction processes involved in remediation techniques.

Cofferdam system: This is a system of barriers made from different substances placed under the soil's surface. The main role of the barriers is protection against the spreading of dangerous substances from the contaminated site. Due to different chemical substances present in the barriers, the pollutants are transformed into environmentally friendly forms. The main types of barrier construction are semi-permeable barrier, non-permeable barrier and non-permeable vertical barrier. Semi-permeable barrier is placed perpendicularly to the direction of migration of contaminated ground waters. Contaminants are stopped and reduced (organic compounds) or immobilized (heavy metals). Non-permeable vertical barrier is placed under the ground and can stop groundwater or change its flow direction. Non-permeable horizontal barrier is placed above the groundwater level and stops the migration of contaminants down to a soil profile. Chemicals that are generally used for barrier construction include $Na_2S_2O_4$, which reduces heavy metal; FeS and $FeCO_3$, which reduce Cr (VI); $Ca(OH)_2$, which has the ability to precipitate U, As, Mo, Se; $CaCO_3$, which has the ability to precipitate Cr (III); $CaPO_4$, which has the ability to precipitate Pb; activated carbon, zeolite, peat, synthetic resin etc. Although the barrier technique is applicable to both organic and inorganic contaminants but the efficacy of the technique depends solely on regular monitoring of the site and also on clarity over flow direction of underground water.

Soil cover/insulation: This is a physical method based on covering the contaminated soil to prevent toxic migration to the environment as a result of rainwater or wind erosion. The layers are comprised of combined material such as synthetic fibre, clay and concrete. The cover is usually made of synthetic fiber, heavy loam or concrete. The construction of the cover is comprised of four layers: A protective layer with growing plants to reduce the erosion of surface cover; a drainage layer, to drain rainfall water percolating through the surface layer; a non-permeable barrier to protect the contaminated area against contact with precipitation waters; a base layer, made from selected mineral material with a suitable grain size and a mechanical parameters. It is generally applied when the underground contamination is so wide that it is impossible to extract and remove the contaminated soil; and the physico-chemical properties of the pollutants do not exhibit any ability for release or migration.

4.4 Different Chemical Soil Remediation Technologies

Chemical methods of soil reclamation aim to degrade the pollutants accumulated in the soil or bring such changes to their physico-chemical properties so as to reduce their overall effect on environment. Chemical methods are developed on the basis of different chemical processes, which include oxidation and reduction, extraction, precipitation of sparingly soluble chemical compounds, pH stabilization. It is a very contaminant specific and efficient technique but on the other hand it associated with high expenses, generation of additional chemical waste as well as difficulties in process control.

4.4.1 Ex-situ Methods

This *ex-situ* method is mostly used for removing inorganic contamination, such as heavy metals, radio-nuclides, toxic anions and others. In some cases, it can be applied to organic contamination.

Soil washing: This method uses a wide spectrum of leaching solutions from water to strong inorganic acids. There are two types of washing firstly separation of fine particles with adsorbed contaminants in a stream of dissolvent (water or solutions of inorganic salts Ca or Mg) and finally extraction of contaminants. It is a fast and efficient method for cleaning up of contaminated soil but high cost, generation of additional chemical waste and invasive in nature results in low level of community acceptance of this technique.

Contaminant immobilization: This is a method for the neutralization of some organic and inorganic compounds in soil. For neutralization purposes, the contaminants' bonded substances are added (e.g. cement), which completely blocks the pollutants in the soil. This method is applied in areas where highly contaminated soil must be removed from its place of origin and its storage is connected with a high ecological risk (for example in the case of radio-nuclides). It is a fast, easily applicable and relatively low cost method but high invasivity to the environment, generation of large volume of treated solid waste and issue related to permanent control of stored waste are the major drawbacks associated with this technique.

Chemical and photochemical reduction: This method allows the total mineralization (by chemical reactions) of organic contaminants or the effective transformation of organic and inorganic contaminants into non-toxic, less toxic or chemically inert forms. The method is suitable for the removal of organic compounds (oil, organic solvents, and pesticides) and inorganic ones (ions or heavy metals and oxyanions). The reagents used in this method are: ozone, hydrogen peroxide, chlorates, chlorine monoxides and other oxidizing or reducing reagents. In the case of the photochemical process, an additional factor is ultraviolet radiation. Low operation cost, wide application range are the advantages associated with this technology, however the

major drawbacks of this technology include high invasivity to soil and environment, incomplete detoxification due to development of temporary products and antagonism effects and finally requirement of special safety measures (for caustic and explosive reagents).

4.4.2 In-situ Methods

Soil flushing: This method is a similar to soil washing and is used for the same group of pollutants (heavy metals), but is applied *in situ*. It is less invasive because of the application of mild extractor hence less efficient to strong contaminants, requires insulation of application site and also continuous monitoring. Like other chemical methods it also produces liquid and semi-liquid waste and can't remove contaminants completely.

Contaminant immobilization: It is used for the immobilization of inorganic contaminants such as heavy metals (it is rarely applied to organic contaminants). The method is based on the introduction into the soil of different substances (cement, resin, zeolites) which bond strongly with the contaminants or create sparingly soluble chemical associations (carbonates or phosphates). The effects of this remediation technique are also the modification of the soil chemical properties, causing the immobilization of heavy metals or their chemical transformation into less mobile forms by pH changes (liming). This method is applied as temporary measure in emergency to medium or low contaminated areas and only to the surface layer of soil (30–50 cm).

4.5 Biological Soil Remediation Technologies and Applications

Traditional physico-chemical and chemical methods for the cleanup of pollutants are often prohibitively expensive. Furthermore, available space for disposal and storage is declining. Perhaps one of the greatest limitations to traditional cleanup methods is the fact that in spite of their high costs, they do not always ensure that contaminants are completely destroyed. As a result, the past two decades have seen a tremendous upsurge in the search for cost-effective and environmentally sound alternatives to traditional methods for dealing with wastes. Research, and technology development and implementation to address waste issues now rank very high among the fastest growing activities in the world. The technologies that have emerged as most promising are those that closely mimic time-tested, natural systems which have restored environments to their natural statuses following undesirable perturbations. Chemicals from natural origins (animal, vegetable, or mineral) are transformed, destroyed, removed, or otherwise stabilized through natural processes so they do not accumulate to levels that threaten ecosystem balance or

Table 4.2 Environmental factors and optimum conditions for microbial activity for soil bioremediation

Environmental factors	Optimum conditions
pH	5.5–8.8
Temperature (°C)	15–45
Moisture	25–28% of water holding capacity
Soil type	Low clay or silt content
Oxygen	Aerobic, minimum air-filled pore space of 10%
Nutrient	N and P for microbial growth
Heavy metals	Total content 2,000 ppm
Contaminants	Not too toxic

sustainability. A growing public awareness and concern about environmental pollutants fostered collaboration between government and industry for the development of safe and cost-effective alternative approaches for dealing with wastes. Of the technologies that have been investigated, bioremediation has emerged as the most desirable approach for cleaning up many environmental pollutants. Bioremediation is an option that offers the possibility to destroy or render harmless various contaminants using natural biological activity. As such, it uses relatively low-cost, low-technology techniques, which generally have a high public acceptance and can often be carried out on site. By definition, bioremediation is the use of living organisms, primarily microorganisms, to degrade the environmental contaminants into less toxic forms. It uses naturally occurring bacteria and fungi or plants to degrade or detoxify substances hazardous to human health and/or the environment. The microorganisms may be indigenous to a contaminated area or they may be isolated from elsewhere and brought to the contaminated site. Contaminant compounds are transformed by living organisms through reactions that take place as a part of their metabolic processes (Vidali 2001). For bioremediation to be effective, microorganisms must enzymatically attack the pollutants and convert them to harmless products. As bioremediation can be effective, only where environmental conditions permit microbial growth and activity, its application often involves the manipulation of environmental parameters to allow microbial growth and degradation to proceed at a faster rate. Microbial growth and activity are readily affected by pH, temperature, and moisture. Although microorganisms have been also isolated in extreme conditions, most of them grow optimally over a narrow range, so that it is important to achieve optimal conditions. If the soil has too much acid it is possible to rinse the pH by adding lime. Temperature affects biochemical reactions rates, and the rates of many of them double for each 10°C rise in temperature. Above a certain temperature, however, the cells die. Plastic covering can be used to enhance solar warming in late spring, summer, and autumn. Available water is essential for all the living organisms, and irrigation is needed to achieve the optimal moisture level. Optimum environmental conditions for efficient performance of microorganisms are presented in Table 4.2 (Vidali 2001).

4.5.1 Bioremediation Strategies

Bioremediation coverts organic pollutants mainly to carbon dioxide, water, and biomass. Some of the pollutants can also be immobilized by binding to the humic substance fraction. Degradation may take place under aerobic, as well as under anaerobic conditions. The aerobic process is predominantly used for bioremediation and it can also be classified as ex-situ and in situ. Selection of appropriate technology among the wide range of bioremediation technologies developed to treat contaminants depends on three basic principles – the amenability of the pollutant to biological transformation, the accessibility of the contaminant to microorganisms and the opportunity for optimization of biological activity. Through appropriate selection of the technologies and adjustment of conditions, the degradation process is enhanced and the degree of degradation is improved which ultimately reduces the cost of treatment (Mohapatra 2008). Ex situ techniques are those that are applied to soil and groundwater at the site which has been removed from the site via excavation (soil) or pumping (water). In situ techniques are defined as those that are applied to soil and groundwater at the site with minimal disturbance. These techniques are generally the most desirable options due to lower cost and fewer disturbances since they provide the treatment in place avoiding excavation and transport of contaminants. However, in situ treatment is limited by the depth of the soil that can be effectively treated. In many soils effective oxygen diffusion for desirable rates of bioremediation extend to a range of only a few centimeters to about 30 cm into the soil, although depths of 60 cm and greater have been effectively treated in some cases (Vidali 2001).

4.5.1.1 Ex Situ Methods

Land farming: It also known as land treatment or land application is an above-ground remediation technology for soils that reduces concentrations of organic pollutants through biodegradation. This technology usually involves spreading excavated contaminated soils in a thin layer on the ground surface and stimulating aerobic microbial activity within the soils through aeration and/or the addition of minerals, nutrients, and moisture. As contaminated soil is treated in thin layers of upto 0.4 m thickness, it requires a large treatment area. To promote degradation enhancement of oxygen supply as well as mixing are done by ploughing, harrowing or milling at regular intervals. The treatment process is cost effective and can be adopted if sufficient land is available (Mohapatra 2008).

Composting: It is applied in bioremediation as a means of degrading toxic organic compounds and perhaps lessening the toxicity of metallic contaminants in organic residues, waste and by product. Composting is similar to those that occur biologically in soil by which organic wastes are degraded by microorganisms. Temperatures are generally higher in composts than in soils, resulting in increased solubility of

contaminants and higher metabolic activity in compost. High level of substrate in composts can lead to co-metabolism of organic contaminants. Mechanical treatment by grinding, mixing, and sieving out non-degradable or unwanted materials such as metals, plastics, glass, stones gives good conditions for biological treatment of compostable materials. However, the nature of the organic contaminant, composting conditions and procedures, microbial communities, and time all affect the performance of compost mechanism (Barker and Bryson 2002).

Biopiles: It is a hybrid of land farming and composting. Excavated soils are mixed with soil amendments, placed on a treatment area, and bio-remediate using forced aeration. Contaminants are converted to carbon di-oxide and water. The basic bipile system includes a treatment bed, an aeration system, an irrigation/nutrient system and a leachate collection system. Soil piles can be up to 20 ft and may be covered with plastic to control runoff, evaporation and volatilization, and promote solar heating. If needed volatile organic compounds are treated before they entered into the air stream (Shukla et al. 2010). Biopiles provide a favorable environment for indigenous aerobic and anaerobic microorganisms.

Bioreactors: In this process contaminated soil are treated either in solid or slurry phase. The principle of solid phase reactors is mechanical decomposition of the soil by attrition and by intensive mixture of the components in a closed container. This ensures that the contaminants, microorganisms, nutrients, water and air are brought into permanent contact. A slurry bioreactor may be defined as a containment vessel and apparatus used to create a three-phase (solid, liquid, and gas) mixing condition to increase the bioremediation rate of soil bound and water-soluble pollutants as a water slurry of the contaminated soil and biomass (usually indigenous microorganisms) capable of degrading target contaminants. In general, the rate and extent of biodegradation are greater in a bioreactor system than *in situ* or in solid-phase systems because the contained environment is more manageable and hence more controllable and predictable. However, the contaminated soil requires pre treatment (e.g., excavation) or alternatively the contaminant can be stripped from the soil via soil washing or physical extraction (e.g., vacuum extraction) before being placed in a bioreactor (Vidali 2001).

4.5.1.2 In Situ Methods

Bioventing: An in-situ remediation technology that uses indigenous microorganisms to biodegrade organic constituents adsorbed to soils in the unsaturated zone. It is based on vacuum-enhanced soil vapour extraction. The pressure differences in the sub-surface cause an inflow of atmospheric air and therefore, oxygen supply, as needed for aerobic degradation of contaminants. It is effective in remediating petroleum products, including gasoline, jet fuels, kerosene, and diesel fuel. If the contaminants to be treated are volatile, the extracted soil vapour has to be treated by adsorption of contaminants on activated carbon by biodegradation within a bio filter (Mohapatra 2008).

Biosparging: It exploits and stimulates indigenous microorganisms to degrade organic contaminants in saturated soil. Through boreholes, air is injected into the saturated zone (below the water table) to increase the activity of the soils indigenous microorganisms through increased oxygen dissolution. The increased oxygen enhances aerobic biodegradation of the contaminants present in the soil or groundwater. Biosparging can be used to reduce petroleum constituents that are adsorbed to soil within the capillary fringe, below the water table or dissolved in groundwater. Biosparging is commonly used at sites with mid-weight petroleum products such as diesel fuel; lighter petroleum products tend to volatilise swiftly and are removed very rapidly through sparging. Soil permeability is a key factor in the effectiveness of the technology (Vidali 2001; Mohapatra 2008).

Bioaugmentation: It involves the addition of microorganisms indigenous or exogenous to the contaminated sites. Two factors limit the use of added microbial cultures in a land treatment unit: (a) nonindigenous cultures rarely compete well enough with an indigenous population to develop and sustain useful population levels and (b) most soils with long-term exposure to biodegradable waste have indigenous microorganisms that are effective degrades if the land treatment unit is well managed (Vidali 2001).

4.6 Bioremediation of Organic Pollutants

Numerous types of organic pollutants are to be encountered in contaminated sites; hence diverse types of microorganisms are likely to be required for effective remediation (Table 4.3). The first patent for a biological remediation agent was registered in 1974, being a strain of *Pseudomonus putida* (Prescott et al. 2002), after that a large numbers of organisms belong to at least 11 different prokaryotic divisions (Glazer and Nikaido 2007) has been added to the list in the successive two decades.

Degradation of organic pollutants by microorganism occurs either in the presence of oxygen by respiration or under anoxic conditions by denitrification, methanogenesis, and by sulfidogenesis (Fig. 4.1).

The most rapid and complete degradation of the majority of pollutants in the environment is brought under aerobic condition. Key enzymatic reactions of aerobic biodegradation are oxidations, catalysed by oxygenease and peroxidases. Oxygeneases are oxidoreductases that use oxygen to incorporate into the substrate. Degradative organisms need oxygen at two metabolic stages – the initial attack on the substrate and the end of the respiratory chain. Under strictly anaerobic conditions, soluble carbon compounds are degraded stepwise, to methane, carbon-di-oxide, ammonia and hydrogen sulfide via a synoptic interaction of fermentative and acetogenic bacteria, with methnogens or sulphate reducers. Anerobic degradation processes have always been considered inferior to aerobic degradation in their kinetics and capacities. However, with high loads of easy to degrade organic materials, anaerobic processes have proved to be efficient and far less expensive than aerobic treatment.

Table 4.3 Microorganisms having biodegradation potential for different organic pollutants (Chatterjee et al. 2008)

Pollutants	Organism	References
Benzene, anthracene, hydrocarbons, PCBs	*Pseudomonas spp*	Kapley et al. (1999) and Cybulski et al. (2003)
Halogenated hydrocarbons, linear alkylbenzene sulfonates, polycyclic aromatics, PCBs	*Alcaligenes spp*	Lal and Khanna (1996)
Benzene, hydrocarbons, pentachlorophenol, phenoxyacetate, polycyclic aromatic	*Arthrobacter spp*	Jogdand (1995)
Aromatics, long chain alkanes, phenol, cresol	*Bacillus spp*	Cybulski et al. (2003)
Halogenated hydrocarbons, phenoxyacetates	*Corynebacterium spp*	Jogdand (1995)
Aromatics	*Flavobacterium spp*	Jogdand (1995)
Aromatics	*Azotobacter spp*	Jogdand (1995)
Naphthalene, biphenyl	*Rhodococcus spp*	Dean-Ross et al. (2002)
Aromatics, branched hydrocarbons benzene, cycloparaffins	*Mycobacterium spp*	Sunggyu, (1995)
Hydrocarbons	*Nocardia spp*	Park et al. (1998)
Aromatics	*Methosinus sp*	Jogdand (1995)
Aromatics	*Methanogens*	Jogdand (1995)
Hydrocarbons, polycyclic hydrocarbons	*Xanthomonas spp*	Jogdand (1995) and Ijah (1998)
Phenoxyacetate, halogenated hydrocarbon diazinon	*Streptomyces spp*	Jogdand (1995)
PCBs, formaldehyde	*Candida tropicalis*	Ijah (1998)
PCBs, polycyclic aromatics, biphenyls	*Cunniughamela elegans*	Jogdand (1995)
PCBs,	*P. chrysosporium*	Borazjani et al. (2005)

4.6.1 Polycyclic Aromatic Hydrocarbons

The applications of microorganism for degradation of organics have been growing partly because of better understanding of microbial processes in soil. Especially sites contaminated with polycyclic aromatic hydrocarbons (PAHs). PAHs are aromatic compounds made up of two or more fused benzene rings. It represents a large group of soil pollutants (e.g. naphthalene, phenanthrene, flouranthene, pyrene, chrysene, benzo(α)pyrene etc.). PAHs are recalcitrant and can persist in the environment for long periods, and described to be toxic, mutagenic, or carcinogenic; therefore, they are an important risk to the environment and human health (Kuiper et al. 2004). The main input sources of PAHs are leaches from old storage tanks, road surfaces, and domestic waste; oil spills; tanker leakage; incomplete fossil fuel combustion; and seepage from natural oil reservoirs.

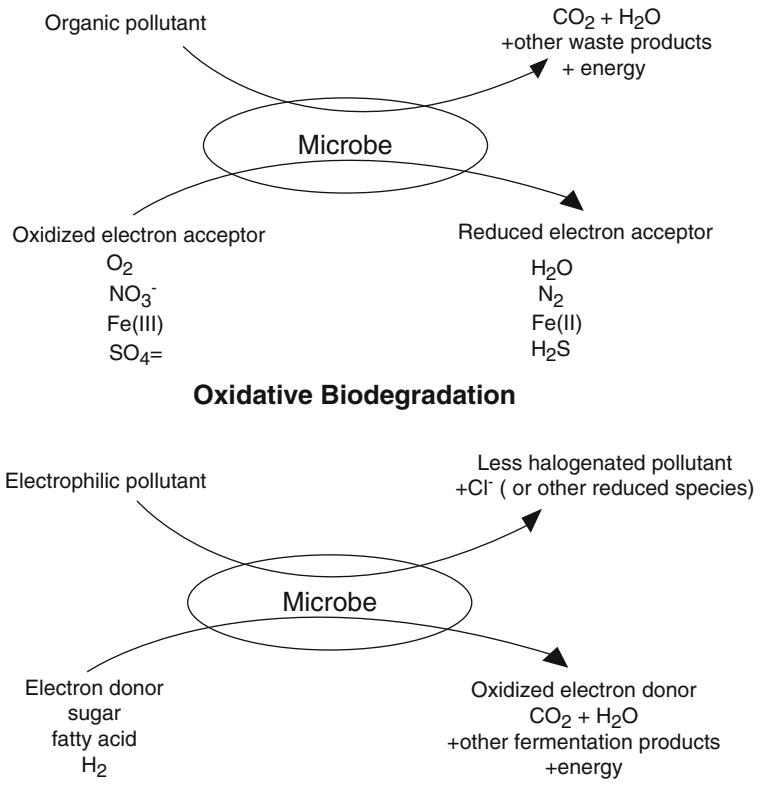
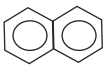
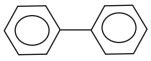
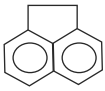
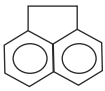
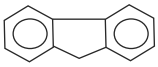
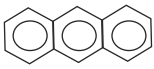
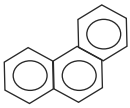
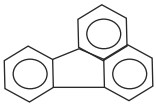
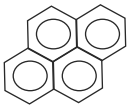
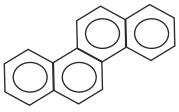
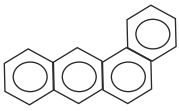


Fig. 4.1 General schemes to biodegrade organic pollutants. In oxidative attacks, (*upper*) pollutants are oxidized by external electron acceptors such as oxygen or sulfate. In reductive attacks (*lower*), electrophilic halogen or nitro groups on the pollutant are reduced by microbes consuming sugars, fatty acids, or hydrogen. The halo- or nitro- group on the pollutant serves as the external electron acceptor (Rockne and Reddy 2003)

Once the pollutants enter the soil, they are trapped into soil pores and immobilized by adsorption to the soil matrix (April and Sims 1990; Mogan and Watkinson 1989). Many PAHs are very hydrophobic, depending upon the numbers of fused ring; therefore, their water solubility is very low. However, uptake of PAHs by microorganism occurs through the water phase and therefore, depends largely upon its solubility (Bouwer and Zehnder 1993). Despite of several limitations; PAHs are conducive to biodegradation by certain bacteria, fungi and algae. Table 4.4 represents a list of PAHs that can be degraded by pure culture of bacteria and their physico-chemical properties. There are three main mechanisms by which microorganisms metabolize PAHs – bacterial degradation, lignolytic fungal degradation, and non-lignolytic bacterial degradation (Fig. 4.2) – but these mechanisms share a common path of "oxidation of the aromatic ring, followed by the systematic breakdown of the compound to PAH metabolites and/or carbon dioxide" (Bamforth and Singleton 2005).

Table 4.4 Polycyclic aromatic hydrocarbons used by pure cultures of bacteria as the sole source of carbon and energy and important physicochemical properties (Reineke 2001)

Compound	Structure	Aqueous solubility (mg/l) at 25°C	Octanol/water co-efficient (log K_{ow})	Degradative pathway	Ionization potential (eV)
Naphthalene		31.7	3.37	++	8.12
Biphenyl		7.0	3.9	++	
Acenaphthene		3.42	4.33	+	7.61
Acenaphthylene		3.93	4.07	+	
Fluorene		1.98	4.18	++	
Anthracene		0.075	4.45	++	7.43
Phenanthrene		1.6	4.46	++	8.03
Fluorathene		0.265	5.33	+	7.85
Pyrene		0.148	5.32	+	7.53
Chrysene		0.002	5.61	+	7.81
Benzo [a] anthracene		0.014	5.61	+	7.56

++ well established; + metabolites identified

The main environmental factors that could affect the feasibility of bioremediation are temperature, pH, oxygen, nutrient availability and bioavailability of PAH in soil. Biodegradation of PAHs was reported in different temperatures including very high to very low. Lau et al. (2003) reported that the laccase and manganese peroxidase

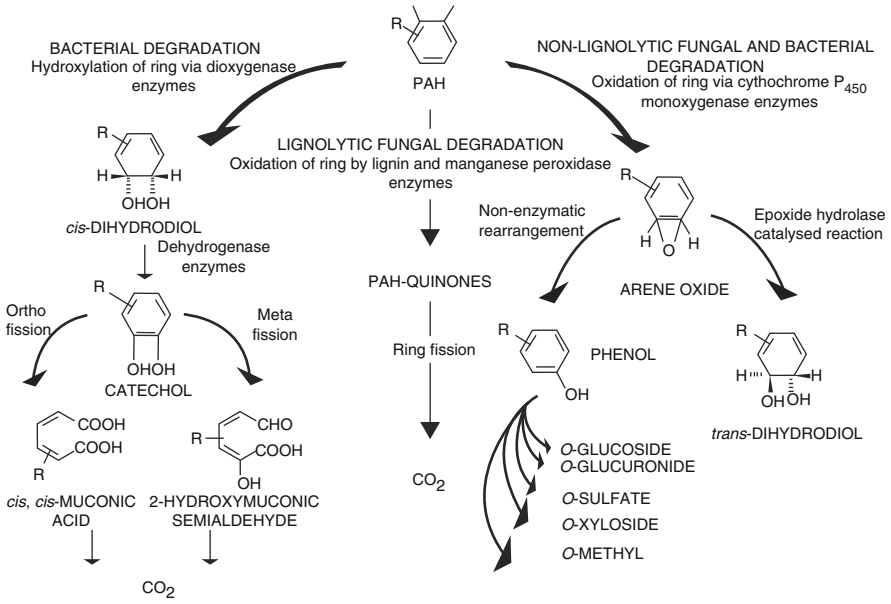


Fig. 4.2 The three main pathways for polycyclic aromatic hydrocarbon degradation by fungi and bacteria (Bamforth and Singleton 2005; Cerniglia 1992)

enzymes of ligninolytic fungi could degrade PAH by over 90% at about 50°C and >75°C respectively, in spent mushroom compost.

In comparison, naphthalene and phenanthrene degradation was reported from crude oil in seawater at temperatures as low as 0°C (Siron et al. 1995). Both alkalophilic and acidophilic environments are able to degrade PAH. In a study by Stapleton et al. (1998), degradation of naphthalene, phenanthrene and anthracene have been recorded in soil of pH 2.0. *Burkholderia cocovenenas*, an organism isolated from petroleum-contaminated soil has been found to degrade phenanthrene ≥80% at near neutral soil pH (Kastner et al. 1998). Although bioremediation of PAHs can proceed in both aerobic and anaerobic conditions, most work has tended to concentrate on aerobic degradation of PAHs. Oxygen is integral to the action of mono- and dioxygenase enzymes in the oxidation of the aromatic ring in PAH (Gibson et al. 1968). Microbial transformation of PAH require similar level of nutrients those required for organic pollutant such as petroleum residues in soil (Bamforth and Singleton 2005). However, high molecular weight PAH-oxidising ligninolytic enzymes of the white rot fungi are produced under nutrient deficient conditions (Bogan et al. 1996). PAHs are hydrophobic compounds with very low solubility (Semple et al. 2003) and can undergo rapid sorption to mineral surfaces in the soil matrix. With increase in soil-PAH contact time, the bioavailability of PAHs in soil decreases (Hatzinger and Alexander 1995).

Table 4.5 Microbial dechlorination pathways "Flanked" signifies an adjacent chlorine

Dechlorination pathway	Chlorines removed
M	Flanked and unflanked *meta*
Q	Flanked and unflanked *para, meta* of 2,3-
H'	Flanked *para, meta* of 2,3- and 2,3,4-
H	Flanked *para*, doubly flanked *meta*
P	Flanked *para*
N	Flanked *meta*
LP	Flanked and unflanked *para*
T	Flanked *meta* of 2,3,4,5- in hepta- and octachlorobiphenyls

Use of surface-active agent (surfactant) can increase availability of PAH. Some microorganisms has been found to produce surfactants, that can enhance desorption of PAHs from the soil matrix (Makkar and Rockne 2003). Overall, the bioremediation of PAH contaminated soil is feasible and at the same time further research is required to remove existing bottlenecks.

4.6.2 Polychlorinated Biphenyl

Polychlorinated biphenyls (PCBs) are a class of chemicals consisting of 209 member compounds, collectively known as congeners. The lipophilic properties of PCBs make them slightly soluble in water and readily soluble in oils, which cause bioaccumulation in the fatty tissues of fish, birds, animals, and humans. Since 1930s, PCB has had a wide range of application from an extender in insecticide to an insulator in transformer production (Ang et al. 2005).

Bioremediation of PCB contaminated soil is a difficult task due to the structure and level of chlorination and occurs primarily by co-metabolic mean. In general, microbial reductive dechlorination of PCBs removes *m*- and *p*- chlorines from highly chlorinated congeners, resulting in predominately *ortho* substituted monothrough tetrachlorobiphenyls (Wiegel and Wu 2000). There are eight major dechlorination pathways known to date, each differing in congener and position reactivity (Bedard 2003). Table 4.5, reviews the known microbial dechlorination processes (Wiegel and Wu 2000). The most extensive dechlorination occurs when process M works in combination with process Q. This activity, known as C dechlorination, voraciously attacks *m*- and *p*- chlorines, resulting in exclusively *ortho* substituted congeners (Zwiernik et al. 1998; Mikszewski 2004). This process is advantageous because lightly chlorinated *ortho* substituted species are non-dioxin-like, do not readily bioaccumulate and microorganism breakdown this less chlorinated congeners at a faster rate than the highly chlorinated congeners.

Microbial degradation of PCB occurs via a meta-cleavage pathway to yield tricarboxylic acid cycle intermediate and (chloro) benzoate (CBA). The initial step in

the aerobic degradation of PCBs is the dioxygenation of PCB congeners by the biphenyl dioxygenase enzyme. In this step the enzyme catalyses the incorporation of two hydroxyl groups into the aromatic ring of a PCB congener, which increases the reactivity of the PCBs, rendering them more susceptible to enzymatic ring fission reactions (Bruhlmann and Chen 1999; Ang et al. 2005). *Phanerochaete chrysosporium* has been found to degrade PCB with variety of congeners and positions of chlorine substitution (Yadav et al. 1995).

It has also been found that addition of co-substrates has accelerated PCB dechlorination activity through a stimulation or "priming" of the microbes responsible for PCB reductive dechlorination (Bokvajova and Burkhard 1994). Perhaps one of the more successful applications of this type of biostimulation has been the addition of less toxic poly brominated biphenyls (PBBs) to stimulate PCB dechlorination (Bedard et al. 1998). It has been found that PBBs are readily debrominated at high rates by sediment enrichments that have been previously contaminated with PCBs. Once PCB sufficiently dechlorinated, mono and di-chlorinated biphenyls are reported to be degraded by bacteria such as *Burkholderia* Str. LB400 (Maltseva et al. 1999). It can break one ring with oxygen and proceed to mineralize the compound. Borazjani et al. (2005) has reported that addition of nitrogen to soil through application of chicken manure can enhance the microbial decomposition of Aroclors 1242 and 1248 by white rot fungus (*P. chrysosporium*). However, complication may arise in biodegradation of higher chlorinated PCBs as they are very much resistant to microbial attack and may take several years or decades for degradation.

4.6.3 Pesticides

To most common organic pesticides used in agriculture are atrazine and organophosphate. Atrazine is the most widely used herbicide in conservation tillage systems, which are designed to prevent soil erosion. First introduced in 1950s and has been widely used for weed control in agricultural production of crops such as maize, sorghum and sugar cane. Although it contains only one chlorine constituent, atrazine is recalcitrant to biodegradation, with a reported half-life of greater than 170 days in soils containing atrazine-degrading microorganisms (Radosevich et al. 1996) and has solubility of approximately 30 mg/l (Protzman et al. 1999). Due to its recalcitrance, atrazine is frequently detected in surface and ground water samples, posing a direct risk to humans via potable water consumption. Like other triazine herbicides, atrazine functions by binding to the plastoquinone-binding protein in photosystem II, which animals lack. Plant death results from starvation and oxidative damage caused by breakdown in the electron transport process. Oxidative damage is accelerated at high light intensity (Arnold et al. 2002). Its alleged endocrine disruptor effects, possible carcinogenic effect, and epidemiological connection to low sperm levels in men has led several researchers to call for banning it in the US (Ackerman 2007). Of the various atrazine-degrading microorganisms isolated, *Pseudomonas sp*. ADP has been most closely studied to elucidate the genes and

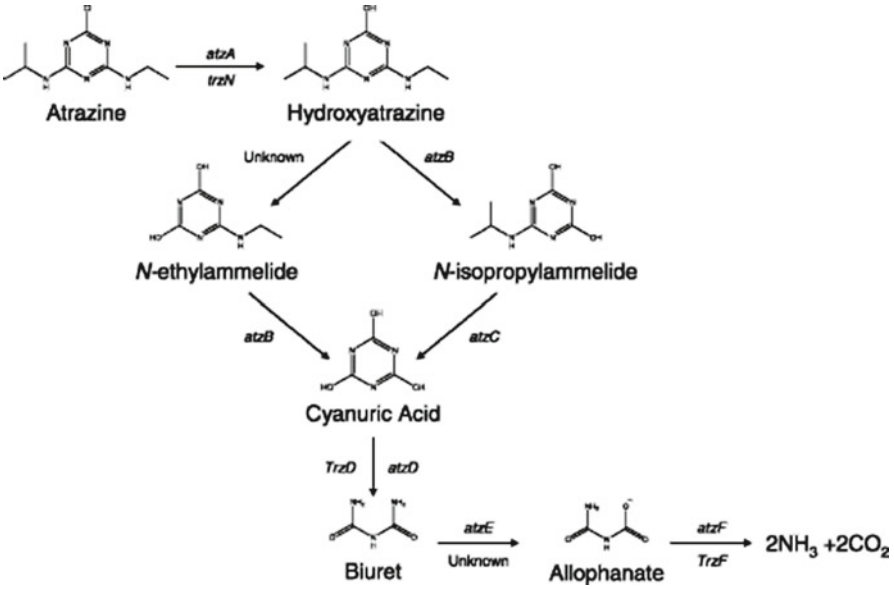


Fig. 4.3 Proposed metabolic pathway responsible for the rapid dissipation of atrazine in s-triazine-adapted soils. *Abbreviations* denote genes coding for the following enzymes: *atzA* atrazine chlorohydrolase, *trzN* triazine hydrolase, *atzB* hydroxyatrazine ethylaminohydrolase, *atzC* N-isopropylammelide isopropylaminohydrolase, *TrzD* cyanuric acid amidohydrolase, *atzD* cyanuric acid hydrolase, *atzE* biuret hydrolase, *atzF* allophanate hydrolase, and *trzF* allophanate hydrolase (Krutz et al. 2009)

enzymes responsible for atrazine metabolism. In the biodegradation of atrazine, *Pseudomonas sp*. ADP converts atrazine to cyanuric acid using the AtzA, B and C enzymes. AtzA first transforms atrazine to hydroxyatrazine and AtzB then catalyzes the hydrolytic deamidation of hydroxyatrazine to yield N-isopropylammelide. Finally, AtzC, which is also a hydrolytic deamidase like AtzB, converts N-isopropylammelide to cyanuric acid. Cyanuric acid is subsequently mineralized to carbon dioxide and ammonia by enzymes that are commonly found in soil bacteria (Fig. 4.3) (Ang et al. 2005; Krutz et al. 2009). A list of bacterial strains capable of catabolizing atrazine is presented in Table 4.6.

Organophosphorus (OP) compounds have been used worldwide as pesticides, petroleum additives and plasticizers, and as pesticides have been in use since the end of the Second World War. More than 100 OP pesticides are in use worldwide, accounting for ~38% of total pesticide usage (Singh 2009). Organophosphates are highly toxic neurotoxins used in insecticides and chemical warfare agents. Paraoxon, parathion, chlorpyrifos disulfoton, ruelene, carbophenothion and dimeton are the potential members of organophosphate family. The neurotoxicological properties of this class of compounds are mainly due to its ability to suppress acetylcholinesterase and as a result, prevent acetylcholinesterase from breaking down acetylcholine at the synaptic junction.

Table 4.6 Some isolated atrazine-catabolizing bacterial strains (Ralebitso-Senior et al. 2002)

Strain	End products	Mineralization
Pseudomonas sp.	Deethylatrazine + de*iso*propylatrazine	–
Rhodococcus sp. strain B-30		–
Pseudomonas sp. strain YAYA6	CO_2	+
Pseudomonas sp. strain ADP	$CO_2 + NH^{4+}$	+
Rhodococcus sp. strain N186/21	Hydroxy*iso*propylatrazine	–
Ralstonia M91-3	Biuret + $CO_2 + NH^{4+}$	+
Rhizobium sp.	Hydroxyatrazine	–
Agrobacterium radiobacter J14A	CO_2	+
Clavibacter michiganese ATZ1	Hydroxyatrazine + *N*-ethylammelide	–
Pseudomonas sp. strain CN1	Cyanuric acid + CO_2	+
Nocardioides sp.	Hydroxyatrazine + *N*-ethylammelide	–
Pseudoaminobacter sp.	CO_2	+

These compounds have also been associated with pathology and chromosomal damage associated with bladder cancer (Webster et al. 2002; Ang et al. 2005). Microbial degradation through hydrolysis of P-O alkyl and P-O-aryl bond, is considered as the most significant step in detoxification of organophosphates in soil. Table 4.7 lists the microbes active in degrading the most important organophosphates.

Phosphotriesterases (PTEs) are a group of enzymes that are found responsible for degradation of OP in microorganisms, animal and plants. Till date lots of research has been done on biodegradation of OP and accordingly our understanding of OP degradation has greatly advanced in recent years. With better understanding of the subject, it is being exploited for several industrial application (Singh 2009).

4.6.4 Chlorinated Aliphatic Hydrocarbons

Chlorinated aliphatic hydrocarbons (CAHs) are manmade organic compounds. They typically are manufactured from naturally occurring hydrocarbon constituents (methane, ethane, and ethene) and chlorine through various processes that substitute one or more hydrogen atoms with a chlorine atom, or selectively dechlorinate chlorinated compounds to a less chlorinated state. CAHs are used in a wide variety of applications, including use as solvents and degreasers and in the manufacturing of raw materials. CAHs include such solvents as tetrachloroethene (PCE), trichloroethene (TCE), carbon tetrachloride (CT), chloroform (CF), and methylene chloride (MC) as presented in Table 4.8 (Sawyer et al. 1994; Merck 1989). Although a number of biological degradation mechanisms have been identified theoretically and observed on a laboratory scale, the bioremediation mechanisms are used for enhanced bioremediation of CAHs generally can be classified into one of the following mechanism categories: aerobic oxidation (direct and cometabolic) & anaerobic reductive dechlorination (direct and cometabolic). For both aerobic and

Table 4.7 Microorganism known for metabolism of organophosphates in culture and in field conditions (Mohapatra 2006)

Organophosphates	Bacteria	Fungi
Diazinon	*Acetomonas sp., Anthrobactor spp., Flavobacterium sp., Hydrogenomonas sp., Erwina cartovora sp., E. ananas, Kurthia sp.,, micrococcous sp., Pseudomonas sp., P. aeruginosa., P. diminutum, P. melophthora, P. glycinea, Streptomyces sp., S. griseus.*	*Aspergillus oryzae, A. niger, Mucor sp., Fusarium sp., Penicillium sp., Trochoderma sp.*
Chlorpyrifos	*Anthrobactor sp., Corynebacterium sp., Flavobacterium sp., Kurthia sp., P. diminutum, Streptomyces sp. Pseudomonas sp., S. griceus, Streptococcous sp., Bacillus sp., B. subtills*	*Aspergillus sp., Claviceps sp., Mucor sp., Rhizopus sp., Penicillium waksmani, Fusarium species, Rhizospous sp.*
Dichlorvos	*Flavobacterium sp., Streptomyces sp., Pseudomonas sp., P. diminutum., Anthrobactor sp., Bacillus sp., B. subtilis., B. coagulans., B. cereus., Erwina sp., E. amylovora, Enterobactor sp., P. fluorescens, p. glycinea, P. tobaci, P. melophthora*	*A. niger, A. smithii, Mucor alternans, Saprolegnia sp., Penicillium waksmani, P. notatum., Phytophthora sp.*
Dimethonate	Clostridium sp., *Flavobacterium sp., Pseudomonas sp., P. diminutum P. fluorescens., Kurthia sp., Micrococcus sp., Nocardia sp., Proteus vulgaris, P. aeruginosa, P. melophthora, P. putida., Xanthomonas sp., Rhizobium sp.*	*A. niger, Fusarium sp., Claviceps sp., Rhizopus sp., Mucor sp., Penicillium notatum*
Malathion	Acinetobacter sp., Anthrobacter sp., *Enterobacter sp., Erwinia sp., Klebsiella pneumonae, Microsomonas, Micrococcus sp., B. magaterium, S. aurofaciens, R. japonicum, R. meliloto, Pseudomonas sp., P. aeruginosa, P. melophthora., Bacillus sp., B. subtills, B. cereus, Streptomyces sp., Streptococcous sp., S. aeruginosa*	*Saprolegnia sp., Mucor sp., Rhizopus sp., Aspergillus sp., Penicillium sp., Penicillium waksmani, Ttrichoderma viride.*

anaerobic transformations, the presence of a cosubstrate as a carbon and energy source is needed. Thus, transformations can be brought about by co-metabolism or through interactions of the CAHs with enzymes and cofactors produced by microorganisms for other purposes. Much of the effort of in situ bioremediation of CAHs is centered on promoting co-metabolism. Promoting CAH co-metabolism in the subsurface may entail adding the appropriate growth substrate and electron donor, such as oxygen, to simulate the microbial population, while effectively contacting target contaminants with the stimulated population. To date, there has not been a documented full-scale application of an in situ co-metabolic process to guide the design and application of this technology. Most of the CAHs are degraded

Table 4.8 Various chlorinated aliphatic hydrocarbons (Sawyer et al. 1994; Merck 1989)

CAH	Common name (s)	Abbreviation	Sources
Chlorinated ethenes			
Tetrachloroethane (ethylene)	Perchloroethane	PCE	Waste solvent
Trichloroethane (–ethylene)	–	TCE	Waste solvent, degradation product of PCE
cis-1, 2-Dichloro ethane (ethylene)	Acetylene dichloride	Cis-DCE	Waste solvent, degradation product of PCE and TCE
trans-1, 2-Dichloro ethane	Acetylene dichloride	Trans-DCE	Waste solvent, degradation product of PCE and TCE
1,1-Dichloroethene (–ethylene)	Vinylidene chloride	1, 1-DCE	Waste solvent, degradation product of PCE and 1,1,1- TCA
Chloroethane (–ethylene)	Vinyl chloride	VC	PVC waste, degradation product of PCE and 1,1,1-TCA
Chlorinated ethanes			
1,1,1-Trichloro ethane	Methyl chloroform	1,1,1-TCA	Waste solvent
1,1,2-Trichloroethane	Vinyl trichloride	1,1,2-TCA	Waste solvent
1,2-Dichloro ethane	Ethylene chloride	1,2-TCA	Waste solvent, degradation product of 1,1,2, TCA
1,1-Dichloro ethane	Ethylidene chloride	1,1-DCA	Degradation product of 1,1,1-TCA
Chloroethane	–	CA	Refrigerant waste, tetraethyl Pb manufacturing waste, degradation product of 1,1,1-TCA and 1,1,2-TCA
Chlorinated methanes			
Tetrachloromethane	Carbon tetrachloride	CT	Waste solvent, fire extinguisher
Trichloromethane	Chloroform, methane trichloride	CF	Waste solvent, anesthetic waste, degradation product of CT.
Dichloro methane	Methylene chloride	MC	Waste solvent, degradation product of CT
Chloromethane	Methyl chloride	CM	Refrigerant waste, degradation product of CT

Table 4.9 CAH degradation mechanism and associated bacteria

CAHs	Degradation mechanism	Bacteria	References
TCE	Aerobic oxidation	*Burkholderia cepacia* G4, PR1301	Munakata-Marr et al. (1997) and Mccarty et al. (1998)
PCE, TCE, DCE, VC, DCA	Anaerobic reductive dechlorination (dehalorespiration)	PER-K23, *Dehalospirillium multivorans*, *Dehalobacter restrictus*, *Dehalococcus ethenogenes*	Hollinger et al. (1993), Smatlak et al. (1996), Tandol et al. (1994), Yager et al. (1997), and ITRC (2000)
PCE, TCE, DCE, VC, DCA	Anaerobic reductive dechlorination (cometabolic)	*Methanosarcina barkeri*, *Desulfomonile tiedjei*	Fathepure et al. (1987)
PCE, TCE, CT	Anaerobic reductive dechlorination (cometabolic)	Methanogens, denitrifiers, sulfate reducers	Workman et al. (1997) and Yager et al. (1997)
CT	Anaerobic reductive dechlorination (cometabolic)	*Shewanella putrefaciens* MR-1	Petrovskis et al. (1995)

via co-metabolism. The potential for aerobic co-metabolism of completely substituted CAHs, such as CT and PCE, is essentially zero, while it is very high for less saturated compounds, such as MC and VC. The general trend for aerobic cometabolism indicates a better potential the lower the degree of chloride substitution. The rates of aerobic co-metabolism are very compound specific. For example, rates for the dichloroethylene (DCE) isomers vary greatly. Aerobic transformations can result in complete degradation of CAHs to carbon dioxide, water, and chloride. Anaerobic conditions in general show an opposite trend, with a greater transformation potential the greater the degree of substitution.

A potential problem with the anaerobic processes is that CAHs are reduced to less substituted intermediates that are often transformed at slower rates and thus may accumulate. TCE can be reduced anaerobically to DCE, which can be transformed into VC (Vogel and McCarty 1985). Recently, however, it has been demonstrated that PCE and TCE can be anaerobically degraded to ethylene (Freedman and Gossett 1989; DiStefano et al. 1991, 1992), which is a nontoxic end product.

This finding has sparked new interest in situ CAH bioremediation via anaerobic processes (Semprini 1995). Table 4.9 listed some bacteria which are reported as potential candidate for biodegradation of CAH.

4.6.5 Explosives

The most immediate and profound risk from explosives is that of potential reactivity. Explosives exist in soils and sediments as small crystals to large chunks. The amount

Table 4.10 List of microorganism capable of degrading explosives in soil (Adrian and Arnett 2007; Crocker et al. 2006)

Explosive	Name of degradation method	Microorganism involved
RDX	Two electron reductive pathway	Enterobacteria, *E. coli*, clostridium, Acetylbutilecum, *Aspergillus niger*
RDX	Anaerobic denitration	*K. pneumonia, C. bifermentans*
RDX	Aerobic denitration	*Rhodococcus rhodochorus*, Williamsia and gorodonia
HMX	Aerobic degradation	*Methylobacterium*
HMX	Anaerobic denitration	*K. pneumonia, C bifermentans*
CL-20	Aerobic degradation	*P. chrysosporium*
CL-20	Anaerobic degradation	*Clostridium*
TNT	Aerobic degradation	*Pseudomonas, Phanerchoaete chryosoporium, mycobacterium, Rhodococus erythropolis*
TNT	Anaerobic degradation	*Clostridium sp.*

of damage caused is in direct proportion to the size of the crystal. The primary constituents of waste streams from explosives operations that result in soil contamination are nitroaromatics and nitramines including TNT (2,4,6-trinitrotoluene), RDX (Hexahydro-1,3,5-trinitro-1,3,5-Triazine), HMX (Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine), Tetryl (Methyl-2,4,6-trinitrophenylnitramine), Picric Acid (2,4,6-trinitrophenol), PETN (Pentaerythritol tetranitrate), TATB (Triaminotrinitrobenzene). The most frequently occurring impurities and degradation products from these include: 2,4-DNT (2,4-dinitrotoluene), 2,6-DNT (2,6-dinitrotoluene), 2A-4, 6-DNT (2-amino-4,6-dinitrotoluene), 4A-2,6-DNT (4-amino-2,6-dinitrotoluene), TNB (1,3,5-trinitrobenzene), DNB (1,3-dinitrobenzene), NB (Nitrobenzene), Picramic Acid (2-amino-4,6-dinitrophenol) (Craig et al. 1995).

Despite of various chemical limitations a plenty of microorganisms (Table 4.10) are found to colonize soil that are heavily contaminated with explosives and degrade the same to less hazardous form.

4.7 Bioremediation of Inorganic Pollutants

4.7.1 Heavy Metal

Heavy metals are the main group of inorganic contaminants and a considerable large area of land is contaminated with them due to mining, industry, agriculture and defense activities. Although metals are present naturally in the earth's crust at various levels and many metals are essential for cells (e.g. copper, iron, manganese, nickel, zinc), all metals are toxic at higher concentrations. Specifically, any metal (or metalloid) species may be considered a "contaminant" if it occurs where it is unwanted, or in a form or concentration that causes a detrimental human or environmental effect (McIntyre 2003).

Table 4.11 Normal and critical concentration of heavy metals in soil (mg/kg)

Element	Normal range in soil[a]	Critical soil concentration[a]	Concentration in metalliferous soils[b]
Cd	0.01–2.0	3–8	11–317
Cr	5–1,500	75–100	47–8,450
Cu	2–250	60–125	52–50,900
Hg	0.01–0.5	0.3–5	100–400
Ni	2–750	100	19–11,250
Pb	2–300	100–400	3,870–49,910
Zn	1–900	70–40	109–70,480

[a] Alloway (1995)
[b] Blaylock and Huang (2000)

Metal concentrations in soil typically range from less than one to as high as 70,000 mg/kg, as presented in Table 4.11.

Irrespective of the origin of the metals in the soil, excessive levels of many metals can result in soil quality degradation, crop yield reduction, and poor quality of agricultural products (Long et al. 2002). Since heavy metals are not biodegradable and may enter the food chain, they are a long-term threat to both the environment and human health (Jarup 2003). It includes the metals/metalloids, such as arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb), mercury (Hg), nickel (Ni), selenium (Se), silver (Ag), and zinc (Zn). Other less common metallic species that can be considered as contaminants include aluminum (Al), cesium (Cs), cobalt (Co), manganese (Mn), molybdenum (Mo), strontium (Sr), and uranium (U) (McIntyre 2003). Pb, one of the most persistent metals, was estimated to have a soil retention time of 150–5,000 years and was reported to maintain high concentration for as long as 150 years after sludge application to soil (NandaKumar et al. 1995). The average biological half-life of Cd has been estimated to be about 18 years (Forstner 1995) and 10 years once in the human body (Knasmuller et al. 1998). Another concern with toxic heavy metals causing concern is that the metals may be transferred and accumulated in the body tissues of animals or human beings through food chain, which will probably cause DNA damage and carcinogenic effects by their mutagenic ability (Knasmuller et al. 1998). For example, some species of Cd, Cr, and Cu have been associated with health effects ranging from dermatitis to various types of cancer (Das et al. 1997; McLaughlin et al. 1999). At high concentrations, metal ions can either completely inhibit the microbial population by inhibiting their various metabolic activities like protein denaturation, inhibition of cell division, cell membrane disruption etc. or organisms can develop resistance or tolerance to the elevated levels of metals. These metals cannot be degraded biologically, and are ultimately everlasting, though the speciation and bioavailability of metals may change with variation in the environmental factors (Shukla et al. 2010). Toxicity of metals in microorganism occurs through the displacement of essential elements 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

Table 4.12 Microorganisms that utilizes heavy metals (Chatterjee et al. 2008)

Elements	Microorganism
Cu	*Bacillus sp., Pseudomonas aeruginosa, Chlorella vulgaris, Pleurotus ostreatus, Phormidium valderium, Volvariella volvacea, Daedalea quercina*
Ni	*Pseudomonas aeruginosa, Zooglea sp., Chlorella vulgaris, Phormidium valderium*
Zn	*Bacillus sp., Chlorella vulgaris, Aspergillus niger, Pleurotus ostreatus, Daedalea quercina*
U	*Pseudomonas aeruginosa, Citrobacter sp., Chlorella vulgaris, Aspergillus niger,*
Co	*Zooglea sp., Phormidium valderium,*
Cd	*Ganoderma applantus, Zooglea sp., Citrobacter sp., Aspergillus niger, Pleurotus ostreatus, Stereum hirsutum, Phormidium valderium*
Pb	*Stereum hirsutum, Citrobacter sp., Chlorella vulgaris, Ganoderma applantus, Volvariella volvacea, Daedalea quercina*
Hg	*Chlorella vulgaris, Rhizopus arrhizus, Volvariella volvacea, G. metallireducens*
Au	*Chlorella vulgaris, G. metallireducens*
Ag	*Aspergillus niger,, Rhizopus arrhizus, G. metallireducens*
Cr	*D. vulgaris, D. Acetoxidans, D. Fructosovorans, D. norvegicium*

inhibit the activity of sensitive enzymes (Nies 1999). In addition, at high levels, metals can damage cell membranes; alter enzyme specificity; disrupt cellular functions; and damage the structure of DNA (Bruins et al. 2000). As a consequence, microorganisms have been forced to develop metal-ion homeostasis factors and metal-resistance determinants (Nies and Silver 1995; Nies 1999; Bruins et al. 2000. Because metal ions cannot be degraded or modified like toxic organic compounds, there are six possible mechanisms for a metal resistance system: exclusion by permeability barrier; intra- and extra-cellular sequestration; active efflux pumps; enzymatic reduction; and reduction in the sensitivity of cellular targets to metal ions (Ji and Silver 1995; Nies and Silver 1995; Nies 1999; Rensing et al. 1999; Bruins et al. 2000). One or more of these resistance mechanisms allows microorganisms (Table 4.12) to function in metal contaminated environments. Microbial transformation of metal serve various functions in stresses environment and it can be 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 iron, sulfur, manganese and arsenic, microbes can obtain energy (Tebo et al. 1997; Santini et al. 2000). On the other hand, reduction of metals can occur through dissimilatory reduction where microorganisms utilize metals as a terminal electron acceptor for anaerobic respiration. For example, oxyanions of As (Stolz and Oremland 1999; Niggemyer et al. 2001), Cr (QuiIntana et al. 2001), Se (Stolz and Oremland 1999) and U (Tebo and Obraztsova 1998) can be used in microbial anaerobic respiration as terminal electron acceptors. In addition, microorganisms may possess reduction mechanisms that are not coupled to respiration, but instead are thought to impart metal resistance. For example, aerobic and anaerobic reduction of Cr(VI) to Cr(III) (QuiIntana et al. 2001; Nkhalambayausi-Chirwa and Wang 2001); reduction of Se(VI) to elemental selenium (Lloyd et al. 2001); reduction of U(VI) to U(IV) (Francis 1998; Chang et al. 2001); and reduction

of Hg(II) to Hg(0) (Brim et al. 2000; Wagner-Döbler et al. 2000) are widespread detoxification mechanisms among microorganisms.

Microbial methylation plays an important role in the biogeochemical cycle of metals, because methylated compounds are often volatile. For example, mercury [Hg(II).]can be biomethylated by a number of different bacterial species (e.g. *Pseudomonas sp., Escherichia sp., Bacillus sp. and Clostridium sp*.) to gaseous methylmercury (Pan-Hou and Imura 1982; Compeau and Bartha 1985; Pongratz and Heumann 1999), which is the most toxic and most readily accumulated form of mercury (Nikunen et al. 1990). Also, biomethylation of arsenic to gaseous arsines (Gao and Burau 1997); selenium to volatile dimethyl selenide (Flury et al. 1997; Guo et al. 1999; Martens and Suarez 1999; Zhang and Frankenberger 1999; Dungan and Frankenberger 2000); and lead to dimethyl lead (Pongratz and Heumann 1999) has been observed in various soil environments.

4.8 Future Perspective

Bioremediation is not a panacea but rather a "natural process" alternative to traditional physico-chemical and chemical treatment methods. What defines bioremediation and distinguishes it from simple augmentation, is the presence of an active microbial degrader population (Menendez-Vega et al. 2007); transforming the bioavailable contaminants in an optimized environment (Bento et al. 2005). A study has been carried out to routinely monitor the decay of target compound or the appearance of metabolites or end products in field but such a simple approach does not differentiate abiotic from biotic processes nor does it give a measure of the physiological status or performance of the degrader population. The possible formation of toxic intermediates could either inhibit the process or lead to the occurrence of an increased risk status of the soils (Phillips et al. 2000; Diplock et al. 2009). Such a gap still exists between advances in laboratory research and commercial field application. Inadequate knowledge to predict pollutant degradation rate and fate accurately in the field condition and lack of designated field research centers and respective technology demonstration are two main reasons of such gap. Thus, to engage a bioremediation strategy, the user must be confident that the target threshold can be met and that the contributing factors to reach this target can be quantified. The physiologic potential of microbial populations should be tested in terms of field's heterogeneity, variability, size and at the same time complexity of using living organisms should be assessed. Strategic methods for performance evaluation of respective bioremediation techniques should be designed for better understanding of the extent and rate of clean up. To speed up the application, development of innovative site characterization techniques those are rapid, reliable and inexpensive are the urgent needs. Widely accepted bioremediation methods and criteria needs to be established followed by development of an accessible, expanded, and well documented database. Overall, more integrated and cross discipline effort would be required for proper assessment and implementation of bioremediation techniques.

4.9 Conclusions

Bioremediation is recognized as an alternative to traditional physico-chemical methods to restore contaminated sites. Being a cost effective, less labour intensive, safe and environment friendly technique rapid development and advances are happening in this field from the past two decades. It is found effective for a wide range of soil pollutants including PAH, PCB, CAH, pesticides, explosives, even heavy metals and radionuclide. However, in many cases, bioremediation method combines several treatment techniques and can last for a long time (years and decades) and are often applied in combination with other techniques; therefore it is difficult to estimate the efficacy of the same. In this context, more interdisciplinary research should be carried out in relation to process optimization, validation, its impact on the ecosystem and the effectiveness and predictability should be demonstrated to make it a generally accepted technique.

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Chapter 5
Role of Microbiologically Rich Compost in Reducing Biotic and Abiotic Stresses

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Abstract Plant abiotic and biotic stress is related to unfavorable and environmental constraints. These stresses represent the principal cause of crop failure, decreasing average yields of major crops by more than 50%. Compost can be considered as a soil conditioner that contributes to soil fertility, structure, porosity, organic matter, water holding capacity and disease suppression. Composts suppress soil borne diseases and this suppression has been widely reported for *Pythium* spp., *Phytophthora* spp., *Rhizoctonia* spp. and *Fusarium* spp. Compost amendments also contribute to controlling foliar diseases, such as *Puccinia* spp., *Alternaria solani* and *Pseudomonas syringae* pv. *syringae*. Disease suppression by composts has been explained mainly by biotic mechanisms. The severity of soil-borne plant diseases is often reduced when microbiologically improved compost used as growth media. Wide variety of rhizosphere micro-organisms have been isolated and used as microbiological inoculants for improving plant growth and health. These include, arbuscular mycorrhizal fungi (AMF), plant growth promoting rhizobacteria (PGPR), nitrogen fixers and phosphorus solubilizing microorganisms (PSM). Utilization of compost with disease suppressive properties is a relatively new biological method of decreasing biotic stress in plant production. The main advantage of exploring the role of microbiologically rich compost in reducing biotic and abiotic stresses is that, it is a novel way to imitate the natural system occurring in ecosystems. Under saline conditions, compost was not completely efficient in correcting the detrimental effects of salt, but was able to mitigate them. Such an amendment may be used to enhance crop

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yield irrigated with saline waters or grown on saline soils. Compost amendments in soil are also efficient in combating the pH stress. The amendment of alkaline soils with composts has a positive effect on plant vigour. Molecular studies also showed a different profile of microorganisms in disease suppressive composts. This study shows a good approach to add-up value to compost, and make it more efficient in reducing biotic and abiotic stresses.

Keywords Abiotic stresses • Biotic stresses • Compost • PGPR • PSM

5.1 Introduction

The past decades have witnessed a dramatic change in agriculture with food production soaring due to the Green Revolution. The Green Revolution entailed the use of improved technologies (particularly the breeding of high yielding food crop varieties), the expansion of irrigation, mechanization, specialization, and the use of chemical fertilizers and pesticides. While the Green Revolution led to dramatic increase in production of food grains especially in Asia and Latin America in the 1960s and 1970s, the increase in production was not sustainable. For example, evidence indicates that rice yield in Asia declined sharply in the 1980s, from an annual growth rate of 2.6% in the 1970s to 1.5% during the beginning of 1981, owing partly to increasing prices of chemical fertilizer and agrochemicals (pesticides and herbicides) (Kassie and Zikhali 2009). More importantly, despite the productivity gains associated with the Green Revolution, poverty and hunger persist while land degradation and agriculture-related environmental damage are prevalent and unabated.

Decreasing hunger requires increased food production, which in turn requires farmer's access to productivity-enhancing inputs, knowledge and skills. However, the majority of the chronically hungry are smallholder farmers in developing countries who practice subsistence agriculture on marginal soils, lack access to inputs and product markets, as well as financial resources to procure costly chemical fertilizer and other agrochemicals that might enhance the productivity of their land. Moreover, the Green Revolution has been criticized for its adverse human health and environmental impacts. For example agricultural intensification, through excessive and inappropriate use of chemical fertilizers and pesticides, has polluted water bodies and degraded soils, led to biodiversity loss by killing beneficial plants, insects and wildlife, and in some cases poisoned farm workers. Irrigation has led to salinization (build-up of salt within the soil) and retreat of groundwater levels in areas where more water is pumped for irrigation than can be replenished by rainfall. Monoculture systems have led to biodiversity loss, including loss of natural predators, and with heightened pest resistance, have required more or stronger agrochemicals to sustain yield levels. All these costs have not been properly internalized in the calculation of production costs of the Green Revolution model. In addition, inorganic fertilizer loses effectiveness when the organic matter of soil is low, which is of particular concern in developing countries due to continuous cultivation and soil degradation.

Sustainable agriculture has emerged as an alternative agricultural system that addresses many of the constraints faced by resource-poor farmers and at the same time ensures environmental sustainability. It refers to the capacity of agriculture over time to contribute to overall welfare by providing sufficient food and other goods and services in ways that are economically efficient and profitable, socially responsible, and environmentally sound. This system involves a combination of inter-related soil, crop and livestock production practices in conjunction with the discontinuation or the reduced use of external inputs that are potentially harmful to the environment and/or the health of farmers and consumers. Instead, it emphasizes the use of techniques of food production that integrate and are adapted to local natural processes such as nutrient cycling, biological nitrogen fixation, soil regeneration and natural enemies of pests. Using local resources to make initial soil and land improvements can get farmers on a virtuous cycle where raising incomes relieve the constraints to adoption of more resource-intensive sustainable practices.

Crop growth and crop yield are affected by abiotic and biotic factors including weather (rain, heat and temperature), soil conditions (water, pH and nutrients), insect populations, disease incidence and management practices (cultivar, irrigation, fertilization and rotation). These factors represent the principal cause of crop failure, decreasing average yields for major crops by more than 50%. In order to increase agricultural production, new crops and intensive agricultural practices have been introduced in India. These changes resulted in more disease problems. Also diseases, which were minor in nature, have become more severe under intensive cultivation system. By breeding and selection of stress tolerant varieties it is possible to increase the genotypic resistance of plants against abiotic and biotic stresses. Phenotypic resistance can be induced by manipulations and management practices such as inoculation of root-zone by beneficial microbes or leaf-sprays with stress resistance related compounds. A wide variety of rhizosphere micro-organisms have been isolated and used as microbiological inoculants for improving plant growth and health. These include, arbuscular mycorrhizal fungi (AMF), plant growth promoting rhizobacteria (PGPR), nitrogen fixers and phosphorus solubilizing microorganisms (PSM).

Composting is a biological process in which various organic biodegradable wastes are converted into hygienic, humus rich product (compost) which could be used as a soil conditioner and an organic fertilizer (Popkin 1995). Compost of various origins are also used to provide biological control against various plant pathogens (Hoitink and Grebus 1994). Aqueous extracts of compost have also been efficient in replacing synthetic fungicides (Zhang et al. 1998). The addition of composts to agricultural soils has beneficial effects on crop development and yields by improving soil physical and biological properties (Zheljazkov and Warman 2004). Poultry manure application in *Java citronella* plants, significantly increased the herbage, essential oil content and dry matter yield (Adholeya and Prakash 2004). Compost is also an inhabiting place for various microbes due to which biological activities are markedly enhanced in the rhizosphere of plants (Tilak and Reddy 2006). Such syntrophic associations are of ecological importance with implied agricultural significance. Utilization of compost with disease suppressive properties is a relatively new biological way of decreasing stress in plant production.

Documented effects of compost addition to a soil include the obvious effects, such as increased organic matter and plant nutrients like phosphorus and nitrogen, as well as increased soil respiration and microbial biomass (Tiquia et al. 2002). Many benefits from organic matter in compost are long term and are found from repeated applications, building up soil organic matter and hence fertility. Assigning an economic value to organic matter is currently difficult but with the possible adoption of carbon credit systems within agriculture in the future the importance of this value looks to increase. There is a need of greater understanding of crop physiological responses to the abiotic and biotic factors for improving non-irrigated crop production for producers.

5.2 Critical Review

Environmental stresses limits agricultural productivity worldwide. These stresses have an impact on different crop species and also significant barriers to the introduction of crop plants into non agricultural areas. Stresses enhance the severity of problems into the plants which will be exposed in the coming decades (Duncan 2000; Cherry et al. 2000).

5.2.1 Plant Stress

Stress is an altered physiological condition caused by factors that tend to disrupt the equilibrium. Strain is any physical and chemical change produced by a stress (Gaspar et al. 2002). The term stress is used with various meanings, the physiological definition and appropriate term as responses in different situations. The flexibility of normal metabolism allows the response initiation to the environmental changes, which fluctuate regularly and are predictable over daily and seasonal cycles. Thus every deviation of a factor from its optimum does not necessarily result in stress. Stress being a constraint or highly unpredictable fluctuations imposed on regular metabolic patterns cause injury, disease or aberrant physiology. Plants are frequently exposed to many stresses such as drought, low temperature, salt, flooding, heat, oxidative stress and heavy metal toxicity, while growing in nature. Tolerance to abiotic stresses is very complex, due to the intricate of interactions between stress factors and various molecular, biochemical and physiological phenomena affecting plant growth and development (Razmjoo et al. 2008).

Stress in plants could be defined as any change in growth condition(s) that disrupts metabolic homeostasis and requires an adjustment of metabolic pathways in a process that is usually referred to as acclimation (Shulaev et al. 2008). Also the complexity of biological responses means that it is often difficult to disentangle cause and effect. This cell water deficit may itself be regarded as a stress that then

affects various metabolic processes (Jones et al. 1989). Mainly two types of plant stresses are present in nature i.e. biotic and abiotic stress.

5.2.2 Abiotic Stress

Abiotic stress is defined as the negative impact of non-living factors on the living organisms in a specific environment. The non-living variable must influence the environment beyond its normal range of variation to adversely affect the population performance or individual physiology of the organism in a significant way. The most basic stressors include: high winds, extreme temperatures, drought, flood, and other natural disasters, such as tornados and wildfires. The lesser-known stressors generally occur on a smaller scale and so are less noticeable, but they include: poor edaphic conditions like rock content and pH, high radiation, compaction, contamination, and other, highly specific conditions like rapid rehydration during seed germination (Palta et al. 2006). Abiotic stresses are believed to cause major problems in agriculture by reducing crop growth and productivity. Because of their sessile nature, plants must endure adverse environmental conditions and consequently evolve a variety of responses to acclimatize to environmental stresses.

If a single abiotic stress is to be identified as the most common in limiting the growth of crops worldwide, it most probably low water supply (Boyer 1982; Araus et al. 2002). However, other abiotic stresses, notably salinity and acidity, are becoming increasingly significant in limiting growth of both forage grasses and the cereals. Exposure of plants to abiotic stresses (salinity, drought, extreme light and temperature) leads to major loss in crop productivity in India and worldwide. A study on global land use pattern reveals that 7% of the world's land area, amounting to 1,000 million hectares, has become saline (Tester and Davenport 2003). Abiotic stress affects plant growth, as well as development processes such as seed germination, seedling growth and vigour, vegetative growth, flowering and fruit set (Sairam and Tyagi 2004). In salinity affected plants, the result is primarily an ionic imbalance and hyper osmotic stress. The effect of this imbalance or disruption in homeostasis occurs at the cell level as well as at the whole-plant level. Massive changes in ionic and water balance cause molecular damage and growth arrest. Finally, in extreme saline conditions, this leads to tissue death and ultimately death of the plant (Zhu et al. 1997; Xiong and Zhu 2002) (Fig. 5.1).

pH causes changes in nutrient solubility which affects the nutrient availability and that way the plant growth. The availability of important metals, like aluminium, iron and zinc as well as micronutrients and other nutrients, depends on the cation exchange ability which is affected by the pH conditions in the soil. In low pH areas nitrogen and phosphorus availability is limited and especially aluminium toxicity is an important factor reducing plant growth. When pH rises over 7, low availability of iron, zinc and copper cause deficiencies (Fageria et al. 1998). On top of nutrient availability, pH has an effect on the whole soil community by affecting the soil properties and microbiology.

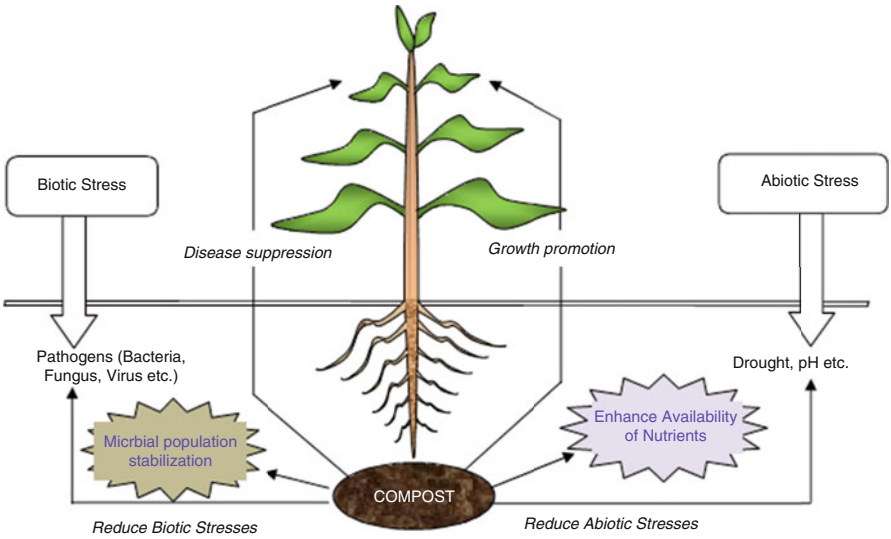

Fig. 5.1 Compost and its possible responses towards reducing plant stresses and in improvement of soil quality by enhancing nutrient availability and stabilizing microbial population

5.2.3 Biotic Stress

Biotic stress is stress that occurs as a result of damage done to plants by other living organisms, such as bacteria, viruses, fungi, parasites, harmful insects, nematode and weeds. It is a major focus of agricultural research, due to the vast economic losses caused by biotic stress to cash crops. Global loss because of pathogens is estimated to be 12% of potential crop productivity. The relationship between biotic stress and plant yield, affects economic decisions as well as practical development. The impact of biotic injury on crop yield impacts population dynamics, plant stress or co-evolution, and ecosystem nutrient cycling. The major biotic stresses affecting crops are fungal diseases although insects, viruses, bacteria and parasitic weeds can also drastically decrease crop production. The relative importance of aerial fungal diseases and their effect on yield varies among years and cropping regions. Foliar diseases caused by biotrophic pathogens, such as rusts, downy mildews and powdery mildews, are major limiting factors in crop production. Several rust species can infect grain belonging to genus *Puccini* such as *Puccinia graminis* on wheat rust, *P. sorghi* on maize and forage legumes, most of them belonging to the genus *Uromyces*, such as *U. appendiculatus* on common bean, lentil and *U. vignae* on cowpea. Lack of natural sources of resistance (Ramteke et al. 2004) makes this disease a good candidate to be solved using various methodologies. Root rot, caused by *Aphanomyces euteiches*, *Rhizoctonia solani*, *Fusarium solani* and wilt, caused by several *formae speciales* of *Fusarium oxysporum* are the most destructive soil-borne diseases of pea, chickpea, lentil, fababean and lupin (Infantino et al. 2006). Most of these attack the seedling

stage of the crop and are referred to as damping-off diseases. For example, damping-off, generally caused by either *Rhizoctonia solani* or *Pythium* spp., can result in up to 80% of plant death (Denman et al. 1995; Wang et al. 2003). *Fusarium* root-rot (caused by *Fusarium* spp.) can also cause severe seedling losses especially in common tomato and lentils (Hamwieh et al. 2005; Schneider et al. 2001). In most growing areas of the world, *Fusarium* wilt (caused by *F. oxysporum*) is a major constraint in the production of tomato, and lentil (Bayaa 1997) in particular. The disease affects seedlings and adult plants where it causes leaf chlorosis, wilting and death. Other important soil-borne diseases such as southern stem rot (*Sclerotium rolfsii*) and the white mold (*Sclerotinia sclerotiorum*) can cause both seedling and pod rots in warmer and cool weather respectively (Kolkman and Kelly 2003).

5.2.4 Approaches to Overcome Plant Stresses

Plants have to exploit their immediate environment to maximum effect. Their inability to move swiftly means that the best way of dealing with many stresses is through physiological or morphological changes.

5.2.5 Approaches to Overcome Abiotic Stress

Traditional approaches to breeding crop plants with improved abiotic stress tolerances have so far met limited success (Richards 1996). This is due to a number of contributing factors, including: (1) the focus has been on yield rather than on specific traits; (2) the difficulties in breeding for tolerance traits, which include complexities introduced by genotype by environment, or G×E, interactions and the relatively infrequent use of simple physiological traits as measures of tolerance, have been potentially less subject to G×E interferences; and (3) desired traits can only be introduced from closely related species. Most cereals are moderately sensitive to a wide range of abiotic stresses, and variability in the gene pool generally appears to be relatively small and may provide few opportunities for major step changes in tolerance. Of potentially larger impact on abiotic stress tolerance is the use of genetic manipulation technologies to generate such step changes. Having said this, more immediately achievable, if modest, increases in tolerance may be introgressed into commercial lines from tolerant landraces using marker-assisted breeding approaches (Dubcovsky 2004), facilitated by recent breakthroughs with positional cloning (e.g. Yan et al. 2003, 2004) that are likely to enable identification of extant tolerance genes within cereal germplasms.

Another approach in battling abiotic stress is to amend the environment, i.e. the soil. With pH stress, it is important to change the nutrient availability as well as the soil properties to change the pH of the soil. This can be done by neutralizing the soil through liming (with calcium or magnesium carbonate). This method has been

widely applied across the globe. Unfortunately, there are negative long term effects with the over use of these chemicals, with no improvement of soil properties and therefore alternative approaches are necessary. One alternative is to mix organic compounds into the soil. This would increase the buffering capacity of the soil, add to the natural source of nutrients and carbon in the soil and biota that recycles the nutrients as well as increase the water holding capacity. Increasing the buffering capacity would improve the soil's ability to keep the pH neutral and thus increase the nutrient availability (Wright et al. 2009).

5.2.6 Approaches to Overcome Biotic Stress

Biocontrol involves harnessing disease-suppressive microorganisms to improve plant health. Disease suppression by biocontrol agents are the sustained manifestation of interactions among the plant, the pathogen, the biocontrol agent, the microbial community on and around the plant, and the physical environment. Biocontrol of soil borne diseases is particularly complex because these diseases occur in the dynamic environment at the interface of root and soil known as the rhizosphere, which is defined as the region surrounding a root that is affected by it. The rhizosphere is typified by rapid change, intense microbial activity, and high populations of bacteria compared with non-rhizosphere soil. Plants release metabolically active cells from their roots and deposit as much as 20% of the carbon allocated to roots in the rhizosphere, suggesting a highly evolved relationship between the plant and rhizosphere microorganisms. In view of current and future directions in biocontrol attentions are being drawn on the bacteria viz., *Pseudomonas* and *Bacillus* and the fungi *Trichoderma* as examples representing a range of life strategies and mechanisms of disease suppression. There are also biotechnological approaches in use to reduce the biotic stress. Molecular markers are particularly useful when targeting characters controlled by several genes. Numerous molecular marker-related techniques have been used in relation to biotic stresses. Random Amplified Polymorphism (RAPD), Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeat (SSR) and derivatives have been reported for biotic (Ouedraogo et al. 2002; Román et al. 2002) stresses. As a result, genetic maps for many species were established in which potential resistance and/or tolerance loci or QTLs have been located. This improved the knowledge of the genetic control of specific resistance and/or tolerance in many crops by providing information on the number, chromosomal location and individual or interactive effects of the QTLs involved. More importantly, these technologies have identified specific molecular markers that may be used in breeding programs through Marker-Assisted Selection (MAS) to enhance biotic stress tolerance. For instance MAS was successfully used for the breeding of resistant soybean to cyst nematode (Diers 2004), of resistant pinto bean to common bacterial blight (Mutlu et al. 2005) and of resistant narrow-leafed lupin (*Lupinus angustifolius L.*) to phomopsis stem blight (Yang et al. 2002) and anthracnose (Yang et al. 2004). Moreover, when

resistance is conferred by single genes and/or easily overcome by new pathogen races, the gene pyramiding strategy facilitated by MAS can be an efficient method.

There are few different approaches, which are presently in use to reduce the plants stresses. Most recent approach is the use of compost for reducing the biotic and abiotic stresses in plants.

5.2.7 Role of Compost to Combat Stresses

Compost amendment to soil is mostly considered as a way to improve the soil fertility and physical structure, as it helps in stabilization of the aggregated framework which may reduces erosion and runoff process (Bresson et al. 2001; Barzegar et al. 2002; Innerebner et al. 2006). Furthermore, it increases the amount of vital nutrients and soil organic carbon (Filcheva and Tsadilas 2002). Utilization of compost also stimulates the soil micro flora and fauna, particularly in degraded and arid environments (Ouedraogo et al. 2001; Ros et al. 2003). However, the response of the microbial community shows variation according to the nature of the organic amendments (Pascual et al. 1998; Garcia-Gil et al. 2000), and the level of compost application (Albiach et al. 2000; Garcia-Gil et al. 2000). The enriched compost carries millions of microorganisms, a tightly knitted soil food web, creating a natural immune system for the plants, acting as a natural predator against most of the known soil borne diseases. Living soil hosts are different from large animals and insects. In particular, the beneficial rhizosphere microbes are important indicator of plant health and soil fertility. Such microbes participate in many key ecosystem processes such as those involved in the biological control of plant pathogens, nutrient cycling and seedling establishment (Jeffries et al. 2003). Positive interactions in the rhizosphere include symbiotic associations with epiphytes and mycorrhizal fungi and root colonization by bacterial biocontrol agents and plant growth promoting bacteria (Bias et al. 2006). However, the microorganism are unevenly distributed, but mainly congregated around nutrient sources and organic matter. Moreover, plant growth shows a direct correlation with the amount of microbial biomass and organic matter level present in the soil (Paul and Clark 1996), which, in turn, could be related to the role of microorganisms in both energy flow and nutrient cycling. The use of microbiologically rich compost for agricultural production could alter these relationships and help in increasing crop productivity in both ways qualitatively and quantitatively. Utilization of compost with disease suppressive properties is a relatively new biological way of decreasing stress in plant production. Number of evidences indicates that single microorganism inoculation do not provide a persistent solution for the long-term sustainable growth of plants, therefore, a consortium of microorganisms is needed for creating the beneficial rhizosphere (Adani et al. 1997). Although the potential benefits of compost and rhizosphere microorganisms for decreasing plant stress caused by abiotic and biotic factors have been shown in various case studies (Gomez 1998), however, there is little knowledge about the importance of this phenomenon in plant rhizosphere ecology.

5.2.7.1 Composting

Composting has been defined as intense microbial activity leading to decomposition of most biodegradable materials, usually mixtures of organic materials, which results in organic residue stability (Adani et al. 1997). Benefits of composting include (1) decrease in mass and volume of organic wastes, (2) recycling of nutrients, (3) maintaining or restoring soil organic matter, (4) reduction in land-filling problems, and (5) biodegradation of toxic compounds and other organic contaminants (Adani et al. 1995; Gomez 1998). Compost can be considered as a soil conditioner that contributes to soil fertility, structure, porosity, organic matter, water holding capacity and disease suppression (Itävaara et al. 1997). Thus, the use of compost has the potential to decrease abiotic and biotic stress of cultivated plants.

5.2.7.2 Compost Microbiology

Microbes play a major role in every system in the environment. Composting involves different groups of microorganisms, which act on the substrates in succession. Although, the initial process is carried out by mesophilic microorganisms and decomposition is mainly carried out by the thermophilic microorganisms. These include fungi, bacteria and actinomycetes (Hultman et al. 2010. Partanen et al. 2010). At this stage, acarines, millipedes and isopods will eat through the waste. The soft tissue of the decaying plants supports growth of nematodes and enchytraieds. As in any food chain, these creatures then attract predators to feed on them – collembolans eat fungi feed on fungal spores. Nematodes, protozoa and rotifers feed on bacteria. The energy released during this feeding frenzy causes a rise in temperature to between 45°C and 70°C. At this temperature, thermophilic microorganisms take over the degradation process. Hemicellulose, cellulose and lignin are degraded rather slowly. Thermophilic bacteria like *Thermonospora* and *Thermoactinomyces* and thermophilic fungi such as *Thermoascus* carry out these reactions. Once the readily degradable materials are degraded, the reaction rate slows down. Eventually the temperature decreases once again to the mesophilic range through heat loss from the surface of the heap and again the mesophiles, either through re-invasion from outside or through germination of heat resistant spores, dominate.

5.2.7.3 Use of Compost to Combat Abiotic Stress

Water is one of the most important ecological factors determining crop growth and development; water deficit plays a very important role in inhibiting the yields of crops (Zhu 2002). Water-limited crop production depends on the intensity and on the pattern of drought, which vary from year to year. It is another important and significant factor restricting plant growth and crop productivity in the majority of agricultural fields of the world (Tas and Tas 2007). It inhibits the photosynthesis of plants, causes changes in chlorophyll contents and components and damage to the

photosynthetic apparatus (Nayyar and Gupta 2006). In addition, it inhibits the photochemical activities and decreases the activities of enzymes in the Calvin Cycle in photosynthesis (Monakhova and Chernyadev 2002).

Water and soil salinization as well as water scarcity or excess, are the main abiotic stresses agricultural production is facing today and also in the near future it could be aggravated due to the already evident global climatic changes. Moreover, the increasing frequency of dry periods in many regions of the world and the problems associated with salinity in irrigated areas frequently result in the consecutive occurrence of drought and salinity on cultivated land. Currently, 50% of all irrigation schemes are affected by salinity (Hu and Schmidhalter 2005). Bialal et al. (2008) performed field trials, which demonstrated that compost is able to improve soil properties and plant growth in saline soil. Highly saline soil amended with high rates of low nutrient compost enhanced plant growth, as compared to unamended moderately saline soil. Whereas, high nutrient compost when used for the same purpose did not perform well, despite low sodium and chloride concentrations. It appears that less salt tolerant plants (corn, sorghum and *E. punctata*) benefited more from usage of compost than salt tolerant plants (Vetiver grass). Compost use does not reduce salinity, except for possible dilution effects, and does also not solve the underlying cause of the salinity problem. However, the use of compost on saline soils still improves the establishment and growth of plants. Therefore, compost use can offer a short-term reprieve for farming on soils with medium to high salinity, or soils with temporarily high salinity (dry period). Their results suggest also that high application of compost may be a very useful tool for ameliorating severely salt affected areas through the establishment of plant cover, including deep-rooted trees that help lowering the water table. Nevertheless, watering of trial plants with industrial effluent demonstrated that compost use is able to aid plant growth in extremely saline conditions.

pH is an abiotic stress problem that globally affects large areas. Acid soils constitute about 30% of the total cultivable area in India. Laterization, podzolisation, intense leaching of bases and accumulation of undecomposed organic matter under marshy conditions contribute towards the soil acidity. These soils occur in the Himalayan region, the eastern and north-eastern plains, peninsular India and the coastal plains under varying environmental conditions of landscape, geology, climate and vegetation. Approximately 100 Mha of land suffers from soil acidity, out of which 51 Mha is under forests. Out of remaining 49 Mha under cultivation, 26 Mha has pH less than 5.5 and 23 Mha has the pH value ranging between 5.6 and 6.5. Most of the acid soils are sedentary and are found on hilly terrains (Singh 2009). These areas are not ideal agricultural land but due to an increased need for food production, especially in developing countries under pressure from industrialization, more unsuitable areas are taken into farming use. Compost is an inexpensive material for soil enhancement because of the unlimited resource of material ranging from cattle manure to household waste and sewage sludge, all easily accessible to farmers. Compost increases the buffering capacity of the soil which makes its body more resilient to pH stress. This allows more of the necessary nutrients to be available for the plants and reduces the risk of aluminum toxicity.

5.2.7.4 Use of Compost to Combat Biotic Stress

Research on the application of composts has demonstrated a variety of disease suppressive effects, which indicate that suppressive effects can result from a combination of physiochemical and biological characteristics (Boulter et al. 2002). Microbial community exists under strong competition for readily degradable organic materials and energy-yielding nutrients in the soil environment (Stone et al. 2004), and the soil microbial community rapidly utilises any readily available nutrients entering the soil system. Typically competition for nutrients such as sugars and amino acids starts with competition for exudates from roots and germinating seeds as plants begin growing through the soil (Hoitink and Changa 2004).

In the first mechanism of biological disease control, beneficial microflora including compost-derived microorganisms compete for nutrients with plant pathogens in the rhizosphere (De Brito et al. 1995; Hoitink and Boehm 1999). Most plant pathogens are saprophytes that readily lose this competition for nutrients in compost-amended soils (Hoitink and Changa 2004). This battle for energy-yielding substances results in the repression of pathogen spore germination and growth; a phenomenon called microbiostasis (Lockwood 1990).

The second mechanism of biological control involves production of antibiotic compounds by beneficial microorganisms that are effective in controlling various plant pathogens, a process known as antibiosis (Hoitink et al. 1996). Antibiosis involves antagonism mediated by specific or non-specific metabolites that result from microbial activity such as lytic agents, volatile compounds, or other toxic substances. For example, the beneficial bacteria *Pseudomonas* spp. produce the antibiotic 2,4-diacetylphloroglucinol which is considered responsible for the suppression of take-all in wheat, *Fusarium* wilt in peas, and cyst nematode and soft rot in potatoes (Weller et al. 2002).

The third mechanism involves predation and parasitism of plant pathogens. Organic amendments stimulate the growth of populations of micro-arthropods such as beneficial nematodes, springtails and mites, which search out and consume pathogen propagules in soils (e.g. sclerotia of pathogenic fungus *Rhizoctonia solani*) (Hoitink and Boehm 1999). Specific isolates of beneficial fungus *Trichoderma* spp. serve as an example of parasitic biocontrol agents, destroying the sclerotia of pathogenic fungus *Rhizoctonia* (Sivasithamparam and Ghisalberti 1998). Increased abundance of arthropods after application of compost products to apple orchards has shown to significantly slow the growth of the pathogenic brown rot fungus (*Monilinia fructicola*) (Brown 2004).

The fourth mechanism of biocontrol involves the induction of systemic resistance in plants by microorganisms present in composts or soils (Zhang et al. 1996). Induced systemic resistance (ISR) or systemic acquired resistance (SAR) is a state of enhanced defensive capacity developed by a plant when appropriately stimulated (van Loon et al. 1998). Induced systemic resistance occurs when microorganisms cause the plant to "turn on" or strengthen its natural defences against disease (Nelson and Boehm 2002). Induced systemic resistance can provide protection against viral, fungal and bacterial plant pathogens; and root, vascular and foliar diseases of plants.

A variety of soil and rhizosphere bacterial and fungal isolates have been reported to stimulate ISR in plants (van Loon et al. 1998). Some of the specific root-colonising microorganisms in composts activate biochemical pathways in plants leading to resistance to root diseases as well as foliar diseases that can be suppressed by ISR (Hoitink et al.; Khan et al. 2004).

Induced systemic resistance can be triggered by exposure of plants to virulent, avirulent, or non-pathogenic microbes or by various chemical agents like salicylic acid, 2, 6-dichloro-isonicotinic acid, or benzo thiadiazole-7-carbothioic acid S-methyl ester (Vallad et al. 2000). For example, various soil-borne non-pathogenic microorganisms, referred to as plant growth promoting rhizobacteria, are capable of stimulating plant defences through the production of microbial metabolites such as salicylic acid, siderophores, antibiotics and lipopolysaccharides, all of which have been implicated in microbially stimulated ISR. Composted paper mill residuals induced resistance to *Fusarium* wilt of tomato, resulting in a reduction in fungal colonisation of root tissues (Pharand et al. 2002). Suppression was associated with reduced fungal colonisation of the tomato roots due to an increase in physical barriers to fungal penetration (callose-enriched, multilayered wall appositions and osmiophilic deposits) (Pharand et al. 2002). Composted pines bark container media suppressed *Pythium* root rot and foliar anthracnose of cucumber (Zhang et al. 1996). Cucumber and Arabidopsis plants grown in composted pine bark had higher levels of β-1, 3-glucanase (Zhang et al. 1998) and peroxidase (Zhang et al. 1996) than those grown in peat. Composted paper mill residuals applied to a sandy soil suppressed leaf spot of field grown cucumber, bacterial spot of field grown snap bean (causal agent *Pseudomonas syringae*), anthracnose (causal agent *Colletotrichum lindemuthianum*) in greenhouse grown snap beans, and the suppression of foliar diseases has been reported as likely due to ISR (Stone et al. 2003; Vallad et al. 2000). Plants produced in compost-amended mixes that induce systemic resistance also have higher concentrations of enzymes related to host defence mechanisms. Composts can provide natural biological control of diseases of roots as well as of plant foliage. Several disease suppressive mechanisms may operate at the same time against different pathogens in compost-amended media. Specific beneficial microorganisms can induce all four mechanisms. The beneficial fungus *Trichoderma hamatum* 382, a fungal biocontrol agent, has been reported to exhibit all four beneficial effects (Hoitink et al. 2001). Physical or chemical aspects of composts that reduce disease severity directly or indirectly by affecting the pathogen or host capacity for growth include: nutrients; organic matter; moisture; and pH. For example, organic amendments of high nitrogen content (e.g. poultry manures, meat meals) have the potential to suppress soil-borne diseases through the toxic effects on plant pathogen growth and survival by ammonia, nitrous acid or volatile fatty acids (Lazarovits 2001). On the other hand such chemical compounds are also toxic to plants and beneficial organisms, and composts applied at high rates that provide excessive N loading have also been implicated in exacerbating numerous diseases such as fireblight, *Phytophthora* dieback, and *Fusarium* wilt (Hoitink and Grebus 1994). Therefore it is important to apply N via composts at environmentally and agronomically responsible levels, which requires understanding of soil factors

regulating the release of nitrogen compounds in soil systems, such as moisture content, pH, soil organic matter (SOM) content and quality, soil texture, buffering capacity and nitrification rate (Lazarovits 2001).

5.3 Analysis

5.3.1 Use of Compost to Overcome Abiotic (Water, Salt and pH) Stress

Plants use some strategies to overcome stress condition. Several strategies were proposed to enhance the growth and yield of agricultural crops exposed to drought conditions. The main strategies were based on: (i) gradually improving the drought tolerance through conventional breeding and selection; (ii) crop introgressions with their wild, more tolerant to drought and salt progenitors; (iii) domesticating halophytes by breeding and selection for improved agronomic characteristics (Shannon 1997). On the other hand, while the use of agro-biotechnological tools to cope with the negative effects of flooding on crop production has received low attention; several strategies are being tested to minimize water and saline stresses (Thomson 2003). In this regard, two main approaches are being used: (i) the exploitation of natural genetic variations, either through direct selection in stressful environments or through mapping quantitative trait loci and subsequent marker-assisted selection; and (ii) the generation of transgenic plants to introduce novel genes or to alter expression levels of the existing genes to enhance salt and drought stress tolerance (Yamaguchi and Blumwald 2005).

In addition, authors also did an attempt and used microbiologically rich compost (JNMsC and TERI-5) to minimize abiotic stress. Both types of composts were prepared in combination with Jatropha press cakes. Jatropha press cake cannot be used in animal feed because of its toxic properties, but they are valuable as organic manure due to their high nitrogen content, which is similar to that of seed cake from castor bean and chicken manure. The nitrogen content ranges from 3.2% to 3.8%, depending on the source. Tender branches and leaves are used as a green manure for coconut trees. All plant parts can be used as a green manure. Extracts from different parts of *Jatropha curcas* show molluscicidal and insecticidal properties. The seed oil, extracts of Jatropha seeds, and phorbol esters from the oil have been used to control various pests, in many cases, successful results. There is a need for research in the utilization of this biopesticidal and manurial property of pressed cakes by investigations on the possibilities of the use and application of the seed cake after the oil extraction, as a carrier for bio-fertilizers which are otherwise applied after mixing (diluting) with inert carriers or organic manures like farmyard manure, cow dung or vermicompost. This holds true especially in the case of mycorrhiza where its conditions and properties are very similar to that of organic manure and for biocontrol of soil-borne diseases. Since its highly recommended as manure, mixing

with bio-fertilizers such as mycorrhiza would further add to its success as soil enricher and as disease suppresser and would benefit more in the agricultural and forestry based-plantations.

A glasshouse experiment approach was used to test pH stress, where neutral (pH 7) acidic (pH 4,5) and alkali (pH 9) soils were mixed with compost at a ratio of 1:4. The composts used contained Jathropha at different ratios, one being prepared only from it (JC) and one containing none, CPP(SUPA). Maize was used as the test plant as it is a commonly used crop with important value in food production. In every soil type the compost amendment enhanced plant growth, but not all composts were equal. The results were most prominent in the alkali soil mixtures. In this soil the pure Jatropha compost caused test plants to die, probably due to the toxic compounds. No such effect was seen in neutral or acidic soil. The best yield was collected from the CPP(SUPA) and JNMsC mixtures, probably due to the rich variety of organic compounds and microbes. Subsequent tests showed that JC had little buffering capacity when compared to the two composts with best results in the plant growth experiment. This shows that multiple compounds hold the key to preparing compost for soil amendments.

5.3.2 Use of Compost to Overcome Biotic (Disease) Stress

This section of the chapter outlines the suppressive effects of a wide range of compost products against a range of diseases caused by a variety of pathogens. The most commonly studied pathogens and their suppression using compost products in container media or as soil amendments are discussed below.

5.3.2.1 Suppression of Soil Born Pathogens Causing Diseases

Fusarium

Fusarium is a soil-borne pathogenic fungus, which causes root rot, stem rot and *Fusarium* wilt of plants. Suppression of *Fusarium* using composts has been reported by several researchers (Punja et al. 2002). The severity of various diseases caused by *Fusarium* has been reduced between 20% and 90% using compost amendments. Microbial activity has been considered a key factor in suppression of *Fusarium* wilt (Cotxarrera et al. 2002). Composts increased microbial populations (Cheuk et al. 2003), and microbial activity in composts and composted peat mixes increased by 50% (Cotxarrera et al. 2002). Several microorganisms or biocontrol agents have been isolated from composts or shown to contribute to suppression of *Fusarium* spp. including populations of non-pathogenic strains of *Fusarium oxysporum* and fluorescent *Pseudomonas* spp. (Kannangara et al. 2000); *Trichoderma* and *Flavobacterum* (Hoitink and Fahy 1986). It has been suggested that fluorescent pseudomonads (beneficial bacteria) secrete iron chelating siderophores, which limit

availability of iron for pathogen germ tube growth (Alabouvette 1999). Disease suppressiveness has been reported to increase with increasing proportions of compost either in soil or potting mix (Boulter et al. 2002). Boulter (2002) found that a higher rate of compost application (97.4 kg/100 m^2) showed a greater capacity to suppress disease compared to a lower rate (48.7 kg/100 m^2), due to increased nutrient availability, and to increased antagonistic or competitive interactions among microorganism populations or their metabolites.

Phytophthora

Phytophthora is a soil-borne pathogenic fungus that causes a variety of problems including root rot, a form of "dieback"; crown rot; or *Phytophthora* blight of plants. Composts have been used successfully for suppression of *Phytophthora* crown and root rots of nursery and fruit crops produced in container media (Aryantha et al. 2000) and field soils (Downer et al. 2001). Addition of compost serves two possible purposes significant for the biological control of *Phytophthora cinnamomi*: it provides a substrate for the growth of fungal antagonists, and creates an environment that promotes enzyme activity (Downer et al. 2001). Several potential biocontrol agents of *Phytophthora* root and crown rots have been identified from compost amended growing media, including *Pseudomonas* spp. (Aryantha et al. 2000); *Pantoea* spp (formerly *Enterobacter* spp) (Krause et al. 2003); *Penicillium* and *Aspergillus* spp. (Downer et al. 2001); actinomycetes (Aryantha et al. 2000) and *Trichoderma* spp. (Downer et al. 2001). Among the microbes, the actinomycetes appeared to be the most active in inhibiting the growth of *P. cinnamomi*, which may also relate to their ability to produce antibiotics.

Pythium

Pythium is a destructive, soil-borne parasitic root fungus, which causes damping-off disease in seedlings and root and crown rot of plants. The suppression of diseases caused by *Pythium* spp. has been well documented (Stone et al. 2001).

The severity of diseases caused by the fungus *Pythium* was reduced by 30–70% when growing media were amended with various compost products. Adequately mature composts have large microbial populations and high microbial activity, which have been directly linked to the suppression of *Pythium* (Ringer et al. 1997) but this may not hold true for all composts (Craft and Nelson 1996). Higher levels of microbial activity induce microbiostatis (suppression of disease organisms due to intense microbial competition) of *Pythium* root rot and damping-off (Boehm et al. 1993) because *Pythium* is a good colonizer but a weak competitor (Kwok et al. 1987). Under conditions of intense competition, the saprophytic and pathogenic abilities of *Pythium* are suppressed, resulting in low disease.

Rhizoctonia spp

Rhizoctonia is a soil-borne pathogenic fungus which causes a range of soil-borne diseases as well as diseases of aerial parts of plants. Composts have been used with varying success to suppress *Rhizoctonia* diseases of several crops (Tuitert et al. 1998). Amendment of soil or container media with composts reduced diseases caused by the soil-borne pathogenic fungus *Rhizoctonia* by up to 70%. Higher levels of microbial biomass and microbial activity have been reported to suppress *Rhizoctonia* by increasing competition between compost-inhabiting microorganisms and *Rhizoctonia solani* (*R. solani*) for cellulose or other available nutrients (Diab et al. 2003). The increase in microbial count (MBC) and microbial number (MBN) indicated growth of saprophytic microorganisms. The high population density of fluorescent pesudomonads, actinomycetes and heterotrophic fungi in growing media amended with adequately matured compost has been found to better suppress *Rhizoctonia* spp. than the use of less matured (immature) composts (Diab et al. 2003). In addition to competition, parasitism was reported as a mechanism for suppressing *Rhizoctonia* spp. In growing media amended with mature compost. The presence of beneficial fungi *Trichoderma* spp. (Kuter et al. 1983) and *Penicillium* spp (Hadar and Gorodecki 1991) has been reported to eradicate *Rhizoctonia*.

5.3.2.2 Suppression of Pathogens Causing Plant Foliage Diseases

Composts are considered able to induce systemic resistance, which can reduce the severity of some plant foliage diseases (Stone et al. 2003). However, Krause et al. (2003) reports that only a small proportion of composts have the ability to suppress foliar diseases. Few studies that show foliar disease suppression is presented below.

Pseudomonas

Pseudomonas is a bacterium. Some pathogenic *Pseudomonas* species cause several plant foliar diseases including bacterial speck and bacterial canker. Compost application has resulted in reduced bacterial speck of Arabidopsis (mustard family) and tomatoes (Vallad et al. 2003). In tomatoes, different types of paper mill residue based composts resulted in the reduction of bacterial speck between 47% and 62% (Vallad et al. 2003).

Colletotrichum

Colletotrichum is a fungus that causes diseases such as anthracnose fruit rot or lesions. Research has shown that amendment of soil or container media with composts can reduce the severity of anthracnose (Abbasi et al. 2002). Composted

cannery waste applied at a high rate (24–30 $t\,ha^{-1}$) reduced the incidence of anthracnose in organic tomatoes by 40% (Abbasi et al. 2002).

Xanthomonas

Xanthomonas is a pathogenic bacterium that causes many plant diseases, including such foliar diseases as bacterial leaf spot or speck, bacterial blight or angular leaf spot, and stem rot. Two studies report a reduction in bacterial leaf and fruit spot of vegetables (radish and tomato) caused by *Xanthomonas* spp. (Krause et al. 2003). Abbasi et al. (2002) found that, in tomato production, application of composted garden organics resulted in reduced bacterial spot incidence on fruit by 28–33%, although the severity of disease on foliage was increased. *Bacillus* spp. has been identified as most effective in suppressing bacterial leaf spot (Krause et al. 2003).

5.3.2.3 Suppression of Pests Causing Plant Diseases

Few studies conducted show a reduction in the incidence of pests being associated with compost application. Increased populations of pest predators have been identified in crops where organic mulches have been applied (Jurgens 2004). Brown and Tworkoski (2004) found populations of pest predators were significantly higher in plots where composted mulch was applied than in plots without composted mulch; and that composted mulch increased the total abundance of arthropods, with increased population of pest predators and fewer herbivorous insects (i.e. pests) (Brown and Tworkoski 2004). Populations of spotted tentiform leafminer (*Phyllonorycter blancardella*) and migrating woolly apple aphid (*Eriosoma lanigerum*) nymphs were reduced in areas treated with composted mulches (Brown and Tworkoski 2004). Mathews et al. (2002) also showed an increase in arthropod predators in a young apple orchard arising from mulch application and associated this specifically with the chemical properties of the composted mulch in providing a food source for pest predators (from decomposing organic matter and associated microbes).

5.4 Future Perspectives

In future a lot of study is needed to understand the role and mechanism of compost against plant stresses. First, understanding mechanisms of pathogen resistance to the action of biocontrol agents is critical to sustain disease suppression with long-term use. Strategies to minimize resistance and prevent its spread should be designed. The second area that is ripe for study is genetic diversity within species of both biocontrol agent and host plant. Exploitation of genetic variation among members of a microbial species that suppresses disease may provide a solution to the variability across space and time that has been observed with many biocontrol agents. The genetics of

the host should be exploited for supportiveness of biocontrol, and hospitality to biocontrol agents should be enhanced through directed breeding or genetic modification of the host plant. The third, and most challenging, area of research needed to explain the biological context for biocontrol is microbial community ecology. A better understanding of the microbial interactions that enhance or detract from biocontrol will determine the long-term success of biocontrol. In particular, attention needs to be paid to non-culturable members of the root associated and soil communities because these microorganisms may be numerically dominant and have not been studied. Molecular methods developed for the study of microorganisms in their environments are key tools for the study of the influences of the microbial community on biocontrol and also in the future there is need to understand the mechanism behind the water stress tolerance of plants grown in compost amended soil.

5.5 Conclusions

Successful biocontrol of plant disease requires an intricate array of interactions. Understanding these interactions at the molecular and ecological levels will make possible the rational development of biocontrol for agriculture. The high population density of fluorescent pseudomonads, actinomycetes and heterotrophic fungi in growing media amended with adequately mature composts has shown to be responsible for greater suppression of various pathogens. Application of genetic analysis to microorganisms involved in biocontrol has led to substantial progress in understanding the microbial metabolites and regulatory genes involved in biocontrol. Ecological analyses have begun to describe the responses of microbial communities to introduction of biocontrol agents. The integrated use of genetic, molecular, and ecological approaches will form the basis for significant future advances in biocontrol research and also an efficient use of limited water resources and better growth under limited water supply are desirable traits for crops in drought environments. Crop production and sustainable development are severely constrained by water limitations during the growing season. In author's recent study about the microbiologically improved compost on yield performance and water use efficiency have shown that proper compost treatment can increase crop yield by significantly soil water conditions. Such composts i.e. JNMsC and cow dung gave significant results and were known to be able to improve soil chemical, physical and biological properties, pot and field trials were conducted to determine whether the use of compost could improve soil properties and plant growth in drought stress conditions.

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Chapter 6
Microbial Chitinases for Chitin Waste Management

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Abstract Chitin is a major structural component of fungi and exoskeleton of insects, crustaceans and other arthropods. It is an insoluble, unbranched, linear chain of β-1, 4-linked N-acetyl D-glucosamine residues and is the second most abundant renewable carbohydrate polymer in nature after cellulose and first in marine environment. The annual production of chitin in aquatic biosphere is around 10^{11} ton. Chitinases produced by chitinolytic bacteria have the potential to convert this waste to pharmaceutically valuable end products such as N-acetyl glucosamine and chitooligosaccharides, and are viable alternatives of chemical processes currently used for the purpose. Chitinases from different bacteria, fungi, plants and animals are glycosyl hydrolases which degrade the insoluble chitin in to soluble chitooligosaccharides and glucosamine. Chitooligosaccharides possess antitumor, antifungal, antibacterial and immuno-enhancing effects. Antagonistic bacteria and chitinases have been exploited as potential biocontrol agents against fungal pathogens in plants. The growing number of application areas for chitin and chitin-derived products demand an equally diverse array of chitin-modifying enzymes for specific needs. The chapter will focus on the applications of chitinolytic enzymes from microbial sources and their possible applications, with special focus on conversion of large quantities of the chitinous substrates into useful biological products.

Keywords Chitinase • Chitin binding protein • Shrimp waste • Chitooligosaccharides • Plant disease control

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6.1 Introduction

Chitin is widely distributed in nature, particularly as structural polysaccharide in fungal cell walls, the exoskeleton of arthropods, outer shell of crustaceans, and nematodes. It is a high molecular weight insoluble homopolymer of β-l,4-linked N-acetylglucosamine (NAG), and one of the most abundant biopolymers found on earth, second only to cellulose. Chitin represents the potential source of renewable biomass and is structurally identical to cellulose except that the hydroxyl (−OH) group on C2 of glucose in cellulose is replaced by an acetamide group (NH.CO.CH3). In the aquatic biosphere alone, more than 10^{11} metric tons of chitin is estimated to be produced annually. A country like India, which has long coastal belt, accounts for 60,000–80,000 ton of chitinous waste annually (Suresh and Chandrasekaran 1998). This huge amount of chitin waste apparently poses environmental problems and could be converted to useful products having several applications. Important applications have been developed for the chitin/chitosan derivatives particularly the low molecular weight oligomers which are highly useful, at low concentration, in inducing immunity in higher organisms. Hence, the recycling of chitin waste has several important implications for the nations endowed with large coast line. Chitin recycling has, therefore, both economic and environmental significance. In this chapter, the importance of chitinolytic bacteria and chitinases obtained from these microorganisms in recycling the chitinous waste are discussed. The possibilities of generating high value bioactive molecules from chitinous wastes, exploiting biotechnological tools, have been highlighted. Considering the vast literature available on chitin-dependent processes, the environmental issues and chemical/enzymatic recycling of chitin, it would not be possible to cover all the information and therefore, large number of omissions in the literature is unavoidable.

6.2 Chitin, Chitosan and Their Physico-chemical Properties

Chitin is crystalline, intractable, highly hydrophobic, and insoluble in water and organic solvents. It is soluble in hexafluoroisopropanol, hexafluoroacetone, chloroalcohols in conjugation with aqueous solutions of mineral acids (Madhavan 1992) and dimethylacetamide containing 5% lithium chloride. Chitin is a non-toxic, non-allergenic, anti-microbial and biodegradable polymer. It has a strong positive charge which allows it to bind with negatively charged surfaces or materials including metals, skin and macromolecules such as proteins. Many current methods to modify chitin and transform it to useful carbohydrate products employ harsh chemical treatments that incur problems of undesirable by-products and limit further usage of the products. The limitation in using the chemical treatments has lead to the development of efficient bioconversion processes based on the exploitation of chitinases. Chitin in nature is available in two conformations; arrangement of

individual polymeric chains in antiparallel (α chitin) or parallel fashion (β chitin). Its degree of crystallinity or its context with other polysaccharides or proteins leads to the evolution of numerous hydrolytic enzymes and proteins that are needed to specifically recognize it. Chitin indeed is a highly versatile and promising biopolymer with numerous industrial, medical, agricultural and commercial uses.

Chitosan, a deacetylated derivative of chitin is soluble in dilute acids like acetic acid and formic acid. Chitosan has numerous biotechnologically interesting properties. In nature, chitosan is present in the cell walls of a limited group of fungi belonging to the order Mucorales, as well as some endophytic biotrophic plant pathogenic fungi (El Gueddari et al. 2002). Chitosan and its derivatives were employed to protect crop plants from phytopathogens, also used in tissue and paper coating due to their antimicrobial properties. Chitosan can bind to heavy metals and/or proteins in waste water treatment processes. Chitosan can be used to produce wound dressings to promote scar-free wound healing. Chitin and chitosans can also be partially or fully depolymerized to yield N-acetylglucosamine and glucosamine oligomers or monomers, respectively.

6.3 Soil Is a Rich Source of Chitin and Chitinolytic Diversity

The presence of chitin in soil is due to the decay of fungal mycelium or insect exoskeletons that inhabit soil. One gram of ploughed soil contains up to 10^6 organisms capable of decomposing chitin (Schlegel 2003). Among the chitinolytic bacteria, several *Actinobacteria* and *Streptomyces* species play active role in chitin decomposition (Kawase et al. 2004; Yu et al. 2008). Paul and Clark (2000) suggest that as much as 90% of soil actinomycetes are capable of breaking down chitin. Heterotrophic bacteria play an important role in breaking down chitin, but the role of fungi is even greater (Brzezinska et al. 2008).

The microorganisms readily utilize shrimp waste; shrimp heads the most useful and shells the least. Culturable chitinolytic bacterial diversity studies of chitin-rich soils collected from two chitin-producing industrial sites (Das et al. 2010) showed dominance of bacteria from the class Gammaproteobacteria and *Bacilli*. A large number of chitinolytic soil bacteria have been isolated from soil (Wang et al. 1997), shellfish waste (Wang and Hwang 2001), shrimp shell-enriched soil (Zhu et al. 2007). Phytospheres, such as rhizosphere and phylloplane, are also important habitats for chitinolytic bacteria (Gonzalez-Franco et al. 2003; Kishore et al. 2005a).

Use of culture independent methods led to identification of novel group of bacterial chitinases in alkaline soils (Tsujibo et al. 2003), sandy soils (Williamson et al. 2000), upland pastures (Metcalfe et al. 2002), intertidal hot springs (Hobel et al. 2005), maize rhizosphere soils (Ikeda et al. 2007), garden and park waste compost (Poulsen et al. 2008) and vermicompost (Yasir et al. 2009). Bacterial chitinases in arable soils are highly diverse and unique groups, based on the comparative analysis

of sequences available in public databases. Environmental parameters such as soil type and pH are responsible for shaping the composition of bacterial chitinases (Terahara et al. 2009).

6.4 Shrimp Waste Contributes to Environmental Pollution

Processing of large bulk of fish, shrimp and other aquatic organisms produces a corresponding large bulk of by-products and wastes. Much of these wastes are made into various value-added products, while considerable quantities are discharged as the processing effluents with large volume of waters used in processing. Fish and shrimp processing effluents are very high in biological oxygen demand, chemical oxygen demand, total suspended solids, fat-oil-grease, pathogenic and other microflora, organic matters and nutrients. Fish and shrimp processing effluents are, therefore, highly likely to produce adverse effects on the receiving coastal and marine environments. Although substantial reduction of the waste loads is possible through application of available simple techniques, due to lack of proper managerial and regulatory approaches this is not in practice in most part of the world. About 40% of the oyster shell waste is dumped in the coastal region causing serious environmental problems (Lee et al. 2005).

Chitinous waste produced commercially from sea food processing is a major environmental threat. Demineralisation and deproteination require large amounts of acid and soda and, thus, a lot of fresh water. The de-acetylation of chitin to generate chitosan requires even larger amounts of soda and, in addition, a lot of energy to heat the process. Alternative ways of chitin isolation and chitosan generation are required to extract these biopolymers without undue environmental stress. In the fishing industry, crab shells have always been treated as common waste, which, at best, was considered suitable for livestock feed or was used in agriculture as an inexpensive, natural nitrogen fertilizer. The effectiveness of crab shells as fertilizer has gained importance in present day agriculture due to the fact that the shells are broken down by enzymes, and the acetylglucosamine units of chitin hinder the development of fungi and nematodes in soil (Jaszkowski 2001). The possible utilization of chitin rich waste (Fig. 6.1) would fetch two major benefits in the countries where seafood is processed on an industrial scale. Apart from decreasing pollution, the chitooligosaccharides produced during the process contribute enormously to the field of biotechnology. However, the production of chitin and its hydrolyzed derivatives, such as acetylglucosamine and chitooligosaccharides from waste of the shellfish industry, has been limited due to the high cost of chitinase and expensive pretreatment processes of the shrimp and crab shell (Cosio et al. 1982). Therefore, the exploitation of chitinases produced by chitinolytic bacteria for efficient bioconversion of chitinous waste has gained tremendous importance. Soil bacteria are excellent sources of chitinases and could be used for catabolic conversion of chitinous waste into useful molecules for application in agriculture, biotechnology and medicine (Kishore et al. 2005b; Bhattacharya et al. 2007).

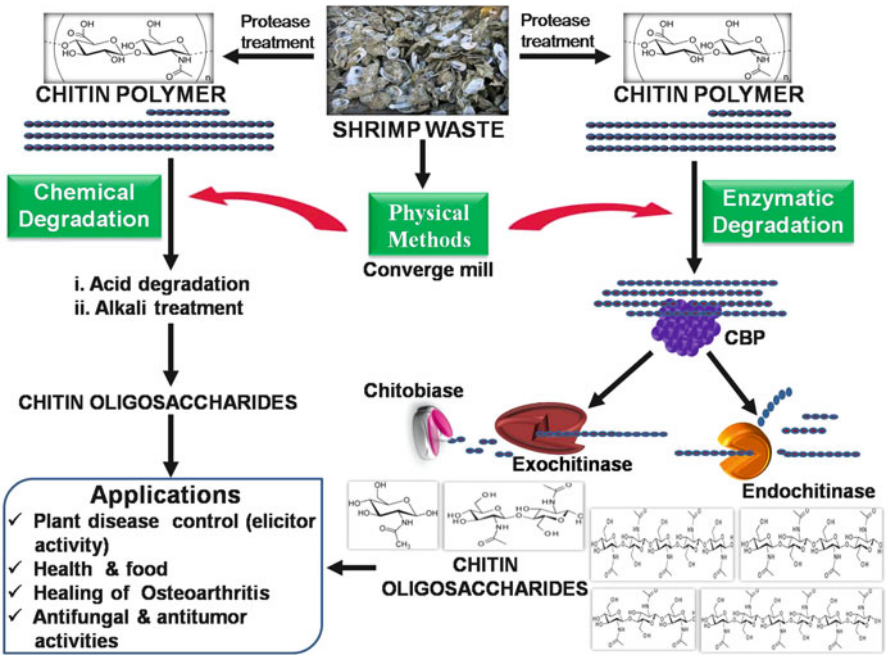

Fig. 6.1 Schematic diagram showing the possible utilization of chitin waste for production of N-acetylglucosamine and chitooligosaccharides. Chitin waste could be deproteinized by treating with proteolytic enzymes to obtain raw chitin. Physical methods of pretreatment of chitinous waste enhances further enzymatic or chemical degradation. Raw chitin or shrimp/crab shells can be subjected to physical force such as grinding and milling in a converge mill (Nakagawa et al. 2011). In the enzymatic degradation, chitin degrading enzymes viz. exo- or endo-chitinases along with auxiliary helper proteins efficiently convert chitin waste to useful oligomers and dimers. Random cleavage of polymeric chitin by endochitinases gives mostly chitooligomers. while the action of exochitinases gives rise to dimers that are further cleaved by chitobiase resulting in monomers. End products of endo and exo enzymes (oligomers and N-acetylglucosamine) have wider applications in various industries as detailed in the text

6.5 Chitinases and Chitin-Binding Proteins Cooperate in Degradation of Chitin

6.5.1 Microbial Chitinases Hydrolytically Cleave Chitin

The crystalline and inaccessible nature of chitin compelled the chitin degrading organisms to develop a tool box of enzymes and accessory proteins to ensure efficient degradation. The degradation typically occurs in two steps involving the initial cleavage of the chitin polymer by chitinases (endo and exo chitinases) into chitin oligosaccharides, and then further cleavage to N-acetylglucosamine monomers by chitobiases.

Numerous chitin hydrolyzing and modifying enzymes efficiently degrade the insoluble chitin polymer. Chitin degradation in a marine environment comprises of at least four major steps: sensing of chitin either by random collisions or by chemotaxis; attachment to the chitin to stay in close proximity of the nutrient; expression of a multitude of enzymes and other proteins required for catabolism of the polymer; and finally, uptake and catabolism of the hydrolysis products of the glycosidases (West and Colwell 1984). Based on the micro array expression profiling studies, the chitin catabolic operon (VC0611–VC0622 genes) in *Vibrio cholerae* was proposed to be regulated by chitoligosaccharides (Li and Roseman 2004; Meibom et al. 2004). In the chitin catabolic system, chitin sensor (ChiS, VC0622) has an important role in regulating the gene cluster. The gene cluster was also found to be available in the revealed genomic sequences of other *Vibrio* species (Ruby et al. 2005). Reports are available on the importance of chitin-and cellulose- binding domains of *Bacillus* on substrate bidning ability and conformational stability (Neeraja et al. 2010a, b).

Degradation of chitin in the environment occur through chitin-degrading enzymes and accessory proteins produced by chitinolytic microorganisms. Till date several chitinases have been cloned and characterized but the reported chitinolytic diversity constitutes a meagre percentage when compared to the enormous unexplored diversity that is hidden due to limitations of proper tools and processes of isolation. Chitinases produced by the Gram-negative soil bacterium *Serratia marcescens* have been studied extensively (Zakariassen et al. 2009). On the other hand, understanding of the chitinases from Gram-positive bacteria of the genus *Bacillus* is scarce although secretion of chitinases have been reported from several species such as *B. circulans* (Hashimoto et al. 2000), *B. subtilis* (Yang et al. 2009), *B. alvei*, *B. pumilus* (Ahmadian et al. 2007), *B. lentus*, *B. licheniformis* (Chuang et al. 2008), *B. cereus*, *B. amyloliquifaciens* (Wang et al. 2002) and *B. thuringiensis* (Barboza-Corona et al. 2008) and other *Bacillus* sp. (Lee et al. 2008).

6.5.2 Chitinases Are Grouped Under Different Families of Glycosyl Hydrolases (GH)

Based on amino acid sequence similarity in the catalytic domain, chitinases are grouped into families 18, 19, and 20 of GH (Henrissat and Bairoch 1993). Evolutionarily, family 18 is more diverse and consists of chitinases from bacteria, fungi, viruses, animals, and some plant chitinases. Family 19 chitinases have been found mostly in plants (classes I, II, and IV) and also in prokaryotes such as *Streptomyces* sp. and *Aeromonas* sp. (Hart et al. 1995; Ueda et al. 2003). Family 20 includes the β-N-acetylhexosaminidases from bacteria, *Streptomycetes*, and humans. Bacterial chitinases are further grouped into three major subfamilies, A, B, and C, based on the amino acid sequence of individual catalytic domains (Watanabe et al. 1993). Apart from these three different GH families, very recently Vaaje-Kolstad et al. (2010) discovered the enzymatic activity of CBP21 (chitin binding protein21), which catalyzes cleavage of glycosidic bonds in crystalline chitin with a mechanism different from the

fundamental mode of action of known GH. According to Carbohydrate Active Enzymes (CAZy) database, CBP21 belongs to family CBM33 (carbohydrate-binding modules) (Boraston et al. 2004).

Based on their mode of action, chitinases were broadly classified into two major categories; endochitinases and exochitinases. Endochitinases (EC3.2.1.14) cleave chitin randomly at internal sites, generating soluble, low molecular mass multimers of GlcNAc, such as chitotetraose, chitotriose, and diacetylchitobiose. Exochitinase was further divided into two subcategories: chitobiosidases and β-(1,4)-N-acetyl-glucosaminidases. Chitobiosidases (EC 3.2.1.29) or chitin-1,4-β-chitobiosidases catalyze the progressive release of diacetylchitobiose starting at the nonreducing end of chitin chains. Its sole products are diacetylchitobioses which are further split into monomers of N-acetyl glucosamine by β-(1,4)-N-acetyl-glucosaminidases (GlcNAcase, EC 3.2.1.30) or chitobiases.

6.5.3 Chitin Binding Domain (ChBD) Facilitates Chitin Hydrolysis

Chitinases and cellulases have one or more carbohydrate binding modules (CBMs) that are beneficial to the enzyme to adhere to and sometimes disrupt the substrate. Chitin binding domains (ChBDs) are found mainly at the N-termini of plant chitinases, but in bacterial or fungal chitinases they are located either at C-terminal or N-terminal end. The three aromatic amino acids tryptophan, phenylalanine and/or tyrosine are found among the residues conserved in the bacterial and plant ChBDs, and are also present in cellulose binding domains (CBDs). These aromatic amino acids have been implicated in the binding of chitinases to chitin (Zeltine and Schrempf 1997) and cellulases to cellulose (Linder et al. 1995). Another common feature of ChBDs and CBDs is that they are connected to the catalytic domains of the enzyme by a linker peptide that is Gly/Pro-rich in plant chitinases (Raikhel and Lee 1993) and Thr/Ser/Pro rich in fungal cellulases (Abuja et al. 1988). Family 19 chitinase from *Aeromonas* sp. No.10S-24 (72.6 kDa) is composed of two ChBDs, two proline- and threonine-rich (PT-rich) linkers, and a catalytic domain. The hydrolytic activities toward insoluble and soluble substrates were significantly reduced by the truncation of two ChBDs besides, the antifungal activity determined from the digestion rate of haustoria of powdery mildew was also reduced (Kojima et al. 2005). The differences in efficiency of chitinases towards various forms of chitin substrates are due to different types of auxiliary CBM within these chitinase molecules. Analogous to cellulases, removal of the ChBDs of the plant and bacterial chitinases decrease their activities on insoluble but not on soluble substrates (Blaak and Schrempf 1995).

Like other enzymes involved in the degradation of polysaccharides, most chitinases have CBD, which assist in specific association to insoluble substrates. Some CBDs have affinity towards chitin (Goldstein et al. 1993) while others have affinity for cellulosic materials (Morimoto et al. 1997). This is explained by the structural

similarities of chitin and cellulose. The binding efficiency of the cellulases is much enhanced by the presence of CBD and the enhanced binding correlates with the better activity towards insoluble cellulose (Kruus et al. 1995). It was demonstrated that the fusion of CBD of endoglucanase improved the activity and conformational stability of *Bacillus licheniformis* DSM 13 chitinase (Neeraja et al. 2010b).

The carbohydrate binding and catalytic modules of both chitinases and cellulases seem to generate a continuous substrate-binding site formed by a surface of aromatic residues, which guide the substrate into the enzyme's active site (van Aalten et al. 2001)

6.5.4 Chitin Binding Proteins (CBPs) Catalyze Oxidation-Mediated Hydrolysis of Crystalline Chitin

Enzyme systems capable of degrading highly crystalline polysaccharides like chitin consist of endo-acting enzymes that cut randomly in the polysaccharide chain and processive exo-acting chito-biohydrolases, which degrade the polymers from chain ends. It was difficult to understand how the GH could act on a polysaccharide chain in its crystalline environment. Therefore, it was speculated that a substrate-disrupting factor could exist which makes crystalline substrate more accessible to hydrolytic enzymes. Microorganisms, which break down chitin, produce a protein that increases substrate accessibility and potentiates hydrolytic enzymes. These proteins are classified as carbohydrate-binding modules (CBMs) and belong to family CBM33. CBP21 produced by the chitinolytic bacterium *S. marcescens* potentiates chitin hydrolysis by chitinases (Vaaje-Kolstad et al. 2005). CBP21 was shown as an enzyme that catalyzes cleavage of glycosidic bonds in crystalline chitin, thus opening up the inaccessible polysaccharide material for hydrolysis by normal glycoside hydrolases (Vaaje-Kolstad et al. 2010). This enzyme acts on the surface of crystalline chitin introducing chain breaks and thereby generating oxidized chain ends which promotes further degradation by chitinases. Swapping of chitin-binding domain in *Bacillus* chimeric chitinases resulted in improved substrate binding affinity and conformational stability (Neeraja et al. 2010a).

6.6 Chromatographic Techniques Facilitate Analysis of Chitooligosaccharides

Thin layer chromatography analysis for qualitative assessment of product formation is relatively insensitive and not quantitative. Analyses of degradation products of *Thermococcus kodakaraensis* chiA from *N*-acetyl-chitooligosaccharides and their chromogenic derivatives with thin layer chromatography indicated that the N-terminal catalytic domain mainly hydrolyzed the second glycosidic bond from the non-reducing end of the oligomers, whereas the C-terminal domain randomly

hydrolyzed glycosidic bonds other than the first bond from the non-reducing end (Tanaka et al. 2001). Chitinases from *S. marcescens* have a characteristic catalytic mechanism which depends on participation of the N-acetyl group of the sugar unit bound to the −1 subsite (Synstad et al. 2004). This substrate-assisted catalytic mechanism implies that family 18 chitinases are expected to have an absolute preference for A-units in the −1 subsite and that the presentation of a deacetylated sugar (D-unit) to the −1 subsite would represent nonproductive binding. Crude chitinase from *Burkholderia cepacia* TU09 was used to digest α and β chitin powder producing GlcNAc in greater than 85% yield from β and α chitin within 1 and 7 days, respectively whereas *B. licheniformis* SK-1 chitinase completely hydrolyzed β chitin within 6 days, giving a final GlcNAc yield of 75%, along with 20% of chitobiose. However, only a 41% yield of GlcNAc was achieved from digesting α chitin with *B. licheniformis* SK-1 chitinase (Pichiyangkura et al. 2002).

Highly sensitive analytical tools are available to characterize the products released by chitin–degrading enzymes. Enzymatic degradation of chitin or chitosan, in most of the cases, gives a mixture of chitooligosaccharides having different degree of polymerization (DP) and degree of acetylation (DA). Different DA and DP chitooligosaccharides contribute to the diverse physicochemical and biological properties. Therefore, production and purification of well-defined chitooligosaccharides remained a daunting task for researchers all over the world, despite little success. Techniques including gel filtration, ultrafiltration, and ion exchange chromatography were employed individually or in combination for separation and purification of chitooligosaccharides. Size exclusion chromatography (SEC) was frequently used for preparative separation of chitooligosaccharides. Sørbotten et al. (2005) successfully separated oligomers up to DP 20 by SEC. Methods that employ HPLC have also been described for the separation and quantification of chito-oligosaccharides (Krokeide et al. 2007). Combination of HPLC and electrospray mass spectroscopy allows the simultaneous separation of α and β anomers, and monitoring of chitooligosaccharide products down to picomoles (Suginta et al. 2005). Mass spectroscopy and nuclear magnetic resonance (NMR) were utilized to determine the size of chito-oligosaccharides produced by the activity of chitinases or chemical depolymerisation of chitin (Howard et al. 2003). Using NMR, it is also possible to determine the pattern of acetylation (Sørbotten et al. 2005) in addition to the DA in a chitooligomer, which is crucial for bioactivities attributed to chitooligosaccharides.

6.7 Chitinases and Chitooligosaccharides Are Useful in Plant Disease Control

Chitin serves as a fibrous strengthening element through increased hydrogen bonding and strength between adjacent polymers and contributes to cell wall rigidity in fungi. The glycosidic bond is the key linkage responsible for the cell wall integrity and any disturbance caused in it would destroy the morphology and

lead to the leakage of the cell contents. Safety and environmental concerns led to the search for an alternative biocontrol agent that can replace synthetic fungicides for control of post harvest diseases (Wilson et al. 1991). Bacterial enzymes like glucanases, chitinases and chitosanases target and degrade the cell walls of phytopathogenic fungi. A simple and rapid application of the hydrolytic enzyme preparations can serve the purpose of plant disease control in a more effective manner (Neeraja et al. 2010c).

Antagonism of fungal plant pathogens by microorganisms specifically by the production of chitinases plays a major role in biological disease control (Chernin et al. 1995). *Puccinia arachidis* produces an endochitinase that inhibits germination of uredospores of the rust fungus *Fusarium chlamydosporum* (Mathivanan et al. 1998). Chitinolytic enzymes from the most studied mycoparasitic fungus, *Trichoderma harzianum*, were inhibitory to a wider range of deleterious fungi (Lorito et al. 1993). The role of extracellular chitinase in the biocontrol activity of *Trichoderma virens* was examined using genetically modified strains (Baek et al. 1999). Sampson and Gooday (1998) reported the chitinolytic activity of two strains of *Bacillus thuringiensi*s on insects and also showed the inhibitory activity of allosamidin (chitinase inhibitor) and restored effect on addition of exogenous chitinase or allosamidin. When *Chi*A was combined with *B. thuringiensis* δ–endotoxin, a synergistic toxic effect was seen on insect larvae (Ragev et al. 1996; Sampson and Gooday 1998). Tanaka and Watanabe (1995) reported the individual and combined actions of glucanases and chitinases produced by *Bacillus circulans* on selective enzymolysis of cell walls of *Pyricularia oryzae*. Wang et al. (2002) showed the inhibitory activities of *Bacillus amyloliquefaciens* V656 on fungal growth. *S. marcescens* is reported to control *Botrytis cinerea, Rhizoctonia solani, Sclerotinia minor and Fusarium oxysporum* (Someya et al. 2000; El-Tarabily et al. 2000). *Aeromonas caviae*, a chitinolytic soil isolate showed to control *Rhizoctonia solani, Sclerotium rolfsii* and *Fusarium oxysporum* f.sp. *vasinfectum* in cotton (Inbar and Chet 1991). Zhang and Yeun (2000) showed the role of chitinase production by *Stenotrophomonas maltophilia* strain C3 in biological control of leaf spot on tall fescue (*Festuca arundinacea*) caused by *Bipolaris sorokiniana*. Field-testing of Bacticide, Phytoverm and chitinase proved to control raspberry midge blight in Siberia (Shternshis et al. 2002).

Biotechnological applications of chitin and chitinolytic enzymes were extensively reviewed by Patil et al. (2000). Partially purified chiA (cloned and expressed in *E. coli*, was found to reduce disease caused by *Sclerotium rolfsii* in beans and *R. solani* in cotton (Shapira et al. 1989). The *Chi* A and *Chi* B genes from *S. marcescens* have been transformed into other bacterial species like *P. fluorescens* and *E. coli* in an attempt to improve their ability to control fungal plant pathogens (Koby et al. 1994).

The use of bacterial biocontrol agents for the control of post harvest diseases of fruits has gained considerable attention and has moved from the laboratory to commercial application (Wilson and Wisniewski 1994). Chitin-based formulations have also been applied for foliar disease control (Kishore et al. 2005a). Manjula et al. (2004) showed that the whole cells of *Bacillus subtilis* AF 1 proved more effective

than the cell free chitinase-based formulations in biological control of citrus fruit rot and groundnut rust. Chitinolytic *S. marcescens* GPS5 reduced the severity of late leaf spot disease of ground nut and on supplementation with 1% colloidal chitin, disease control efficiency of the formulation increased (Kishore et al. 2005b). Increase in seedling emergence and dry weight of pigeonpea in the field with chitin-supplemented formulations of *Bacillus subtilis* AF 1 was demonstrated by Manjula and Podile (2005).

6.8 Chitin, Chitosan and Chitooligosaccharides Have Medical and Pharmaceutical Applications

Chitin and chitosan oligosaccharides have attracted much attention for their various physiological activities, including antifungal activity, antitumor activity, and immuno-enhancing effect (Prashanth and Tharanathan 2005; Nam et al. 2001). Various applications of chitin and chitosan were reviewed by Ravi Kumar (2000). Mouse and human fibroblast cell cultures exposed to chitin gel matrixes of pore size 100–500 mm were found to proliferate indicating the feasibility of using these porous chitin matrixes for cell transplantation to regenerate tissues (Chow et al. 2001). Lee (2009) reviewed the immunologic effects of chitin *in vivo* and *in vitro* and discussed new aspects of chitin regulation of innate and adaptive immune responses. Exogenous chitin activates macrophages and other innate immune cells, and also modulates adaptive type 2 allergic inflammations. These studies further demonstrate that chitin stimulates macrophages by interacting with different cell surface receptors such as macrophage mannose receptor, toll-like receptor 2 (TLR-2), C-type lectin receptor Dectin-1, and leukotriene B4 receptor (BLT1).

Chitosan, deacetylated form of chitin, and their derivatives have various medical and pharmaceutical applications (Akiyama et al. 1995; Denarie et al. 1996; Vander et al. 1998). The transfection efficiency of chitosan as a gene delivery vehicle has been studied by Sato et al. (2001). Wang et al. (2001) demonstrated the direct use of P-chitosan in combination with monocalcium phosphate and calcium oxide to produce a fast setting mixture with improved mechanical properties suitable as a phosphate cement to fill bone cavities. On extending the study to a rabbit model, no adverse effects were attributed to the P-chitosan–calcium phosphate cement, with histological evidence suggesting biocompatibility, bio-absorbability and osteoinductivity. *N*-Acetyl-D-glucosamine (GlcNAc) and D-glucosamine (GlcN) have been promoted as a treatment or as nutriceutical agents for patients with osteoarthritis and inflammatory bowel disease (Talent and Gracy 1996; Salvatore et al. 2000). In contrast to GlcN hydrochloride or sulfate, both of which have a bitter taste, GlcNAc has a sweet taste that facilitates its use in daily consumption. Coming to cosmetic applications, chitosan is the only natural cationic gum that becomes viscous on being neutralized with acid. These materials are used in creams, lotions and permanent waving lotions and several derivatives have also been reported as nail lacquers (Mark et al. 1985).

6.9 Eco-friendly Way of Clearing Sea Shell Wastes

The economic importance of chitinases can be assessed by the fact that chitinases can utilize chitinous wastes in the environment, and in some cases these chitin wastes can be used for cost-effective production of microbial chitinases (Gohel et al. 2006). The production of inexpensive chitinolytic enzymes is an element in the utilization of shellfish processing wastes (Chang et al. 2003). Rattanakit et al. (2002) developed a chitinase formulation by using shrimp shellfish waste as a substrate for solid state cultivation of *Aspergillus* sp. SI-13. Microbial reclamation of shellfish wastes for the production of chitinases has been reported where they prepared substrate by boiling and crushing of shellfish waste (Wang et al. 2001). Labrie et al. (2001) demonstrated the effect of chitin waste-based composts on oomycete plant pathogens. Rojas-Avelizapa et al. (1999) selected and characterized *Bacillus thuringiensis* strains which grow solely in a proteo-chitinous substrate (milled shrimp waste). Sabry (1992) tested 40 strains of bacteria for their potentiality to degrade chitin and utilize shrimp-shell waste to produce chitinases. The most active organisms were reported from *Alcaligenes* and *Bacillus* sps. The production of chitinolytic enzyme by *Pseudomonas aeruginosa* K-187, using shrimp and crab shell powder as the carbon source has also been reported (Wang et al. 1997). Due to the rich content of protein along with chitin in sea shells, disposal of which causes serious environmntal issue, Waldeck and his co-workers (2006) characterized new chitinase-deficient *Bacillus licheniformis* strains able to efficiently deproteinate shrimp shell waste, eventually resulting in chitin of superior quality.

Shrimp waste is the most inexpensive substrate for enriching the chitin/chitosan modifying enzymes. *S. marcescens* QMB1466 is a chitinase overproducing mutant that shows considerable chitinolytic activity towards crystalline chitin substrates such as those found in crustacean shell. This strain has been used in the development of a bioconversion process of shellfish chitin to single-cell protein (Cosio et al. 1982). Green et al. (2005) employed this strain for the degradation of chitin substrates (prawn shell of *Nephrops* sp) into commercially valuable products, with particular interest in the suitability of bioprocessed chitin as an inducer of chitinolytic activity in a conceptualized, integrated biotechnological process. *Bacillus subtilis* NPU 001, a strain isolated from soil samples, excreted a chitinase when cultured in a medium containing 2% (w/v) shrimp and crab shell powder as the major carbon source (Chang et al. 2010).

6.10 Damage to Environment by Shrimp Waste Could Be Reduced by Chitinases and Chitinolytic Microbes

The non-toxic nature and absorbent properties of chitin make it environmentally safe for bioremediation and an excellent pharmaceutical tool for drug discovery and delivery. In addition, chitin is abundant and inexpensive, making it attractive material

for industrial processes and large-scale applications. Considering the success of chitinases/chitinolytic organisms as biocontrol agents in the present scenario enables us to extend the efficacy of these enzymes in bioconversion of environmental waste. Extensive reports on bacteria that are able to degrade shrimp waste are periodically identified and improved using recombinant DNA technology. The sea shell waste, which poses environmental problems, could also be subjected to enzymatic processes to generate commercially useful products. Exploiting the vast chitinolytic diversity would lead to the identification of novel chitinases, which would efficiently degrade the large biomass rich in chitin. Industries are searching for economically feasible chitinases that can make useful material out of the second largest-renewable carbohydrate polymer.

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Chapter 7
Diversity of Microbial Carbonic Anhydrases, Their Physiological Role and Applications

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Abstract Carbonic anhydrase (CA) is a zinc metalloenzyme catalyzing the reversible hydration of CO_2 to bicarbonate, a reaction that supports various biochemical and physiological functions. Although ubiquitous in highly evolved organisms from the eukarya domain, the enzyme has received scant attention in prokaryotes from the bacteria and archaea domains. Unraveling of microbial genome sequences suggest that CA is widespread in metabolically and phylogenetically diverse prokaryotes. Evidence for the presence of carbonic anhydrase was obtained for freshwater, marine, mesophilic, thermophilic, aerobic, anaerobic, pathogenic, symbiotic, acetogenic, autotrophic, heterotrophic and photosynthetic species. In prokaryotes, carbonic anhydrases are involved in diverse biochemical and physiological processes, including photosynthesis, respiration, CO_2 and ion transport, and CO_2/bicarbonate balance required for biosynthetic reactions. Besides the biochemical and physiological importance of CA in CO_2 metabolism, this enzyme has found a new dimension in the field of biomimetic CO_2 sequestration. Anthropogenic CO_2 emission has led to adverse impact on climate and has been implicated in global warming. In the global effort to combat the predicted disaster, several CO_2 capture and storage technologies (CCS) are being considered. A novel biomimetic approach for CO_2 scrubbing using CA provides a viable means to accelerate CO_2-hydration reaction and has been found to be feasible for fixing large quantities of CO_2 into calcium carbonate in presence of suitable cations at moderate pH values *in vitro*. Thus, biomimetic $CaCO_3$ mineralization for carbon

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capture and storage offers potential as a stable CO_2 capture technology. Cost-efficient production of the enzyme by bacterial overexpression and production of value-added CO_2 by-product are critical for development of economically feasible CA based CO_2 capture processes.

Keywords Diversity • Microbial carbonic anhydrase • Physiological roles • Classes of carbonic anhydrase • Biomimetic CO_2-sequestration

7.1 Introduction

The planet earth came into being about 4.5 billion years ago with a primeval atmosphere that was hostile to life. Carbon dioxide (CO_2) was the dominant active gas in the earth's early atmosphere which comprised of 80% water vapor, 10% carbon dioxide, 5–7% hydrogen sulfide, and small amounts of nitrogen, carbon monoxide, hydrogen, methane and inert gases. There was absence of free oxygen (O_2) in the ancient environment. The atmosphere was anaerobic until the advent of water splitting, oxygen evolving photosynthesis (Kasting 1993). This was an important turning point in Earth's history because the CO_2 in the atmosphere was being converted to oxygen (O_2). Plants, algae and cyanobacteria are able to fix CO_2 during photosynthesis. Photosynthesis produced oxygen and reduced the amount of carbon dioxide in the atmosphere as carbon became locked up in fossil fuels and in carbonate sedimentary rocks. It is believed that the amount of oxygen present in the atmosphere increased slowly over the years and so did the number and variety of life forms on earth. Organisms used oxygen for a process called respiration to derive energy by breaking down of sugars. The ability to use O_2 allowed the diversity of life to expand vastly. In all domains of life, from microorganisms to mammals, carbon dioxide (CO_2) is the end product of respiration. Today, CO_2 makes up only about 0.036% of the atmospheric gases.

Carbon dioxide (CO_2) and its hydration product bicarbonate (HCO_3^-) are substrates and products of various metabolic reactions in the cells of different living forms. The interconversion of CO_2 and HCO_3^- is spontaneously balanced to maintain the equilibrium between $CO_2 \leftrightarrow HCO_3^-$ (dissolved inorganic carbon; DIC), carbonic acid and carbonate of which HCO_3^- is physiologically most important as it is a substrate for several carboxylating enzymes which catalyze the committed steps of different biosynthetic pathways such as biosynthesis of fatty acids, amino acids and nucleotides (Mitsuhashi et al. 2004). HCO_3^- is negatively charged and highly soluble in aqueous solution but poorly soluble in lipids, while CO_2 is highly soluble in both aqueous solution and lipids.

Therefore, although CO_2 is membrane permeable and can freely diffuse in and out of the cell, HCO_3^- must be transported across the cell membrane. Conversion of HCO_3^- to CO_2 may facilitate its transport into the cell while conversion of CO_2 to HCO_3^- may be important for trapping CO_2 inside the cell to provide substrate for different carboxylating enzymes (Smith and Ferry 2000). Although this uncatalyzed

hydration-dehydration of CO_2-HCO_3^- proceeds at significant rates, the interconversion is slow at physiological pH and thus requires enzymatic catalysis which in biological systems is accelerated by carbonic anhydrase (CA).

The enzymatic interconversion of CO_2 and HCO_3^- not only allows the cell to concentrate CO_2 to the levels required for cellular enzymes but also helps the cell to maintain the proper intracellular levels of CO_2/HCO_3^- to carry out cellular processes. The ubiquitous enzyme, carbonic anhydrase catalyses the reversible hydration of CO_2 to support various physiological functions involving DIC. Carbonic anhydrases are widely distributed in organisms belonging to all branches of evolutionary tree, from eukaryotes such as vertebrates, invertebrates, and plants to prokaryotes such as archaea and bacteria. CAs are known to participate in diverse biochemical and physiological processes, including photosynthesis, respiration, CO_2 and ion transport, and pH regulation (Smith and Ferry 2000).

7.2 Carbonic Anhydrase

Carbonic anhydrase (EC 4.2.1.1), also known as carbonate dehydratase and carbonate hydro-lyase, is a Zn^{2+}- metalloenzyme that catalyzes the interconversion of carbon dioxide and bicarbonate (7.1). Since this simple conversion of a membrane-permeable gaseous substrate into a membrane-impermeable ionic product is vital to many fundamental biological functions, such enzymes are thus physiologically important and widely distributed in nature.

$$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+ \tag{7.1}$$

CA is ubiquitously found in all animals and photosynthesizing organisms examined for its presence, as well as in Bacterial and Archeal domains (Smith and Ferry 2000). This enzyme is found in many different tissues and is involved in a number of different physiological processes, including bone formation, calcification, ion transport, acid-base balance and CO_2 transport (Gilmour and Perry 2009).

CA was first discovered in human erythrocytes (Meldrum and Roughton 1933), followed by the identification of several isozymes in virtually all mammalian tissues and cell types, where they function in various physiological processes such as respiration and transport of CO_2/bicarbonate between metabolizing tissues and lungs, pH regulation, ion transport, calcification, tumorigenicity and biosynthetic functions (such as gluconeogenesis, lipogenesis and ureagenesis) (Supuran 2008). Carbonic anhydrases are abundant in plants and unicellular green algae, where they are essential for photosynthetic CO_2 fixation (Badger and Price 1992). Carbonic anhydrases are also present in physiologically and phylogenetically diverse prokaryotes indicating a far greater role for this enzyme in nature than previously recognized. Investigation of function of prokaryotic CAs during the last few years implicated a variety of physiological roles in addition to transport of CO_2 or HCO_3^-, maintaining internal pH and CO_2/HCO_3^- balance required for biosynthetic reactions (Zimmerman and Ferry 2008).

The kinetics of CA involves a two step process, the first step involves the attack of Zinc bound hydroxide ion on CO_2 (Eq. 7.1a).

$$Zn^{2+}OH + CO_2 \leftrightarrow Zn^{2+} + HCO_3 \tag{7.1a}$$

$$Zn^{2+} + H_2O \leftrightarrow H^+ + Zn^{2+} - OH \tag{7.1b}$$

The second step involves regeneration of the active site by ionization of the zinc bound water molecule and removal of the proton from the active site [Eq. 7.1b].In the second step, the zinc ion act as Lewis acid to lower the pKa of the water molecule from 14.0 to 7.0, most CA have K_{cat} values greater than 10^4 s^{-1} which requires an intermediate PSR (Eq. 7.1c) to transfer the proton from the metal bound water molecule to the external buffer "B" (Eq. 7.1d).

$$PSR - Zn^{2+} - H_2O \leftrightarrow Zn^{2+} - OH^- + PSR - H^+ \tag{7.1c}$$

$$PSR - H^+ + B \leftrightarrow PSR + B - H^+ \tag{7.1d}$$

Proton transport from the active site is the rate limiting step for enzyme with $K_{cat} > 10^4$ s^{-1}. K_{cat} reflects the rate of proton transport, whereas the catalytic efficiency (K_{cat}/K_m) is more reflective of the hydration step and is insensitive to the rate of proton transfer (Tripp et al. 2001).

7.2.1 Distinct Classes of Carbonic Anhydrases

A remarkable feature of carbonic anhydrases is the existence of five distinct gene families designated as α, β, γ, δ, and ζ that have no significant sequence homology among them indicative of independent evolution (Hewett-Emmett and Tashian 1996; Tripp et al. 2001). Also, the crystal structures for three of its classes (α, β and γ) reveal completely different tertiary and quaternary structures (Fig. 7.1), but the active site shows essential features of remarkable similarity. All these classes share the common property of binding a zinc ion at their active sites, which is important for the catalytic activity. Thus, the carbonic anhydrase classes constitute a spectacular case of convergent evolution, in which the same catalytic activity has evolved via several independent, evolutionarily very ancient ways (Smith and Ferry 2000).

All the animal CAs belong exclusively to α-class. In humans, atleast 16 different tissue- and organ-specific α-CA isoforms or CA-related proteins (CARPs) have been described (Supuran 2008). In addition to animals, α-CAs have been identified in plants, algae, fungi and some bacteria (Bahn and Mühlschlegel 2006; Moroney et al. 2001; Tripp et al. 2001). However, no α-CA has so far been described in archaea. The β-class CAs were first discovered in plants and subsequently found to be present in algae, fungi, archaea and bacteria. The γ-class CAs are predominantly distributed in the bacteria and archaea domains (Smith and Ferry 1999). In eukaryotes, however, they are found only in photosynthetic

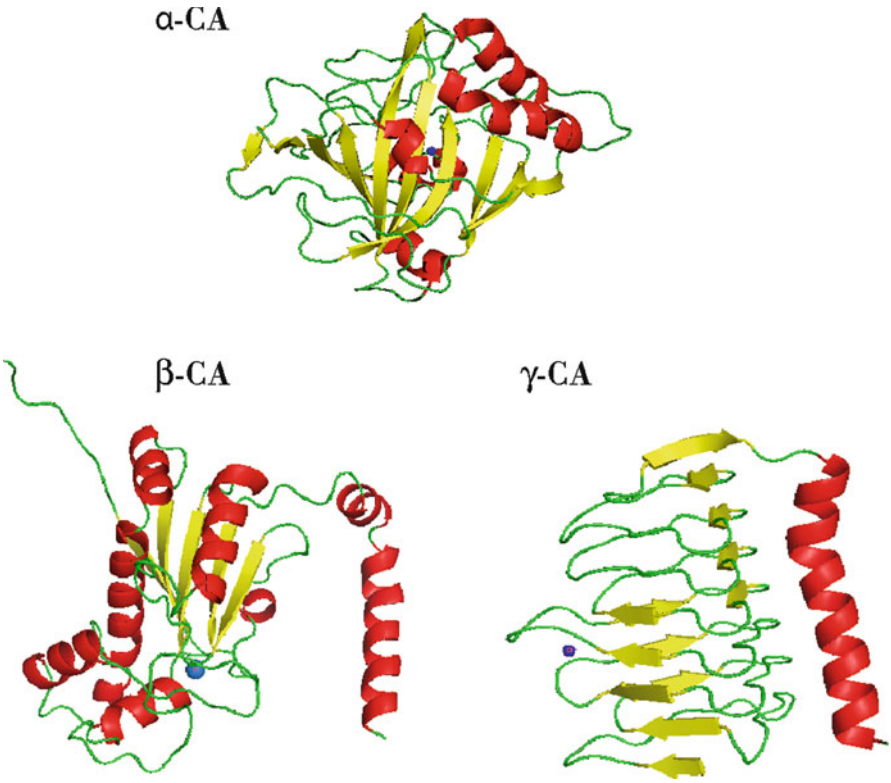

Fig. 7.1 Diverse structures of α-, β- and γ- carbonic anhydrases

organisms. The recently discovered dimeric δ- & ζ-CAs are so far known to be present in a few marine diatoms (Table 7.1).

The widespread occurrence of CAs in various organisms all over the phylogenetic tree clearly indicates that these ancient enzymes are involved in critical biological processes. But why did the different classes of CA have evolved and what specific advantages one class may provide over the other is not yet known. Next unresolved question is the occurrence of more than one type of CA in a single organism. Several organisms contain putative CA encoding genes from more than one class and some even contain genes from all the three known classes. For example, analysis of the *Arabidopsis* genome database revealed at least six α-, five β- and three γ-CA encoding genes (Moroney et al. 2001). Although this multiplicity indicates towards the diverse role of CAs, specific function of each of these isoforms remains to be investigated.

Analysis of the completed genome sequences of several bacteria such as *Borrelia burgdorferi*, *Chlamydia trachomatis*, *Mycoplasma pneumoniae*, *Mycoplasma genitalium*, *Thermatoga maritima*, and *Treponema pallidum* and archaea such as *Archaeoglobus fulgidus* indicated absence of open reading frames

Table 7.1 A summary of different classes of carbonic anhydrases

Class	Distribution	Inhibition	Subunit composition	Zinc coordination
α	Mammals, plants, algae, protozoa and bacteria	Highly susceptible to sulfonamides	Monomeric	Three histidine[b]
β	Plants, algae, fungi, bacteria and archaea histidine	Less susceptible to sulfonamides	Dimeric to octameric	Two cysteine and one histidines
γ	Plants[a], algae[a], bacteria and archaea	Highly susceptible	Trimeric to sulfonamides	Three histidines[c]
δ	Marine diatoms	Not known	Dimeric	Three histidines
ζ	Marine diatoms	Not known	Dimeric	Not known

[a]Putative genes exist in EST database

[b]Histidine residues are contributed by the same subunit

[c]Histidine residues are contributed by two different subunits

showing homology to any of the CA classes (Smith and Ferry 2000). So, the next unresolved question is whether there are still some classes of CA that remains to be discovered. Whether these organisms possess a different undiscovered class of carbonic anhydrase or perhaps they do not require this enzymatic activity remains to be answered.

7.2.2 Structure of Carbonic Anhydrases

The alpha (α) class CA has been reported to have evolved around 200–300 million years ago (Hewett-Emmett and Tashian 1996). Its occurrence has been reported in humans, animals, higher plants, green algae, eubacteria, and viruses (Smith et al. 1999). The enzyme consists of monomeric and trimeric polypeptide units (Tiwari et al. 2005) having ten stranded, antiparallel β sheet (Strop et al. 2001). The mechanism of catalysis of α-CA in several isozymes of the α-class has been widely studied (Tu et al. 1998). His-64 accepts a proton from active-site water molecules which intervenes between the zinc-bound water molecule and His-64. The His-64 PSR can be replaced with other residues that function as PSRs, a result consistent with the proposal that proton transfer occurs through different structures of intervening water chains. Crystal structures of human CA II have shown His-64 in either an "in" (toward zinc) or "out" position. This fluctuation of His-64 facilitates proton transfer between active site waters and solvent water at the mouth of the active site cavity. The aqueous phase molecular dynamics of the wild-type enzyme in three protonation states indicate that His-64 primarily assumes the "in" orientations and it was concluded that fluctuations between the two orientations of this residue may have limited influence on proton transfer (Tripp et al. 2001). According to Fitting Marcus theory, intramolecular proton transfer involves a reorganization of the active site cavity. Lu and Voth in 1998 reported that the reorganization includes water molecule that is not directly involved along the pathway i.e. movement of His-64 from the "out" to the "in" orientation involves breaking H-bonds between the side chain and water. Recent studies have demonstrated that esterase activity is due to the zinc-hydroxide functionality of the enzyme and it is inhibited by sulphonamide inhibitor acetazolamide, with IC_{50} values in the range of 28–1,210 nM (Innocenti et al. 2008).

The first crystal structure of β-class carbonic anhydrase illustrated the polypeptide structure of protein as dimer, tetramer, hexamer, and octamer having four stranded β sheet core with α helices (Mitsuhashi et al. 2000). In β class CA, Zn ligates with two conserved cysteine and one conserved histidine (Kimber and Pai 2000). The β class CA has been reported in angiosperms and green algae (Eriksson et al. 1996), archaebacteria and eubacteria (Hewett-Emmett and Tashian 1996). Kimber and Pai in 2000 reported that the β class is composed of two subclasses. The "plant type" has been represented by the *P. sativum* CA enzyme whereas the "Cab type" was represented by the *M. thermoautotrophicum* CA enzyme. Structural dissimilarities between the two subclasses and their varied responses to inhibitors

suggested a difference in their kinetic mechanism. Kinetic analyses indicated a zinc hydroxide mechanism for the β class CA (Smith and Ferry 2000). In the crystal structure of *P. sativum* zinc bound acetate shows the binding of bicarbonate in the active site (Kimber and Pai 2000). The zinc bound oxygen of acetate hydrogen bonds with Asp162 $O_{\delta 1}$, and the second oxygen hydrogen bonds to Gln-151 indicating that these residues play an important role in catalysis. The bond between acetate and Asp162 $O_{\delta 1}$ is identical to the hydrogen bond between the zinc bound oxygen of acetate and Thr199 $O_{\gamma 1}$ of the α class CA II isozyme. Thr199 $O_{\gamma 1}$ functions to orient the zinc bound hydroxide for nucleophillic attack on CO_2 (Lindskog 1997). It was suggested that Asp 34 in the active site of Cab could function similarly to Asp 162 of the plant type subclass (Strop et al. 2001).

It is suggested that origin of γ class CA dates back to 3.0–4.5 billion years (Smith and Ferry 2000). The occurrence of γ class CA in *Methanosarcina thermophila* an acetate utilizing methanogenic anaerobe was first reported by Alber and Ferry in 1994. The polypeptide structure of γ CA consists of a homotrimer having left handed parallel β helix (Tiwari et al. 2005). Kisker et al. (1996) reported that the active site of the enzyme is composed of three histidine ligands along with two water molecules forming the coordination sphere with the central metal ion. The only well characterized γ class enzyme is "Cam" from the archaeon *Methanosarcina thermophila* (Alber and Ferry 1994). In the γ class CA the metal binding site consists of three histidine residues in a tetrahedral geometry similar to that of the monomeric α class (Kisker et al. 1996). However, in Cam, two of the histidine are donated by one monomer (His-81, His-122) and the other from an adjacent monomer (His-117). High resolution crystal structures with bicarbonate bound to the active site led to proposed roles for other active sites residue. Kinetic analyses of the Q 75A variant indicated that Gln-75 is important for CO_2 hydration activity. Gln-75 function is analogous with Thr-199 in HCA II isozyme by hydrogen bonding and orienting the zinc bound hydroxide for attack on CO_2. The zinc ions are located between the subunit and ligated to His-81 and His-122 from one subunit and His-117 from a neighbouring subunit. A putative water molecule completes distorted, tetrahedral co-ordination geometry. A superposition of the metal centres in *M. thermophila* CA and human CA II shows remarkable similarities, illustrating that in this case, convergent evolution of catalytic function includes the evolution of similar zinc binding sites. The presence of cobalt in the active site of the enzyme has been reported by Roberts et al. (1997). Experiments have demonstrated an enhancement in dehydration activity of the enzyme when cobalt is present in its active site (Iverson et al. 2000).

7.3 Prokaryotic Carbonic Anhydrases

The emergence of genome sequencing and the availability of antisera to α, β and γ-classes of CAs have allowed a comprehensive search for the presence of CAs in metabolically and phylogenetically diverse prokaryotes (Smith et al. 1999).

Evidence for the presence of carbonic anhydrase was obtained for freshwater, marine, mesophilic, thermophilic, aerobic, anaerobic, pathogenic, symbiotic, acetogenic, autotrophic, heterotrophic and photosynthetic species. These results demonstrated that CAs are not only far more prevalent in multitude of prokaryotes, than previously recognized, but that the β and γ-classes are predominant.

In fact, so far only few α-class CAs have been identified in the Bacteria domain and none in the Archaea domain. Interestingly, many prokaryotes contain CA genes from more than one class, some even contain genes from three of the known classes (i.e., α, β and γ). In addition, some prokaryotes contain multiple genes encoding CAs from the same class (Table 7.2). For example, *Pseudomonas aeruginosa* contains six CA sequences, three from β-class and three from γ-class. The presence of multiple CA genes within a species underscores the importance of this enzyme in prokaryotic physiology; however, the role(s) played by these CAs are still largely unknown (Smith and Ferry 2000).

Table 7.2 Distribution of putative carbonic anhydrase genes in some prokaryotes

Prokaryotic taxa	α-class*	β-class*	γ-class*
Archaea			
Methanobacterium thermoautotrophicum^{e}	-	2	1
Methanococcus jannaschii^{e}	-	-	1
Methanosarcina thermophila^{e}	-	-	1
Pyrococcus furiosus^{e}	-	-	1
Pyrococcus horikoshii^{e}	-	-	1
Sulfolobus solfataricus^{c}	-	2	1
Bacteria			
Caulobacter crescentus$^{\alpha}$	-	2	-
Magnetospirillum magneticum$^{\alpha}$	2	2	2
Rhodobacter sphaeroides$^{\alpha}$	-	3	-
Rhodospirillum rubrum$^{\alpha}$	1	1	-
Granulibacter bethesdensis$^{\alpha}$	-	2	1
Ralstonia eutropha$^{\beta}$	2	2	2
Neisseria gonorrhoeae$^{\beta}$	2	2	-
Neisseria meningitides$^{\beta}$	-	1	-
Bordetella pertussis$^{\beta}$	-	1	-
Pseudomonas putida$^{\gamma}$	-	1	1
Pseudomonas aeruginosa$^{\gamma}$	-	3	3
Aeromonas hydrophila$^{\gamma}$	1	1	2
Vibrio cholerae$^{\gamma}$	1	1	1
Yersinia pestis$^{\gamma}$	-	2	-
Salmonella typhimurium$^{\gamma}$	-	4	1
Shewanella denitrificans$^{\gamma}$	-	1	2
Klebsiella pneumoniae$^{\gamma}$	-	3	-
Acidithiobacillus ferrioxidan$^{\gamma}$	-	2	-
Erwinia carotovora$^{\gamma}$	2	2	-

(continued)

Table 7.2 (continued)

Prokaryotic taxa	α-class*	β-class*	γ-class*
Escherichia coli[γ]	-	2	2
Haemophilus influenzae[γ]	-	1	-
Helicobacter pylori 26695[ε]	1	1	-
Helicobacter pylori J99[ε]	2	2	-
Synechococcus sp. PCC7002[@]	1	1	1
Synechococcus elongatus PCC7942[@]	1	5	-
Synechocystis sp. PCC6803[@]	-	4	-
Anabaena variabilis[@]	1	4	-
Corynebacterium glutamicum[x]	-	1	1
Mycobacterium avium[x]	-	2	-
Mycobacterium bovis[x]	-	3	-
Mycobacterium tuberculosis[x]	-	3	-
Streptomyces coelicolor[x]	-	4	-
Chlorobium tepidum[#]	-	-	1
Deinococcus radiodurans[$]	-	1	-
Enterococcus faecalis[+]	2	-	-
Clostridium acetobutylicum[+]	-	2	1
Bacillus subtilis[+]	-	3	-
Streptococcus pyogenes[+]	-	2	-

*Numbers indicate the number of putative carbonic anhydrase genes present. (-) indicates no putative genes have been identified in the completed genome. Species from the Archaea domain belongs to the following kingdoms: [e]euryarchaeota, and [c]crenarchaeota.

Species from the Bacteria domain belongs to the following kingdoms: proteobacteria (a class)[α], proteobacteria (β class)[β], proteobacteria (γ class)[γ], proteobacteria (ε class)[ε], cyanobacteria[@], actinobacteria[x], cytophagales/green sulfur[#], thermus/deinococcus[$], firmicutes (Gram-positive)[+]

7.4 Distribution and Physiological Functions of Carbonic Anhydrases in Prokaryotes

Two general roles have been suggested for known carbonic anhydrases: (i) transport of CO_2 or HCO_3^- and (ii) to provide CO_2 or HCO_3^- for enzymatic reactions. Physiological roles have been investigated largely in photosynthetic microbes: however, carbonic anhydrase occur across the spectrum of prokaryotic metabolism in both the Archaea and Bacteria domains and many individual species contain more than one class, indicating that carbonic anhydrase have far more extensive and diverse roles in prokaryotes, than previously recognized. These results suggest novel roles for this enzyme in prokaryotic physiology (Smith and Ferry 2000).

7.4.1 Photosynthetic Prokaryotes

A significant portion of the total carbon fixed in the biosphere is attributed to the autotrophic metabolism of prokaryotes. The efficiency of carbon fixation in cyanobacteria and many autotrophic bacteria is enhanced by a polyhedral microcompartment called the *carboxysome*, where RuBisCo and CA are usually confined (Moroney and Ynalvez 2007). It is proposed that HCO_3^-, which is actively transported and accumulated in the cytoplasm, diffuses through the proteinaceous shell and is dehydrated to CO_2 by a carboxysome-associated carbonic anhydrase. The close proximity of the carbonic anhydrase to encapsulated RuBisCo is thought to be a key factor in speeding the fixation of CO_2 (Kaplan and Reinhold 1999). It is also possible that the carboxysomal shell provides a barrier to the escape of CO_2 from RuBisCo. All three classes (α, β and γ) of CAs have been identified in cyanobacteria. CAs are thought to play a central role in the CCM and in fact CA activity is found both at the plasma membrane and in the carboxysomes. The physiological function documented for different classes of CAs in cyanobacteria is discussed in the following section:

7.4.1.1 α-CAs

In *Anabaena* and *Synechococcus*, the expression of an extracellular α-type CA (EcaA) is regulated by the level of CO_2 in the growth medium and is highest in cells grown at elevated CO_2 levels (1% CO_2) and low but still detectable levels are detected following 24 h low CO_2 (0.01%) exposure (SoltesRak et al. 1997). It is proposed that EcaA has a role in inorganic-carbon (Ci) accumulation by maintaining equilibrium levels of CO_2 and HCO_3^- in the periplasm.

7.4.1.2 β-CAs

In the cyanobacterium *Synechococcus* PCC7942, the cells accumulate bicarbonate in the cytoplasm and convert it into CO_2 in carboxysomes, the polyhedral bodies in which β-CA (IcfA) is localized; and this process brings about an elevation in the CO_2 concentration around the CO_2-fixing enzyme, RuBisCo. Similarly, in *Synechocystis*, a β-CA referred to as CcaA (cyanobacterial carboxysome localized) has been shown to be associated with the carboxysome, where it has been proposed to dehydrate HCO_3^- to CO_2 for the CO_2-fixing enzyme RuBisCo (So and Espie 1998).

7.4.1.3 γ-CAs

In the cyanobacterium *Synechococcus* PCC7942, a γ-CA homologue, CcmM is also localized in carboxysomes. The sequence of *ccmM* encodes a bifunctional gene

product of 539 amino acids of which the first 219 are 35% identical to those of Cam, the prototypical γ-CA from *M. thermophila* while the remaining 320 amino acids show 45–51% identity to the C-terminal region of *Anabaena* Rubisco activase. *Synechococcus* cells deleted in *ccmM* show high CO_2 requiring (HCR) phenotype and have empty carboxysomes suggesting that CcmM is required for correct carboxysome assembly and for optimal growth on low levels of CO_2. CA activity has not been reported for the heterologously expressed CcmM in *E. coli* but it is suggested that CcmM plays a role in the elevation of CO_2 concentration around the CO_2-fixing enzyme, RuBisCO (Price et al. 1993). The CcmM protein is proposed to transport bicarbonate from the cytoplasm to the centre of the carboxysome where a conserved β-CA, the CcaA protein, efficiently converts bicarbonate into carbon dioxide and thus, RubisCO could fixate through the Calvin cycle. In addition it is proposed that CcmM may prevent leakage of CO_2 out of the carboxysome.

7.4.2 Non-photosynthetic Prokaryotes

In prokaryotes, carbonic anhydrases are involved in diverse biochemical and physiological processes, including photosynthesis, respiration, CO_2 and ion transport, and CO_2/bicarbonate balance required for biosynthetic reactions. Although the wealth of information known about the function of this enzyme in prokaryotes relates to its role in the fixation of CO_2 by phototrophic microbes, relatively little is known about the physiological roles of CAs in non-photosynthetic organisms; but in general, loss of CA activity may result in growth defects. In some cases, such as acetogenic bacteria (Braus-Stromeyer et al. 1997) and methanogenic archaea (Smith and Ferry 1999), organisms that utilize CO_2 in central metabolic processes (analogous to CO_2 fixation in photosynthetic organisms), CA activity may be required to insure adequate levels of CO_2 or HCO_3^- as a substrate for other enzymes. Bicarbonate is a substrate for various carboxylation reactions of physiological importance. Carboxylating enzymes that use bicarbonate as a carboxylate source include 5-phosphoribosyl-5-amino-4-imidazole carboxylase (EC 4.1.1.21), phosphoenolpyruvate carboxylase (EC 4.1.1.31), carbamoylphosphate synthetase (EC 6.3.4.16), pyruvate carboxylase (EC 6.4.1.1), and acetyl-CoA carboxylase (EC 6.4.1.2). These enzymes catalyze key steps of pathways for the biosynthesis of not only physiologically essential but also industrially useful metabolites, such as amino acids, nucleotides, and fatty acids. The heterogeneity of CA in non-photosynthetic aerobic bacteria was contemplated by the isolation and characterization of this enzyme from diversified genus of bacteria under optimized parameters for enhanced production of CA in *Enterobacter* sp. and *Aeromonas* sp. (Sharma et al. 2008). The presence of CA for the first time in members of γ-proteobacteria group was established by purification and characterization of extracellular CA from *Pseudomonas fragi* (Sharma et al. 2009). The presence of this extracellular CA in Pseudomonas fragi isolated from $CaCO_3$ enriched soil sample has been envisaged to play an important role in bicarbonate ion transport. It was

found to be a trimeric protein with a subunit molecular weight of 31.0 kDa. The physiological role of this enzyme is significant in terms of its evolutionary and phylogenetic relationship and the important physiological role of this enzyme that determines the survival of the organism in an extreme environment corresponding to karst ecosystem. The physiological functions documented for different classes of CAs in several microorganisms of environmental, medical and industrial importance is discussed in the following section:

7.4.2.1 α-CAs

In contrast to the widespread distribution of CAs, only a few non-photosynthetic prokaryotes have been shown so far to possess active α-type CA isoforms. They include *Helicobacter pylori* (Marcus et al. 2005), the CO_2-requiring pathogen *Neisseria gonorrhoeae* (Chirica et al. 1997), the purple non-sulfur phototrophic bacterium *Rhodopseudomonas palustris* (Puskas et al. 2000) and the nitrogen-fixing bacterium *Mesorhizobium loti* (Kalloniati et al. 2009). A common feature of all bacterial α-type CAs is the presence of an N-terminal signal peptide, responsible for the periplasmic or extracellular localization of the enzyme and a possible physiological role in CO_2 uptake.

In *N. gonorrhoeae* α-CA plays a role in the acquisition and transport of CO_2 in the cytoplasm. The enzyme has been shown to be optimally expressed under low CO_2 conditions (Forkman and Laurell 1966). Transfer to high CO_2 concentration (10%) rapidly results in non-detectable levels of mRNA and protein. In *R. palustris*, the periplasmic α-type CA is expressed under anaerobic conditions and has been proposed to be essential for bicarbonate uptake.

In *H. pylori* the periplasmic α-CA together with cytoplasmic urease is an integral component of the acid acclimation response that allows this neutralophilic bacterium to colonize in the stomach (Marcus et al. 2005). The products of urease activity in the cytoplasm are $2NH_3$ plus CO_2, which diffuse very rapidly across the inner membrane into the periplasmic space. Here, the α-CA whose expression is upregulated by low environmental pH catalyzes the hydration of CO_2 and provides protons for both NH_4^+ formation and bicarbonate, which can buffer the periplasm at a pH value close to its pKa of 6.1, within the range of bacterial growth ability (Marcus et al. 2005). A very similar role has been proposed for α-CA (CAA1) of nitrogen-fixing bacterium *M. loti* in the symbiotic nitrogen-fixing bacteroids where large amounts of NH_3 and CO_2 diffuse across the inner membrane into an acidified symbiosome space, where NH_3 is protonated to NH_4^+ for transport across the symbiosome membrane. CAA1 has been proposed to participate in buffering of the bacteroid periplasm at a pH favorable for NH_3 protonation, facilitating its diffusion and transport to the plant. The possible role of CAA1 in maintaining an acidic periplasm during symbiotic nitrogen fixation is supported by the observation that the expression of the respective gene is upregulated both upon the onset of nitrogen fixation and at alkaline pH values

in free-living bacteria (Kalloniati et al. 2009). Smith and Ferry (2000) concluded that esterase activity is associated only with alpha class carbonic anhydrase and has not been documented with other known classes of CA. The experimental support was duly justified by the work of Armstrong et al. (1966) who determined esterase activity in α-class HCA and by Ekinchi et al. (2007) who established the simultaneous *in vitro* inhibition of hydratase and esterase activities of human carbonic anhydrase – I and II in presence of certain drugs. The presence of esterase activity in bacterial CA belonging to alpha class was first reported by Chirica et al. (2001) in *N. gonorrhoea*, and further in CA from *E. taylorae* and *A. caviae* (Sharma et al. 2008) and in extracellular CA from *P. fragi* (Sharma et al. 2009). The conclusive evidence for absence of esterase activity in beta and gamma CA was provided by Smith and Ferry (1999) and Smith et al. (1999) in Cab from *Methanobacterium thermoautotrophicum* and Cam (γ-CA) from *Methanosarcina thermophila*, respectively.

7.4.2.2 β-CAs

Representatives of β-class CAs are widely distributed in phylogenetically diverse prokaryotes from the Bacteria and Archaea domain. Two members of the β-CA have been identified in *E. coli*. The first to be characterized was CynT, which is encoded by a part of the *cyn* operon whose function allows *E. coli* to utilize cyanate as a nitrogen source for growth as a consequence of cyanase-catalysed decomposition to ammonia (Eq. 7.2).

$$NCO^- + 3H + HCO_3^- \leftrightarrow NH_4^+ + 2CO_2 \tag{7.2}$$

The cyanase gene, *cynS* is co-transcribed with *cynT* and it is suggested that CynT catalyzes hydration of CO_2 generated by cyanase to prevent depletion of the HCO_3^- required for further degradation of cyanate or other metabolic processes (Guilloton et al. 1992, 1993). *E. coli* encodes a second β-class CA called Can (previously YadF) that is required for growth with CO_2 levels available in ambient air and a nitrogen source other than cyanate (Hashimoto and Kato 2003; Merlin et al. 2003); however, Can is not required for growth if higher levels of CO_2 are supplied. Thus, it was proposed that Can function to maintain bicarbonate at levels needed for various metabolic processes other than utilization of cyanate as a source of nitrogen. Similarly, growth of *R. eutrophus* at air levels of CO_2 is principally dependent on sufficient CA activity, which is provided by the β-CA Can (Kusian et al. 2002). Therefore, the physiological role of Can in *R. eutropha* seems to correspond to that of Can in *E. coli*.

Much evidence has been gathered supporting the existence of CA activity in pathogenic bacteria. It has been shown that the *S. typhimurium* β-CA gene which is 24% identical to that of *E. coli* CynT was induced upon infection of macrophages, indicating that CA may play a role in virulence (Valdivia and Falkow

1997). In a competition assay, *S. typhimurium* strains with a targeted disruption of the β-CA gene showed decreased spleen colonization. Growth of *M. tuberculosis* is dependent on a β-class CA (Rv3588c) (Covarrubias et al. 2005). *H. pylori*, the causative agent of peptic ulcer disease, expresses α-class CA (hpαCA) attached to the surface and a β-class CA (hpβCA) found in the cytosol. *H. pylori* has the unique ability among bacteria to grow in the stomach presenting highly acidic conditions, at pH values as low as 1.4. Therefore, the pathogen has evolved specialized processes for survival in acid, which maintain its cytoplasmic pH around 6.4. It is postulated that hpβCA along with hpαCA and urease play an important role in the urea and bicarbonate metabolism, as well as acid resistance of the bacterium (Chirica et al. 2002).

A prototypic "cab-type" β-CA (Cab) from the thermophilic methanogen, *Methanobacterium thermoautotrophicum*, is the first documented carbonic anhydrase from a CO_2-reducing chemolithoautotrophic methanoarchaeon (Smith and Ferry 1999a). These microbes have a high demand for CO_2 in both catabolic and anabolic reactions, suggesting that CA may be essential to deliver CO_2 to the cell and concentrate it in the vicinity of CO_2-utilizing enzymes, analogous to its role in photosynthetic organisms in which the enzyme is essential for efficient CO_2 transport into the cell and elevation of the CO_2 concentration near the active site of RuBisCO. For example, Cab could convert bicarbonate to CO_2, the substrate for the formylmethanofuran dehydrogenase (Vorholt and Thauer 1997) catalyzing the first committed step in the reduction of CO_2 to methane during methanogenesis. The central anabolic pathways for *M. thermoautotrophicum* are the autotrophic pathways for acetyl coenzyme-A biosynthesis and the incomplete reductive tricarboxylic acid cycle in which some of the CO_2 fixation enzymes utilize HCO_3^-. Thus, to provide substrate for carboxylation reaction, interconversion between CO_2 and HCO_3^- is another potential role postulated for Cab.

Production of amino acids in *Corynebacterium glutamicum,* an industrially important microorganism is dependent on the synthesis of oxaloacetate, a key intermediate, by two bicarbonate-dependent enzymes, pyruvate carboxylase and phosphoenolpyruvate carboxylase. The genome of *C. glutamicum* harbors two genes encoding putative β and γ-class CA's (*bca* and *gca* respectively). Deletion of *bca* severely restricted the growth in air leading to the proposed role for this CA in growth and maintenance of favorable intracellular levels of CO_2/bicarbonate (Mitsuhashi et al. 2004).

The genome of a rhizobacterium *Azospirillum brasilense*, which frequently colonizes the roots of many non-legume crops harbours ORFs encoding one β-CA (*bca*) and two γ-CAs (*gca1* and *gca2*). The β-class CA is expressed under normal atmospheric conditions to produce functionally active carbonic anhydrase. The expression of β-class CA in response to growth phase, CO_2 concentrations and pH led to the proposed role of β-CA in scavenging CO_2 from the ambient air and in fulfilling the requirement for higher CO_2 hydration in the cultures in *A. brasilense* growing exponentially at neutral to alkaline pH (Kaur et al. 2009).

7.4.2.3 γ-CAs

Although widely distributed in bacteria and archaea domains, the physiological role of γ-CAs is not well documented. "Cam" (carbonic anhydrase methanosarcina) from methane-producing (methanogen) species *Methanosarcina thermophila* is the prototype of the γ-CA and the only enzyme from this class that has been extensively characterized biochemically and structurally. The *Methanosarcina* are the metabolically most diverse methanogens as they obtain energy for growth by producing methane from the reduction of CO_2 with H_2 or by the conversion of methyl groups of acetate, methanol or methylamine to methane. The methyl group of acetate is converted to methane and the carboxyl group to CO_2 that are metabolic end products. In *M. themophila* a metabolic switch from acetate to CO_2 results in the elevation of Cam activity suggesting that it is important for the utilization of acetate as a growth substrate (Alber and Ferry 1994). Acetate-utilizing methanogens have a requirement to exchange acetate and CO_2 albeit in the opposite direction. Thus, it has been postulated that an acetate/HCO_3^- antiporter activity is facilitated by the conversion of CO_2 to HCO_3^- catalyzed by Cam. Also, a 34-amino acid N-terminal putative signal sequence present in the Cam sequence possibly translocates Cam across the cytoplasmic membrane to the exterior of the cell. Thus the second postulated function of Cam on the basis of its possible location outside the cell membrane is to facilitate the removal of cytoplasmic CO_2 by conversion to HCO_3^- outside the cell membrane. This function may be important mechanism for 'driving' the conversion of acetate to CO_2 and methane, thus improving the thermodynamics of the pathway (Alber and Ferry 1994).

E. coli encodes several gene products (CaiE, PaaY, and YrdA) which are homologous to γ-CAs, with fully conserved active-site residues. CaiE is encoded by a gene in the *caiABCDE* operon which encodes proteins involved in carnitine metabolism. CaiB and CaiD function as carnitine dehydratase and carnitine racemase respectively and CaiE is involved in the generation or regeneration of a coenzyme for CaiB/CaiD (Eichler et al. 1994). *Pseudomonas putida* PhaM is encoded by a gene found within a catabalon involved in the aerobic degradation of phenylacetic acid (Ferrandez et al. 1998). The *phaM* gene is located upstream in an operon with *phaN* which encodes a transcriptional repressor. Disruption of the *phaM* gene, did not affect catabolism of phenylacetic acid leaving the role of PhaM undefined. However, PhaM shares significant identity to the *E. coli* CaiE suggesting that it may play a similar role, although no carbonic anhydrase activity been reported for either CaiE or PhaM. In amino acid producing microorganism, *C. glutamicum* deletion of the γ-CA (*gca)* encoding gene had no discernable effect leaving a role for this putative γ-class undefined (Mitsuhashi et al. 2004).

A putative γ-CA encoding gene (*gca1*) of the non-photosynthetic rhizobacterium, *A. brasilense* (Kaur et al. 2010) which showed significant homology with other members of γ-CA family when cloned and expressed in *E. coli* did not show CA activity. Lack of detectable CA activity as found in case of recombinant Gca1 protein from *A. brasilense* was also observed in recombinant γ-CA of *Arabidopsis* (Parisi et al. 2004), two cyanobacterial CcmM orthologs (Cot et al. 2008), *E. coli*

proteins YrdA, CaiE, and PaaY (Merlin et al. 2003), γ-CA-like proteins from *C. glutamicum* (Mitsuhashi et al. 2004) and *C. reinhardii* (Mitra et al. 2004).

The *gca1* in *A. brasilense* was co-transcribed with an upstream *argC* which encodes a putative *N*-acetyl-γ-glutamate-phosphate reductase thought to be involved in arginine biosynthesis. The transcription of *argC-gca1* operon was upregulated in stationary phase and in atmosphere having elevated CO_2 levels. The unique operonic organization of *gca*1 and *argC*, observed in *A. brasilense* is syntenous with some of its closely related α-proteobacteria, viz. *Magnetospirillum, Rhodospirillum, Granulibacter* etc. These observations indicated that the γ-CA-like gene in *A. brasilense*, instead of being involved in CO_2 hydration, may have a role in arginine biosynthesis.

7.5 Application of Carbonic Anhydrase in Carbon Sequestration

"The greenhouse effect occurs naturally providing a habitable climate. Atmospheric concentration of some of the gases that produce the greenhouse effect are increasing due to human activities and world's climate scientist believe this is an instrumental cause for global warming. Over one third of the human induced greenhouse gases come from the burning of fossil fuels to generate electricity; nuclear power plants do not emit these gases" (Sathaye et al. 2006). The unpredictable warming of the climate system has been supported by the increase in global average temperature of air and ocean, widespread melting of snow and ice as well as rise in global sea level (IPCC 2009). Human induced climate change has already affected multiple systems, both physical and biological (Rosenzweig et al. 2008). Changes in polar temperatures, both in Arctic and Antarctic, have been attributed conclusively to human activity, with impacts on ecosystems, indigenous communities, ice sheet and ice shelf stability, and sea level rise (Gillett et al. 2008). Human activities since the start of the industrial era around 1750 have increased the levels of green house gases in the atmosphere. Carbon dioxide is the major anthropogenic green house gas. Solomon et al. (2009) have put the current atmospheric CO_2 concentration levels at 385 ppmv and have predicted that an increase upto 450–600 ppmv over the coming century will result in certain regions resembling "dust bowl" era and inexorable sea level rise. Most of the observed rise in temperature since the mid twentieth century is very likely due to the observed increase in anthropogenic Green House Gases concentration. The global atmospheric concentration of carbon dioxide has increased from a pre industrial value of about 280 ppm to 390 ppm since 1800 (Bloom et al. 2010). The annual carbon dioxide emissions have increased from an average of 23.5 Gt CO_2 per year in 1990s to 26.4 Gt CO_2 per year in 2000–2005. The IPCC in their report (2007) have predicted that, by the end of twenty-first century climate change will result in a possible temperature rise between 1.1°C and 6.4°C causing disappearance at Arctic summer sea ice and melting of glaciers at both the poles, the simultaneous effect being increase in sea level, at an average rate of 28–43 cm. Electricity generation is one of the major sources of CO_2 emissions, providing one

third of the total emission. In context to increased CO_2 concentration in atmosphere the recommendations of scientists to policymakers of Kyoto Protocol emphasized the immediate need for methods to achieve large-scale decrease in emission of CO_2 from the atmosphere (Jacob 2005).

Besides the biochemical and physiological importance of CA, the enzyme has found a new dimension in the field of biomimetic CO_2-sequestration. Anthropogenic CO_2 emission has led to adverse impact on climate and has been implicated in global warming. In the global effort to combat the predicted disaster, several CO_2 capture and storage technologies (CCS) are being considered. CA has been exploited as a biocatalyst to sequester CO_2 through the conversion of CO_2 to HCO_3^- in the mineralization of $CaCO_3$. 'Mineral sequestration' is new and relatively less studied method, and has good potential to sequester substantial amount of CO_2 from the environment permanently. Carbonate minerals, such as calcite, aragonite, dolomite, and dolomitic limestone, comprise a massive CO_2 reservoir, estimated to contain an amount of carbon equivalent to $1{,}50{,}000 \times 10^{12}$ metric tons of CO_2 (Wright and Colling 1995). The main advantage of the process is that the formed mineral carbonates are end products of geologic processes and are known to be stable over geological time periods (millions of years). Besides, the reaction products are environmentally benign. Thus, carbonate sequestration process provides a geologically proven safe and long-term repository of CO_2 disposal. However, the rate of conversion of CO_2 to stable carbonate minerals is expected to be slow (hundreds of years).

Mineralization of CO_2 to calcium carbonate occurs through a reaction between calcium ions and aqueous CO_2 (Sullivan et al. 1993). The rate-controlling step in the fixation of gaseous CO_2 into carbonate ions is the hydration of CO_2. The conversion of H_2CO_3 to H^+ and HCO_3^- is very rapid, the rate being virtually diffusion controlled. Dissociation of bicarbonate ions to yield carbonate ions is slower than dissociation of H_2CO_3, but is still much faster than the rate controlling step. Thus, if a viable means to accelerate the hydration of CO_2 could be found, it should be feasible to fix large quantities of CO_2 into calcium carbonate at moderate pH. To address this problem of rate, a new approach known as 'biomimetic' approach to accelerate an aqueous processing route to carbonate formation has been employed in recent years (Bond et al. 2001).

A biomimetic approach is the one in which a particular aspect of a biological process or structure is identified and applied to solve a specific non-biological problem. In recent years a biocatalyst, carbonic anhydrase (CA) which catalyzes the reversible hydration of CO_2 to bicarbonate in biological systems and is linked to calcium carbonate biomineralization in mollusk shells (Miyamota et al. 1996), avian egg-shell (Fernandez et al. 2004), unicellular coccolithophore algae, *Emiliania huxleyi* coccolithogenesis (Quinn et al. 2006) etc., has been employed for transformation of CO_2 into carbonate compounds. Among other carbon capture and storage technologies, biotechnology of using CA for biomimetic $CaCO_3$ mineralization holds much promise because it is a viable and environmentally benign technology (Liu et al. 2005). The feasibility of using CA as a catalyst for hydration of CO_2, as well as its precipitation in the form of calcium carbonate has been demonstrated (Mirjafari et al. 2007; Liu et al. 2005).

Biosequestration of CO_2 into calcium carbonate was achieved using purified carbonic anhydrase from *Citrobacter freundii* (Ramanan et al. 2009). Recently, *Chlorella* sp. was used as a model organism for evaluating bio-mitigation of CO_2 and simultaneous biodiesel production. Production of precursors for biodiesel, Biomass generation, Calcite formation and CO_2 sequestration was successfully achieved through the micro algae, *Chlorella* sp. The characterization of oil content through GCFID and further confirmation through FAME indicated the presence of palmitic acid (C16:0), docosapentaenoic acid (C22:5), and docosahexaenoic acid (C22:6). The oil generated by the microalgae can serve as alternate fuel, since it has high calorific value 29 kJ g^{-1}. The superior quality of calcite formed by the microalgae has been characterized by FT-IR, SEM and XRD. The study encompasses the bio-sequestration of CO_2, calcite formation and oil production simultaneously using photobioreactor from a single algal species (Fulke et al. 2010). The immobilization of carbonic anhydrase enzyme and the microbial strains producing this enzyme on inert biopolymeric materials was established (Prabhu et al. 2009). In this study immobilization by adsorption was found to be an effective strategy for both enzyme and microbial adsorption; however Liu et al. (2005) reported the successful immobilization of commercial bovine carbonic anhydrase through entrapment method using chitosan alginate beads. The principle of single enzyme nanoparticle for biomimetic CO_2 sequestration has been established. Individual enzyme molecules are stabilized within a biopolymeric silica network of nanometerscale thickness. A key result is that stabilization of the activity was achieved with minimal substrate mass-transfer limitation compared to CA entrapped in larger scale materials. The development of stabilized SENCA as soluble individual SEN-CA particles also provides the opportunity to further process them into other forms (Yadav et al. 2010).

Recently, enhanced biomimetic sequestration of CO_2 into $CaCO_3$ was investigated using purified carbonic anhydrase from indigenous bacterial strains (Sharma and Bhattacharya 2010). The effect of physical factors, anions, and metal ions on the efficiency of the enzyme under conditions simulating an onsite scrubber was critically analyzed. Successful CO_2 sequestration was visualized at pH ~ 9.2. The sequestration process has to be carried out near ambient conditions, as recourse to caustic conditions will not be environmentally favorable. Thus the whole experimental setup needs to be adjusted in the pH range 7.5–9.5, in order to strike proper balance with respect to enzyme activity, precipitation of calcium carbonate and environmentally amenable conditions. A much higher rate of $CaCO_3$ precipitation was observed in presence of enzyme catalyzed reaction compared to uncatalysed reaction. Similar reports have been documented by Favre et al. (2009) and Mirjafari et al. (2007).

Liu et al. (2005) have reported successful calcium carbonate formation using different brines at pH 8.5–8.7. Ramanan et al. (2009) studied the carbonation reaction using tris buffer at pH 8.3. Formation of calcium carbonate using tris buffer at various pH has also been demonstrated by Favre et al. (2009). The enzyme carbonic anhydrase was found to accelerate the formation of solid carbonate by a factor >10 at 5°C when a buffer of stock solution at pH 10.5 was used. Depending on the conditions, the formation of two different phases was observed: vaterite, favored at pH

10.5 at 5°C, without enzyme, and calcite at lower pH, or at 20°C. The precipitation rate of calcium carbonate in presence of either microbial CA or bovine CA is faster than that in presence of other biological factors (bovine serum albumin, carboxymethyl chitosan, and glutamic acid). Thus, suggesting a novel and holistic approach categorizing microbial CA as a marker for biomimetic CO_2 sequestration and environmental amelioration (Li et al. 2010).

Carbonic anhydrases from indigenous strains (*P. fragi*, *M. lylae*, *and M. luteus* 2) were combined under equimolar ratio to generate enzyme consortia and used to evaluate the potential for biomimetic carbon dioxide sequestration against commercial BCA under simulated conditions of a onsite scrubber. The amalgamation of indigenous CAs with varying properties has been successfully used to demonstrate enhanced biomimetic sequestration of CO_2 into $CaCO_3$ compared to commercial bovine CA under process parameters simulating an on-site scrubber (Sharma and Bhattacharya 2010). The recombinant beta carbonic anhydrase has also been shown to be useful in evolving an efficient technique for producing homogeneous nano-size calcium carbonate particles (Kaur et al., personal communication) which can have important biomedical applications.

7.6 Future Prospective and Conclusions

Although several reports on the characterization of carbonic anhydrases have come from different laboratories around the world; we do not yet understand the specific roles of different types of carbonic anhydrases with their diverse structures in the cellular metabolic processes. Recent discoveries have demonstrated that carbonic anhydrases are indeed widespread and nearly ubiquitous in metabolically and phylogenetically diverse prokaryotes from the Bacteria and Archaea domain. The multiplicity of carbonic anhydrases in many prokaryotes underscores their importance in prokaryotic physiology and suggests novel roles for this enzyme. The presence of this enzyme and its important role in well established pathogens like; *Salmonella*, *Helicobacter*, and *Neisseria* enthralls a griping prospective for the role of this enzyme in pathogenesis. A better understanding of the role of carbonic anhydrases in the cellular metabolism may lead to increased use of this enzyme for commercial exploitation. The developments in the technology for large scale production of this enzyme, its immobilization on appropriate materials and design of bioreactors might facilitate its application in addressing environmental problems such as reducing CO_2 from the flue gas. Biomimetic CO_2 sequestration opens up an original and applicative approach for this enzyme that might facilitate environmental amelioration linked with the current scenario of global warming and climate disparity. Although juvenile, the approach has been proved in principle and a number of parameters deemed to influence CA activity have been worked out, it presents an enterprising application of this enzyme for tackling anthropogenic CO_2 emissions reflecting global warming.

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Chapter 8
Screening and Evaluation of Protease Inhibitory Peptides in *Microcystis* spp. – Dominant Water Blooms

Suvendra N. Bagchi, Palash K. Das, and Shubhro K. Ghosh

Abstract Cyanobacteria or blue-green algae are known to produce a diverse array of secondary metabolites with a multitude of biological activities. There are approximately 600 odd biologically active peptides produced by cyanobacteria, of which a number of them inhibit mammalian serine protease activity. It is established that planktonic cyanobacteria in eutrophic water bodies make water blooms by accumulation of buoyant colonies in the form of aggregates over water surface. In this chapter we mention about a cost effective and sensitive screening protocol for evaluation of anti-protease blooms comprising *Microcystis* spp. with *Microcystis aeruginosa* PCC7806 lab cultures as reference material. Bovine trypsin and chymotrypsin were soaked in filter paper discs and these discs were placed on agar solidified plates containing casein, gelatin and skimmed milk. The enzyme activities led to the digestion of the substrates and thus formed clearing zones. To evaluate the bloom's ability to inhibit proteases, a methanolic extract prepared from freeze dried bloom cells was added to filter paper discs along with proteases. Concentration dependent decrease in diameter of clearing zones was the measure of extent of inhibition. Further identification of protease inhibitory metabolites was carried out directly in the dried bloom samples by MALDI-TOF MS. Upon matching with the available database the MS data revealed the presence of peptides: aeruginosin, cyanopeptolin and microviridin, the known inhibitors of animal serine proteases.

Keywords Microcystis • Protease inhibitors • Peptides • Bioassay • Trypsin • Chymotrypsin • MALDI-TOF MS

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8.1 Introduction

Cyanobacteria produce a large number of biologically active secondary metabolites which have been isolated from a variety of cultured strains and field samples. The most prominent amongst them are the toxins and protease-inhibitors that are associated with cytotoxicity and antineoplastic, allelopathic, antifungal and insecticidal properties, besides causing immunosupression and promotion of cell differentiation activities etc. Generally, cyclic peptides and depsipeptides are the most common structural types, but a wide variety of other types like linear peptides, guanidines, phosphonates, purines and macrolides etc. have also been reported (Namikoshi and Rinehart 1996). Peptides and peptidic metabolites are the most studied amongst those produced by cyanobacteria. In the last two decades cyanobacterial peptides have gained a special recognition because of their toxic nature. Health agencies around the world are monitoring water bodies with a history of cyanobacterial blooms. Besides their detrimental effect, cyanopeptides are also characterized as compounds with potential pharmacological applications. So far, over 600 peptides have been reported from a variety of taxa of cyanobacteria and a diverse geographical region (Welker and von Döhren 2006). Approximately 80 structural archetypes of the peptidic compounds have been defined, mostly isolated from Oscillatoriales and Nostocales, followed by Chroococcales and Stigonematales and a few from Pleurocapsales (Boone and Castenholz 2001). These penta-, hepta- and depsipeptides are both cyclic and non-cyclic in structure, comprising of unusual amino acids. Based on the functions the peptides are divided into two major groups: non-toxic (protease-inhibitory) and toxic (Weckesser et al. 1999). There are limited bioassays to screen protease inhibitory metabolites. The common chemical detection methods rest on cumbersome and expensive extraction methods eliminating the minor peptides in process. Here, a rapid and reliable screening of bloom material is highlighted and using minimum processing hassles the congeners of the peptides can be determined using matrix assisted laser desorption ionization time-of-flight mass spectrophotometry (MALDI-TOF MS) based methods.

8.2 Critical Review

8.2.1 Protease-Inhibitory Peptides of Cyanobacteria

The cyanobacterial non-toxic peptides have biological activities which range from cytotoxic, antimicrobial including anti-fungal (Patterson et al. 1994) and animal protease-inhibition (Radau 2000). Mammalian serine proteases *viz*. trypsin, chymotrypsin, elastase, thrombin are strongly inhibited by these peptides. Protease and inhibitor complexes have been crystallized for a trypsin-inhibitor aeruginosin 98 B and another elastase-inhibitor scyptolin A (Matern et al. 2001) and examined

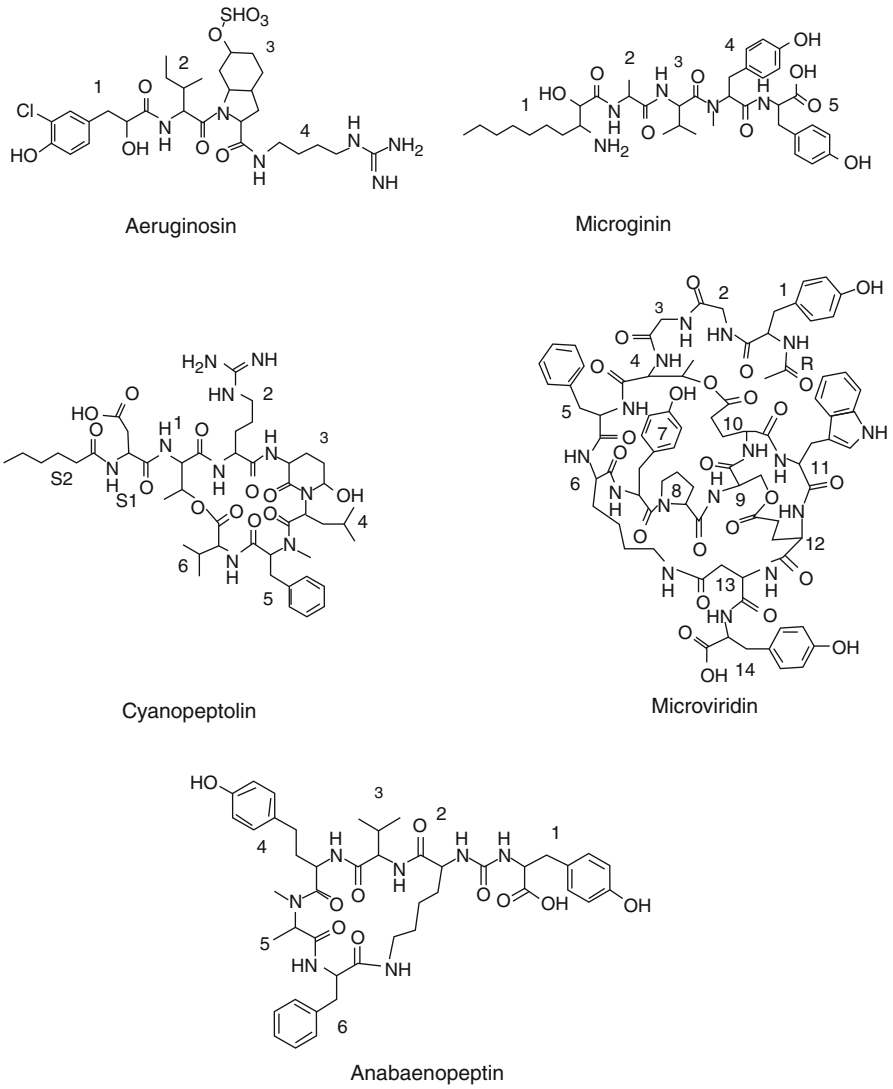

Fig. 8.1 General structure of anti-protease cyanopeptides (Adapted from Welker and von Döhren 2006)

for molecular mode of action. Some of the major classes of protease-inhibitory peptides are:

(a) Aeruginosins
These linear peptides are recognised as derivatives of hydroxyl-phenyl lactic acid (Hpla) at the *N* terminus, the amino acid 2-carboxy-6-hydroxyoctahydroin-dole (Choi) and substituded arginine at the *C*-terminus (Murakami et al. 1995) as shown in Fig. 8.1. *C*-terminal arginine is often derivetised to agmatine by

decarboxylation, arginiol by reduction of carboxyl group to alcohol or to argininal by cyclization of arginiol (Banker and Carmeli 1999; Ishida et al. 1999; Matsuda et al. 1996). At position number 2 variable amino acids, such as Tyr, Phe, Leu, or Ile with usually in D-configuration are present. Further their derivatization such as chlorination, sulfation, bromination and glycosylation do occur at Choi and Hpla moieties (Shin et al. 1997; Welker et al. 2004). With 27 chemical variants known differing mainly in chlorination and sulfation, aeruginosins have been isolated from *Microcystis* and *Planktothrix*. A variant spumigin with mPro instead of Choi from *Nodularia*, and two others, suomilide and banyasides from *Nodularia* and *Nostoc* were also added to this class (Ploutno and Carmeli 2005).

(b) Microginins

These linear peptides are recognized by presence of decanoic acid derivative 3-amino-2-hydroxy decanoic acid (Ahda) and of two Tyr residues at the *C*-terminus (Okino et al. 1993) as shown in Fig. 8.1. Position number 2 is most variable with seven different amino acids can exchange. Microginin vary in amino acid length from four, e.g. microginin 91A (Ishida et al. 2000) to six e.g. microginin 299 C (Ishida et al. 1998). With 38 variants known, microginins have been reported from *Microcystis* and *Planktothrix*. Two peptides differing in decanoic acid derivatives (dimethyl decanoic acid and oxo-dimethyl decanoic acid) are carmabins A and B isolated from *Lyngbya* (Hooper et al. 1998), and another one nostogoinins obtained from *Nostoc* (Ploutno and Carmeli 2002).

(c) Anabaenopeptins

These are cyclic peptides recognized by presence of Lys at the 5th position and formation of a ring by *N*-6-peptide bond between Lys and the carboxyl group of the sixth amino acid (Harada et al. 1995). A side chain of one amino acid is attached to the ring by an ureido- bond as shown in Fig. 8.1. Besides the positions 5 and 3 with methylation and an S-oxygenated amino acids, the ring and side chain amino acids vary from three to five. With 32 variants known, anabaenopeptins have been reported from *Anabaena*, *Nostoc* and *Plectonema* (Harada et al. 1995; Reshef and Carmeli 2002; Fujii et al. 1997).

(d) Cyanopeptolins

These cyclic peptides are recognized from the amino acid 3-amino-6-hydroxy-2-piperidone (Ahp) and cyclization of the peptide ring through ester bond between β-hydroxyl group of Thr and carboxyl group of terminal amino acid (Martin et al. 1993). These peptidolactones as shown in Fig. 8.1 have side chains of variable lengths attached via amino group of Thr. Commonly the side chain either consists of one or two amino acids and an aliphatic fatty acid or a glyceric unit at the *N*-terminal. Further derivatization is seen as O-methylation, sulfation and chlorination. Cyanopeptolins represent the largest class of protease-inhibitory cyanopeptides with 82 variants, majority of which have been reported from the order Chroococcales, Oscillatoriales and Nostocales.

(e) Microviridins
These represent the largest known cyanobacterial oligopeptides with a multicyclic structure established by secondary peptide and ester bonds and a side chain of variable length as shown in Fig. 8.1. The peptide ring consists of seven amino acids with an ester bond between the 4-carboxy group of Asp at position 10 and hydroxyl group of Thr at position 4, and another between Lys at position 6 and the 4-carboxy group of Glu at position 7. Variants within microviridins are primarily due to substitution on the side chain. Till date about 10 variants of microviridin A-J; have been isolated from *Microcystis*, *Nostoc* and *Planktothrix* (Fastner et al. 2001; Welker et al. 2004; Rohrlack et al. 2004).

8.2.2 Cyanobacteria-Grazer Interaction and Protease Inhibitors

According to von Elert et al. (2004), interest in this area comes from terrestrial systems where the importance of plant protease inhibitors for the control of herbivores is well-documented in chemical ecology (Bowles 1998). This issue has not been applied to alga-herbivore interactions in fresh water systems even though it is well known that a number of protease inhibitors are produced by cyanobacteria (Weckesser et al. 1996), and that daphnids show reduced somatic growth and reproduction when cyanobacteria dominate the phytoplankton (von Elert et al. 2003). These cyanobacterial protease inhibitors have only been shown to inhibit vertebrate proteases and it is not known whether they interact with digestive proteases from zooplankton, as in most instances sequence matching have not been done. Further, characterization of crustacean digestive proteases has been confined to decapods' enzymes, and only few sequence data of proteases from a lower marine crustacean have been reported (Johnson et al. 2002; Rojo et al. 2010; Schwarzenberger et al. 2010).

It is generally accepted that cyanobacteria can cause major disruptions of the aquatic ecosystem (Christoffersen 1996), in particular, affecting the grazers, *Daphnia* and *Moina*, which are the key species in freshwater food chains (Lampert 1987; Muller-Naverra et al. 2000). The first reports suggested that cyanopeptolins were antagonistic to mammalian proteases (Weckesser et al. 1996) but one of them called cyanopeptolin SS, apart from being cytotoxic, was also reported to be toxic to *Daphnia* (Jakobi et al. 1996). The other peptides like Oscillapeptin J from *Planktothrix rubescens* (Blom et al. 2003) and microviridin J from *Microcystis* (Rohrlack et al. 2003) were also recognized as being toxic to zooplankton and inhibitory to mammal proteases. Microviridin J was found to interrupt the intermolting cycle in *Daphnia* by inhibiting its digestive proteases (Rohrlack et al. 2004). Presence of trypsin and chymotrypsin in guts of *Daphnia* (Agrawal et al. 2005b) and a direct evidence for presence of cysteine protease along with trypsin and chymotrypsin in *Moina* and their inhibition by *Microcystis* extracts have provided ample evidence towards phytoplankton-grazer interaction at level of digestive and developmental abatement (Agrawal et al. 2001, 2005a).

8.2.3 *Bioassays and Chemical Detection of Cyanobacterial Peptides*

Thin layer chromatography (TLC) was applied as one of the earliest methods involving derivatization of the peptides to colored or fluorescent products for detection (Pelander et al. 2000). In some cases TLC and gas chromatography (GC)/MS were used (Tsuji et al. 2001). The most widely acclaimed spectroscopic method for peptide analysis is liquid chromatography coupled to MS. In MS, molecules are ionized and resolved on basis of mass and charge (Graves and Haystead 2002). Thus MS offers the advantage of monitoring only those specific masses that are identifiable from databases. Different MS methods have been deployed in combination with the liquid chromatography system e.g. fast atom bombardment (FAB MS), electrospray ionization (ESI MS) and atomic pressure ionization (API MS) (Harada et al. 2004). LC-ESI MS is a technique used for the simultaneous detection and quantification of peptides and the characterization of breakdown products. A major constrain in quantitative analysis of peptides is the lack of standards (Rapala and Lahti 2002). Tandem mass spectrometry (MS/MS) identifies unknown compounds more accurately, where specific peptides are fragmented into individual identifiable amino acids (Graves and Haystead 2002). MALDI-TOF MS enables the identification of peptides present in single cells or in colonies of cyanobacteria (Fastner et al. 2001). This technique involves isolation of cyanobacterial sample in a matrix of high laser light absorbing small organic molecules such as α-cyano-4-hydroxy-cinnamic acid (CHCA) etc., followed by ionization of the analyte by nitrogen laser beam pulse of short duration. Time-of-flight mass analyzer measures the mass to charge ratio (m/z) of ions and the measurements obtained in delayed extraction mode allow determination of monoisotopic mass values. The post source decay resulting from the fragmentation of molecular ions is routinely employed to obtain sequence information of the peptides (Mikalsen et al. 2003). The main advantage of this technique is that it enables complete analysis of small samples without any purification. Surface-enhanced laser desorption ionization mass spectrometry (SELDI-TOF MS) is a chip based variant of MALDI-TOF MS which involves retention of the target compound on a solid phase chromatographic surface (chip), which are subsequently ionized by laser and detected by TOF MS. The technique enables the user to analyze peptides in biological materials like, blood, serum and tissues with a minimum sample preparation (Yuan and Carmichael 2004).

Lethality bioassay using live animals and zooplankton *Artemia salina*, and a variety of animal cell lines such HeLa are the simplest methods and offer rapid screening of bioactive cyanopeptides (Rapala and Lahti 2002). For peptide bioassays most of the methods are in-house developed and can not be universalized in all laboratories or with all samples and test organisms. Unfortunately, unlike microcystins, immunological detection methods (ELISA etc.) are also not optimized for other peptides.

8.3 Analysis

8.3.1 Methods

Axenic culture of *Microcystis aeruginosa* PCC7806, containing trypsin and chymotrypsin inhibitory peptides used as reference strain was provided as lyophilized powder through courtesy Professor J. Weckesser, Institute of Biology II, Albert-Ludwigs University, Freiburg, Germany.

Periodically cyanobacterial blooms aggregated around shores due to wind flow especially in early morning were collected by skimming across the water surface using 25 μm plankton nets. The bloom material was then transferred to 1.5 L wide mouth plastic bottles. Precaution was taken not to collect old and disintegrated cells and other phytoplankton. Samples were brought to laboratory at 10°C. The visible contaminating particles were removed using Pasteur pipette and water samples were allowed to stand for at least 6 h in order to allow the buoyant colonies to float, and were collected using Pasteur pipette. The colonies were washed with distilled water before identification and extraction. Morphological identification of cyanobacteria present in the concentrated bloom samples was done at 40× (objective) and cell inclusions were detected at 100× (Olympus, Optical Corporation Ltd, Japan). The cyanobacterial colonies were microphotographed using a Nikon SLR camera. As far as possible, identification of *Microcystis* to species level was done using the morphological identification keys as given by Via-Ordorika et al. (2004) and an approximation of *Microcystis* dominance was enumerated using hemocytometer.

The collected blooms of dominant *Microcystis* spp. were centrifuged for 10 min at 5,000 × *g* at 25°C to remove excess water, and the bloom material that floated on the surface of centrifuge tubes were collected using Pasteur pipette and lyophilized using a freeze-drier at −20°C under vacuum until the material completely dried as powder, which were then transferred into airtight glass storage bottles and kept in a refrigerator until further use. For protease-inhibition tests, 1 g dried mass of the blooms was extracted with 100 ml of 75% methanol in three cycles, in the first cycle, 50 ml of 75% methanol was added to the bloom material and stirred over a magnetic stirrer at 150 rpm for 1 h and then centrifuged at 5,000 × *g* for 10 min at 25°C, and the pellet obtained upon centrifugation was again stirred with addition of 25 ml of 75% methanol for 1 h and then centrifuged. This step was repeated once more and the supernatants were pooled and filtered under vacuum using a GF/C filter paper (4.25 cm diameter; Millipore) and dried under vacuum. The residue of 1 g freeze-dried bloom material was dissolved in ~0.2 ml methanol and diluted with water to 2 ml. As required it was appropriately diluted in 10% methanol. *M. aeruginosa* PCC7806 500 mg dry cells were also extracted as above except that methanol extraction was performed only twice.

For the screening of protease-inhibitors of the natural and laboratory grown cyanobacteria a method was developed using trypsin and chymotrypsin as animal

proteases and casein, gelatin and skimmed milk as natural substrates that were hydrolyzed. For the preparation of gelatin and skimmed milk plates, 1 g of each was added separately to 100 ml of 100 mM Tris-HCl buffer (pH 8.0) containing 1 g agar, and this mixture was autoclaved and the molten agar-gelatin/skimmed milk was then poured into sterile Petri dishes under aseptic conditions and allowed to solidify. For preparation of casein plates, 1 g of Hammerstein's casein was mixed with 50 ml autoclaved Tris-HCl buffer. Casein was dissolved by stirring the mix in a water bath maintained at 80°C until a milky white solution without solid casein residue was obtained. To this solution at 80°C 50 ml of previously autoclaved 1% (w/v) molten agar in Tris-HCl buffer added, stirred to mix and then plated on sterile Petri dishes.

Filter paper discs (5 mm) were punched from Whatman filter paper #1. To each disc 10 µl of trypsin or chymotrypsin solution from a stock of 10 mg ml^{-1} prepared by dissolving, trypsin (bovine pancreas) in 0.001 M HCl containing 0.02 M $CaCl_2$ (pH 3.1) and chymotrypsin (TYPE IV, bovine pancreas) in 100 mM Tris-HCl buffer (pH 7.8), was applied using a micropipette. For an analysis of the ability of the cyanobacterial sample to antagonize trypsin/chymotrypsin action, these enzyme discs were loaded with 50 µl the methanolic extracts in decreasing concentration of dry weight of original bloom material (625–20 µg) in 50 µl. The same criterion was applied for *M. aeruginosa* PCC7806 cells. The discs were allowed to dry at room temperature under sterile conditions, carefully placed with sterile forceps on the substrate plates and incubated at 37°C for 6–8 h. Discs impregnated with enzyme and 10% methanol served as controls. After incubation in control sets clearing zones due to hydrolysis of natural substrates were clearly visible. The reaction was terminated by addition of 5 ml each of 5% TCA in case of casein and skimmed milk and 2% $HgCl_2$ in case of gelatin. After 5 min appearance of clearing zones in control and in the experimental sets was recorded to extrapolate a minimum inhibitory concentration in terms of dry weight of original cyanobacterial sample.

MALDI-TOF MS method was performed to identify the variants and congeners of protease-inhibitory peptides in the bloom material which showed positive results in above assays. The procedure adapted for analysis was: 100 µg of freeze dried bloom material was dissolved in 10 µl of water/ethanol/acetonitrile (1:1:1 v/v). All samples were analyzed in a saturated α-cyano-4-hydroxycinnamic acid matrix, solubilized in 50% acetonitrile and 0.3% trifluoroacetic acid (TFA). A mixture of 1 ml matrix and 1 ml sample was prepared directly on the plate. The samples were analyzed using a MALDI VOYAGER DEPRO time-of-flight mass spectrometer (Perspective Biosystems, USA) fitted with a nitrogen laser, giving a 337 nm output. The ions were accelerated with a voltage of 20 kV. Measurements were performed in a delayed extraction mode, allowing the determination of mono-isotopic mass values. A low mass gate of 400 improved the measurement by filtering out the most intensive matrix ions. The mass spectrometer was used in the positive ion detection and reflector mode. Peptide spectrum thus obtained was matched with the library of known cyanobacterial peptides.

8.3.2 Interpretation of Results

The latest manual of identification of *Microcystis* spp. is published by Via-Ordorika et al. (2004). Basically the characteristics are similar to those described by Desikachary (1959) except that some new species are included (Fig. 8.2). These colonies were either spherical or had irregular arrangement, mostly free floating with presence of hyaline thick mucilage sheath and with obligatory presence of gas vesicles. The characterization up to species level was carried out for which colony appearance and size, shape and presence of mucilage sheath and gas vesicles were taken as criteria. Colonies that were irregular in outline with diffused mucilage, distinct holes and a net like appearance were assigned to *M. aeruginosa*. The colony size ranged between 200 and 400 μm. The colonies of *M. viridis* exhibited typical packet like shapes, irregularly agglomerated with narrow hyaline mucilage. They contributed to the major phytoplankton population of the blooms which formed a scattered thin film over the water surface. Colony size ranged from 50 to 300 μm. *M. botrys* colonies were spherical or often joined irregularly without holes, mucilage distinct and thick, usually appeared as minor morphospecies with colony size in the range of 50–150 μm. *M. panniformis* colonies were comparatively large with colony size from 150 to 350 μm, mostly spherical, irregular or flattened with small indistinct holes with an indistinguishable diffused mucilage layer. *M. icthyoblabe* colonies were large with a size range from 200 to 400 μm, mostly irregular and compact colonies without holes and indistinguishable mucilage layer. They also constituted the minor phytoplankton populations.

As shown in Fig. 8.3, different dilutions of the methanolic extracts from a representative bloom material clearly revealed that the inhibition of proteolytic activity was concentration dependent, because with decrease in concentration of the methanolic extracts there was an increase in diameter of the clearing zones. In Petri dishes, 156 μg bloom dry matter was found to be minimum amount for trypsin and 312 μg for chymotrypsin inhibition. In comparison, in a similar concentration dependent inhibition assays using *M. aeruginosa* PCC7806, the minimum amounts inhibitory for both the enzymes was equivalent to 40 μg original dry wt of cells. This suggests that on dry weight basis the lab culture produced more inhibitory substances than the bloom. Further, the results also established that trypsin and chymotrypsin hydrolyzed gelatin, casein and skimmed milk equally well and the inhibition of proteolysis by cyanobacterial methanolic did not discriminate the natural substrates.

In order to analyze the types of protease-inhibitory compounds, bloom samples displaying positive inhibitory tests on disc assays were analyzed by MALDI-TOF MS. Based on the mass spectrum of known compounds each peptide was identified by using a library of having similar mass. All the signals in the spectra matched with the available database for aeruginosins, cyanopeptolins and microviridins (Czarnecki et al. 2006; Welker and von Döhren 2006). Further, as shown in the Fig. 8.4, the representative bloom samples (#1-5) showed the presence of two variants of microviridins at *m/z* 1689.59 Da and 1705.57 Da, aeruginosin at *m/z* 655.37 Da, two cyanopeptolins identified as cyanopeptolin-S at *m/z* 926.42 Da and cyanopeptolin

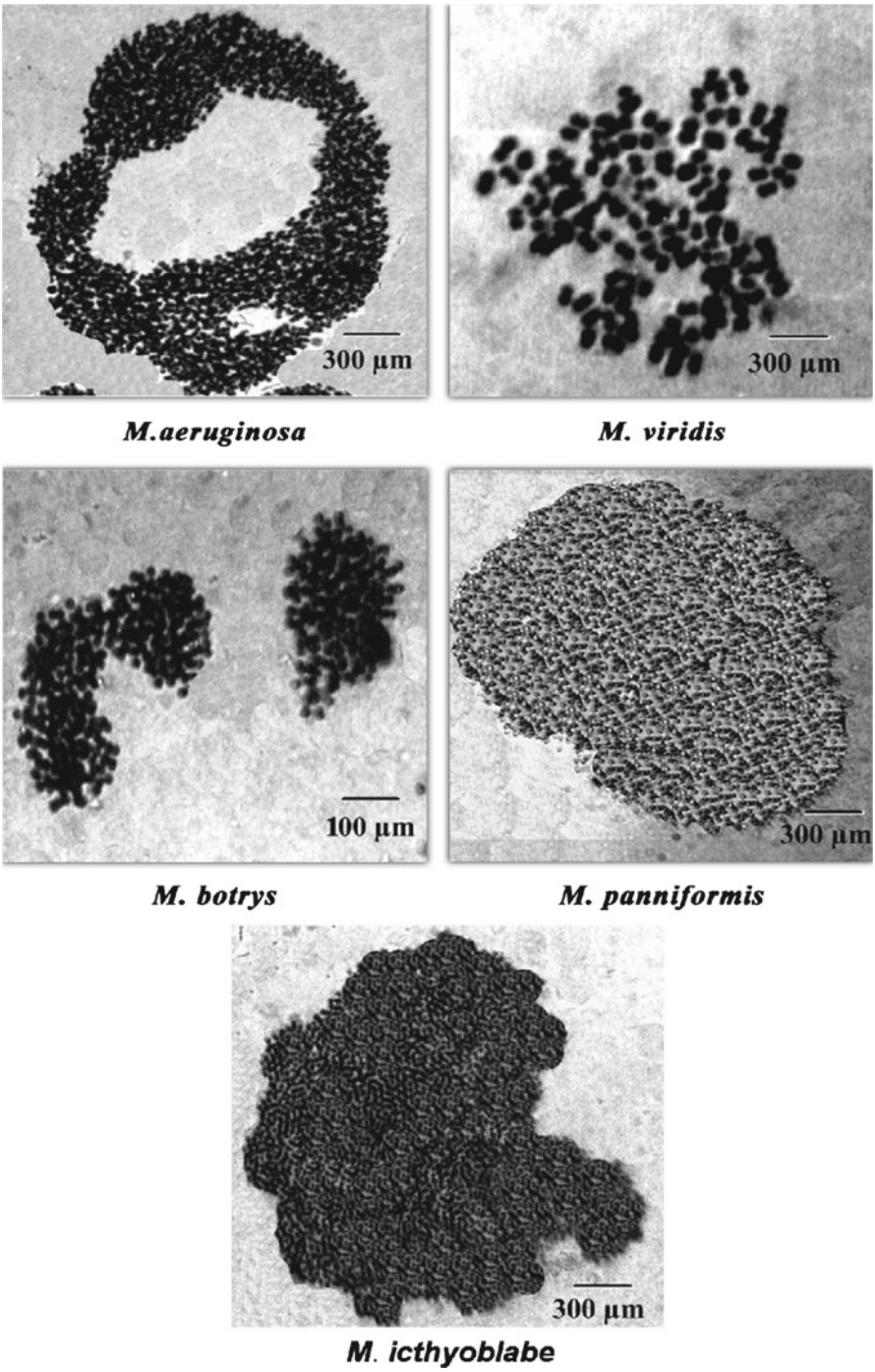

Fig. 8.2 Morphological features of *Microcystis* spp. colonies in natural samples

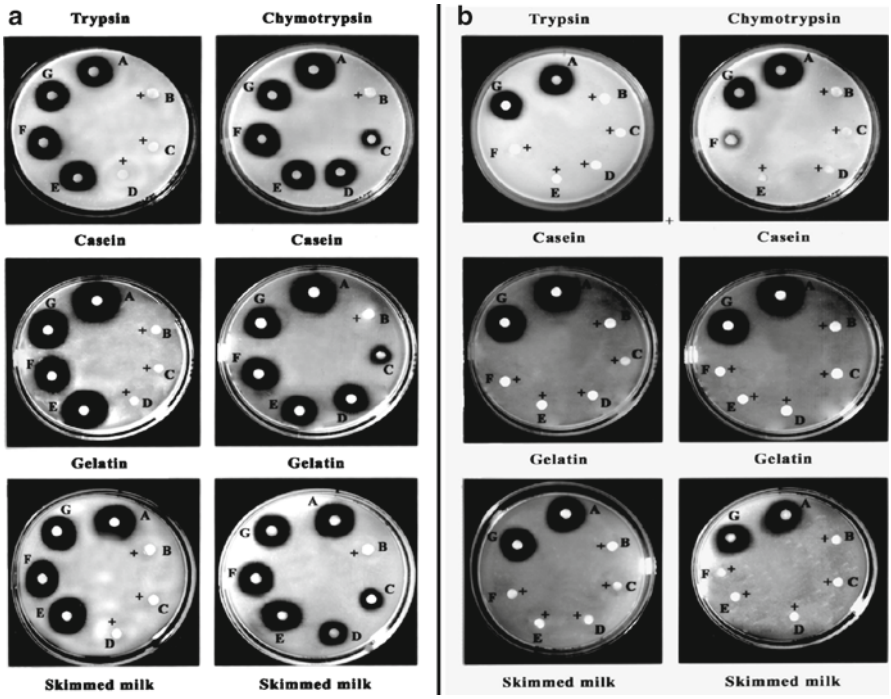

Fig. 8.3 Effect of addition of different amounts of methanolic extracts of (**a**) bloom sample and (**b**) *M. aeruginosa* PCC7806 on clearing zone formation. A -Controls with 10% methanol, and B-G –625, 312, 156, 80, 40 and 20 μg dry matter, (+) Inhibition

958 at *m/*z 959.51 Da, and another cyanopeptolin 911 at *m/*z 912.55 Da. A majority of samples were also toxic as they also gave positive signals for microcystins.

8.4 Future Perspectives

Several hypotheses have been put forward on the physiological and ecological relevance of cyanopeptides with protease inhibitory properties. The allelopathic role of cyanobacterial peptides has been correlated mostly with the reduction of photosynthetic activity and growth rates of other planktonic autotrophs (Smith and Doan 1999), eventually leading to cyanobacterial dominance (von Elert and Jüttner 1997). More relevant interaction seems to be with the predators and grazers that co-habit with the natural phytoplankton population. The peptides *viz.* cyanopeptolins, aeruginosins, microviridins and microginins are known inhibitors of serine/threonine proteases not only of mammalian origin but also of crustacean zooplankton (Agrawal et al. 2001, 2005b; von Elert et al. 2004; Czarnecki et al. 2006). Recently, Schwarzenberger et al. (2010) detected nine digestive trypsin- and

chymotrypsin-like serine proteases in *Daphnia* gut whose expression at mRNA level were regulated by ingested *Microcystis* containing protease inhibitors. Interestingly, one category of protease inhibitor while inhibited corresponding proteases, also up-regulated compensatory alternate proteases of different category or produced insensitive isoforms of the same enzymes. Resistant grazers (with respect to digestive proteases) would efficiently graze on the toxic blooms and this can be an effective environmental management to combat this problem.

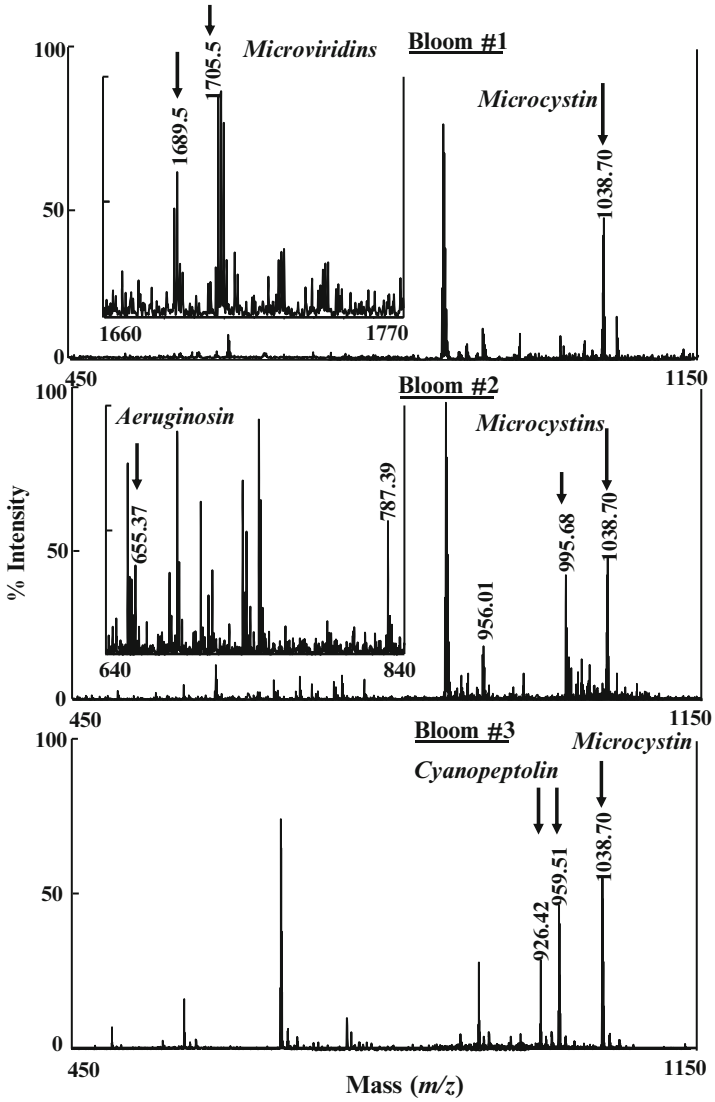

Fig. 8.4 Whole cell MALDI-TOF MS of representative blooms showing presence of cyanopeptides indicated by *arrow sign*

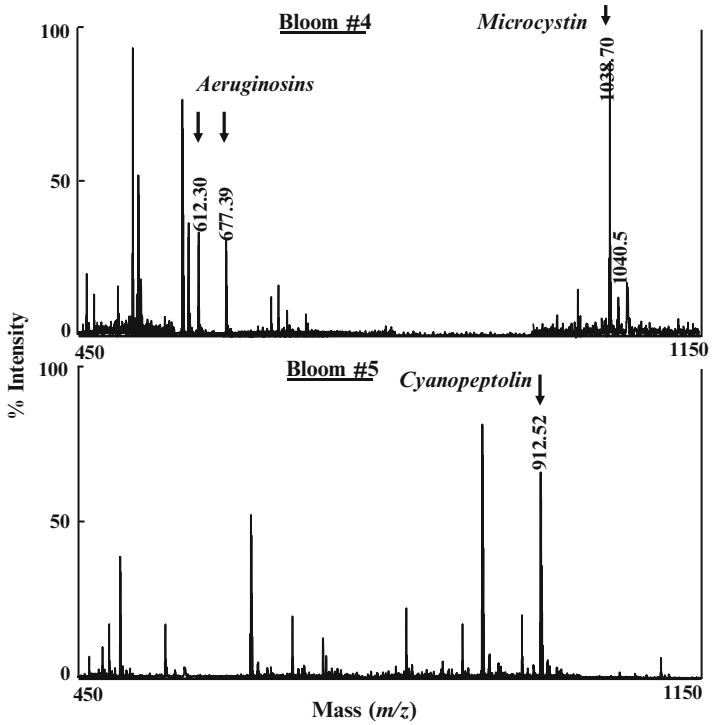

Fig. 8.4 (continued)

Apart from serine proteases, crustacean zooplankton, *Moina*, has been reported to produce cysteine proteases and interestingly there were also respective inhibitors found in *M. aeruginosa* PCC7806 (Agrawal et al. 2005a). This brings out a possibility of highly organized interaction of diverse protease inhibitors of a single species or even strain of cyanobacteria targeting wide range of zooplankton and surviving the dynamic invertebrate grazing community at large.

Another hypothesis relates cyanobacterial peptides to bacterial quorum sensing mechanism, which is supported by the fact that cyanobacteria evolved long before the animal kingdom and the natural selection did not minimize the pool of peptide structures to a few very efficient ones but, in fact, favoured the production of more variants (Kaebernick et al. 2000). Recently, a novel class of proteases were discovered in *M. aeruginosa* PCC7806 (Ghosh et al. 2008a), which were resistant to the indigenous protease inhibitors but were sensitive to the inhibitors produced from another axenic strain of *Microcystis*. Possibly, bloom formation is a feature in which cells with identical sets of protease inhibitors and corresponding insensitive proteases aggregate, whereas the others are rejected. This may enable them to collectively tackle the ever grazing problem of diverse aquatic fauna. There could be some yet to be defined other physiological advantage of the colonized cells in a given ecosystem. Additional work can exercise on factors that can dismantle the

aggregation of the identical chemotypes, and this may avert colonization and proliferation of toxic blooms.

Information on genetic make-up of the peptide synthesizing genes has started to gather paving way to explain the genotypic variations in different chemotypes. This adds to the understanding on geographical and seasonal diversity of cyanobacterial genotypes translated in chemotypes. So far as microcystins are concerned we have found prevalence of Arg-Arg variant prevalent in tropical waters, whereas more Leu-Leu variants were recorded from the colder temperate waters (Ghosh et al. 2008b). These chemotypic divergences are most likely governed by different microcystin gene sets. *Anabaena* 90 was found to contain at least three anabaenopeptilide synthesis genes having seven modules, giving possibility of large variants of this protease inhibitor governed by specific gene sets (Rouhiainen 2004). Moreover, Ziemert et al. (2010) have shown that microviridin precursor gene (*mdnA*) can give rise to 15 new variants of the peptide than what is already known. Natural diversity of this gene cluster can give rise to high versatility among microviridins with possibly varied functions. Genotyping and chemotyping of *Microcystis* blooms have gone much beyond the microbiological features and this may help elucidate the selection pressures that had led to evolution of diverse class of non-toxic peptides. These are believed to be the future research to address the basic issues of interaction between primary produces and consumers, and also to apply this knowledge to regulate the toxic and otherwise nuisance microalgal and cyanobacterial blooms in ever growing eutrophic water systems world-over.

8.5 Conclusions

Natural populations of cyanobacteria are mixture of chemotypes that produce versatile combination of individual peptides and peptide classes. The non-toxic peptides and other similar metabolites have immense ecological advantages for producing cyanobacteria to exert anti-grazing pressure, to impart allelopathic effects on competing organisms, to enable inter-species communication (quorum sensing), and to provide chemotypic signature to the strains based on their specific peptide synthesizing gene sets (genotype). At the moment no correlation could be established between morphospecies and nature of peptide cocktail that the organism produces. Even in one bloom several chemotypes may combine or it may be comprised of predominantly one chemotype. Moreover, chemotype diversity has no relationship with the physico-chemical parameter of the water body or with the age of the bloom, making it very difficult to recognize the chemotypes unless bioassays and the chemical determination techniques are suitably applied directly in bloom samples. Here, to screen the blooms a novel bioassay technique is made available to the researchers where there is no need of any sophistication or substantial cost. As most of the inhibitors are cyclic-, linear- or depsipeptides in composition and because a state-of-the-art database on the possible variants, congeners and derivatives are available (http://www.

oeaw.ac.at/limno/files/pdf/FINAL%20report%20PEPCY.PDF), MALDI-TOF MS technique can be used directly on natural samples to semi-quantitatively determine the composition of individual congeners and variants of the peptides. Collectively, the two methods can decipher the chemotype of individual *Microcystis* species or the cyanobacterial bloom of a given water body.

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Chapter 9
The Oxalate-Carbonate Pathway: A Reliable Sink for Atmospheric CO_2 Through Calcium Carbonate Biomineralization in Ferralitic Tropical Soils

Michel Aragno and Eric Verrecchia

Abstract Calcium carbonate has a residence time in soils in the order of 10^{4-6} years. Therefore, sequestration of atmospheric carbon as $CaCO_3$ is almost irreversible, as compared to organic carbon. Calcium oxalate, a metabolite often accumulated in plants, is chemically highly stable. However, it is readily oxidized by oxalotrophic bacteria, with simultaneous alkalinization and biomineralization of calcium carbonate, which eventually accumulates in soil. In tropical countries, on mainly acidic, non calcareous soils, this represents a significant sink for atmospheric CO_2: at the present rate of increase, one full-grown Iroko tree may stabilize concentration of CO_2 in the air column above a 2,400 m^2 surface. This process may be applied in reforestation and agroforestry, as well as in conservation and sustainable development of tropical forests and soils.

Keywords Calcium oxalate • Calcium carbonate • Tropical soils • Biomineralization • Oxalotrophy • Carbon sink

9.1 Introduction

During the last century, the main gas responsible for the increase in the greenhouse effect and its consequent climatic changes, was atmospheric carbon dioxide, whose concentration increased by 35%. Among the measures that allow this increase to be

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limited are (i) a decrease in the use of fossil carbon, and (ii) CO_2 capture by plants through photosynthesis. However, biomass represents only a transitory, short-term carbon sink (residence time between 1 and 1,000 years), although it was the object of numerous studies during last 20 years.

Carbon transfer from atmospheric CO_2 to calcium carbonate (limestone) combining autotrophic capture and biomineralization reactions represents at much more efficient sink, since the residence time of limestone may be in the order of $10^{4–6}$ years (Retallack 1990). This would require a non-calcareous calcium source, together with the oxidation of organic material to CO_2 and alkalinisation. Such a process was first described on a theoretical basis to explain calcrete formation in semiarid conditions in Galilee, Israel (Verrecchia 1990). This was the first mention of calcium oxalate oxidation resulting in biomineralization of calcium carbonate. At the time, Fungi were thought to be the main organisms biomineralizing calcium oxalate (Verrecchia et al. 1993). Later, bacteria were suspected to be responsible of oxalate oxidation (Verrecchia and Dumont 1996), but the mechanism remained a "black box". Finally, the occurrence of this phenomenon was observed in a tropical forest ecosystem, i.e. in acidic tropical soils under calcium-oxalate forming trees (Braissant et al. 2002, 2004; Cailleau et al. 2004, 2005; Verrecchia et al. 2006), and the mechanism of oxalate oxidation by bacteria elucidated.

9.2 Calcium Oxalate in Plants

A number of plants, including trees, accumulate large amounts of calcium oxalate, a low-soluble salt. It accumulates in the vacuoles of idioblasts (specialized crystal-containing cells), mainly as the monohydrated salt whewellite. The reason for this accumulation is subject to controversy: either calcium ions are taken up by the plant to neutralize the otherwise strong oxalic acid, or oxalic acid is produced to maintain calcium concentration at a sub-toxic level (Franceschi and Nakata 2005). Otherwise, the presence of calcium oxalate crystals with their acute edges could protect the plant webs against grazing. Depending on the plant species and growth conditions, the calcium oxalate content may be high (3–85% $w/w_{dry\ matter}$; Franceschi and Nakata 2005). Whatever the calcium oxalate accumulating plant, this process results in a co-accumulation of both calcium and oxalate in a stable, crystalline form.

9.3 Calcium Carbonate Accumulation in Tropical Acidic Soils

Most soils in the intertropical forest of the Old and New worlds are acidic and do not contain carbonates. However, Cailleau et al. (2005) observed large accumulations of limestone in African ferralitic soils under certain tree species, particularly the Iroko tree, *Milicia excelsa (Moraceae)*, an overforested hardwood tree. In such

soils, the pH under the tree was distinctly alkaline, reaching values between 8 and 9, whereas the soil at a distance from the tree was acidic with pH values < 6. This species accumulates large amounts of Ca-oxalate in its webs. Therefore, it was postulated that calcium carbonate originated from the oxidation of calcium oxalate, since no distinct accumulations of calcium oxalate have ever been observed in the geological record. However, calcium oxalate is chemically and physically highly stable, with a very low solubility ($Kps = 4*10^{-9}$ M^2 in water at pH 7.0). The question then arose of putative microbiological processes accompanying plant litter degradation, which would allow weathering of oxalate crystals and complete oxidation of the organic anion to CO_2 and OH^-.

9.4 Oxalate Metabolism in Bacteria

Although only second to CO_2 as the most oxidized natural carbon compound, oxalate may be utilized both as energy and a carbon source by several bacteria i.e. the oxalotrophs. They include the anaerobic *Oxalobacter formigenes* and *Oxalophagus oxalicus*, as well as a number of aerobes belonging to different taxonomic groups, both Gram-positive and Gram-negative (Sahin 2003). Most aerobic, oxalotrophic bacteria are metabolically versatile organisms able to grow on a number of other substrates. They are widespread in nature, even in environments not subjected to a high oxalate input.

In an aqueous suspension of Ca-oxalate crystals, free oxalate concentration is 63 μM. Although low, this concentration is saturating for bacterial needs, since the half-rate uptake affinity is in the order of 1 μM. So, Ca-oxalate is readily dissolved in the presence of oxalotrophic, aerobic bacteria (Fig. 9.1) without requiring the secretion of acids or chelating compounds.

So far, the only catabolic, oxalate-oxidizing pathway described in oxalotrophic bacteria (Quayle 1961, 1963) consists of oxalate anions being taken up and transferred to coenzyme A by the enzyme formyl-CoA-transferase (FRC, Fig. 9.2). Then, oxalyl-CoA is decarboxylated to formyl-CoA by oxalyl-CoA decarboxylase (OXC, Fig. 9.2) with uptake of one proton. Finally, the transfer of CoA to a new oxalate anion liberates formate. Formate is secreted in anaerobes (Allison et al. 1985), whereas it is utilized as a low potential respiratory substrate in aerobes, through NAD-dependent formate-dehydrogenase (FDH). It is then oxidized to CO_2 (Fig. 9.2). In parallel, oxalate is assimilated through either one of two pathways, the serine pathway (Blackmore and Quayle 1970) or the glycerate pathway (Quayle et al. 1961). The overall equation of oxalate oxidation by aerobes is therefore:

$$\left(COO^-\right)_2 + ½\,O_2 + H_2O \rightarrow 2\,CO_2 + 2\,OH^-$$

combining carbon oxidation to CO_2 and alkalinisation.

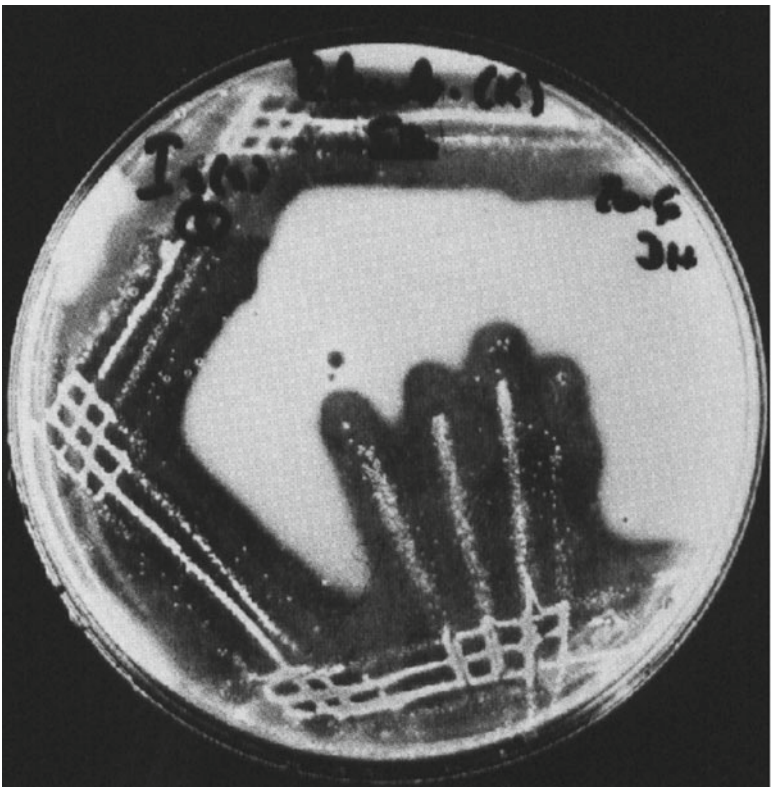

Fig. 9.1 Dissolution of Ca-oxalate precipitate by a strain of *Cupriavidus necator* on mineral agar + Ca-oxalate as sole C-source (From Tamer and Aragno 1980)

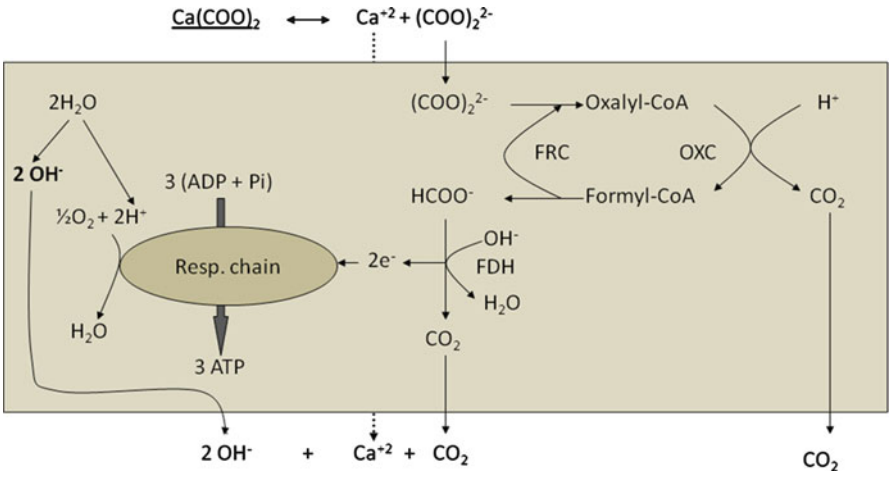

Fig. 9.2 Aerobic oxalate catabolism (e.g. in *Cupriavidus oxalaticus*) FRC formyl-CoA-transferase (EC 2.8.3.16), OXC oxalyl-CoA-decarboxylase (EC 4.1.1.8), *FDH* formate-dehydrogenase (EC 1.2.1.2)

9.5 The Oxalate-Carbonate Pathway

Calcium carbonate biomineralization is therefore possible in acidic tropical soils, combining:

- weathering of non calcium carbonate-bearing rocks, i.e. rocks with calcium-bearing minerals, such as plagioclases or apatite;
- transfer of Ca^{2+} ions to the trees;
- CO_2 fixation by photosynthesis, and biosynthesis of oxalate;
- formation of Ca-oxalate crystals in vacuoles of idioblasts;
- degradation of oxalate-containing plant webs during the littering process, through saprophytic fungi, and liberation of Ca-oxalate crystals;
- solubilisation of Ca-oxalate crystals through oxalate uptake by oxalotrophic, aerobic bacteria, and consecutive release of Ca^{2+} ions;
- catabolic oxidation of oxalate to CO_2, with concomitant release of hydroxide ions;
- precipitation of calcium carbonate at pH > 8.5, accompanied by the liberation of one CO_2 per oxalate molecule.

Therefore, the overall equations of Ca-carbonate formation from Ca-oxalate are :

$$Ca(COO)_2 \leftrightarrow Ca^{2+} + (COO)_2^{2-}$$

(Ca-oxalate dissolution – weathering)

$$(COO)_2^{2-} + \tfrac{1}{2}\, O_2 + H_2O \rightarrow\ 2\, CO_2 + \ 2\, OH^-$$

(Oxalate catabolism through aerobic respiration)

$$Ca^{2+} + \ CO_2 + \ 2\, OH^- \rightarrow\ \underline{CaCO_3} + H_2O$$

($CaCO_3$ precipitation)

$$Ca(COO)_2 + \tfrac{1}{2}\, O_2 \rightarrow\ \underline{CaCO_3} + \ CO_2 \uparrow$$

9.6 Oxalotrophic Bacteria in Biomineralizing Trees' Ecosystems

Preliminary studies have shown a diversity of aerobic, oxalotrophic bacteria in decaying wood, litter and soils below biomineralizing trees. Interestingly, whereas the most studied oxalotrophs were Gram-negative bacteria like *Cupriavidus necator* (=*Ralstonia eutropha)* and *Methylobacterium extorquens,* most isolates from such soils were Gram-positive Actinobacteria related to the genus *Streptomyces* (Braissant et al. 2004). Even if most oxalotrophic bacteria are otherwise versatile organisms living in environments devoid of oxalate, the relative abundance of the FRC gene – a gene coding for formyl-CoA transferase and characteristic of oxalate catabolism by

oxalotrophs – was shown to be significantly higher in soils under a biomineralizing tree than in a distant soil (Khammar et al. 2009; M. Clerc and D. Bravo, personal communication).

9.7 Importance of Fungi

Fungi may participate to the biomineralization process in different ways. First, a number of saprophytic and mycorrhizal Eumycetes, particularly wood-decaying ones, produce calcium oxalate crystals, commonly as dihydrated weddelite (Fig. 9.3, Verrecchia et al. 1993). In some cases, it was shown that the same fungi were also able to dissolve and oxidize calcium oxalate (Guggiari et al. 2010). Extracellular oxalate oxidase (EC 1.2.3.4) could be implicated in this phenomenon. This enzyme was supposed to intervene in lignin degradation by ligninolytic fungi through the production of hydrogen peroxide, a co-substrate of manganese peroxidase (Kuan and Tien 1993; Aguilar et al. 1999). So, the temporary accumulation of calcium oxalate by these fungi could belong to their ligninolytic strategy.

Ligninolytic and cellulolytic fungi also participate in the decay of plant webs containing Ca-oxalate crystals, exposing them to further degradation by bacteria (Fig. 9.4, Braissant et al. 2004).

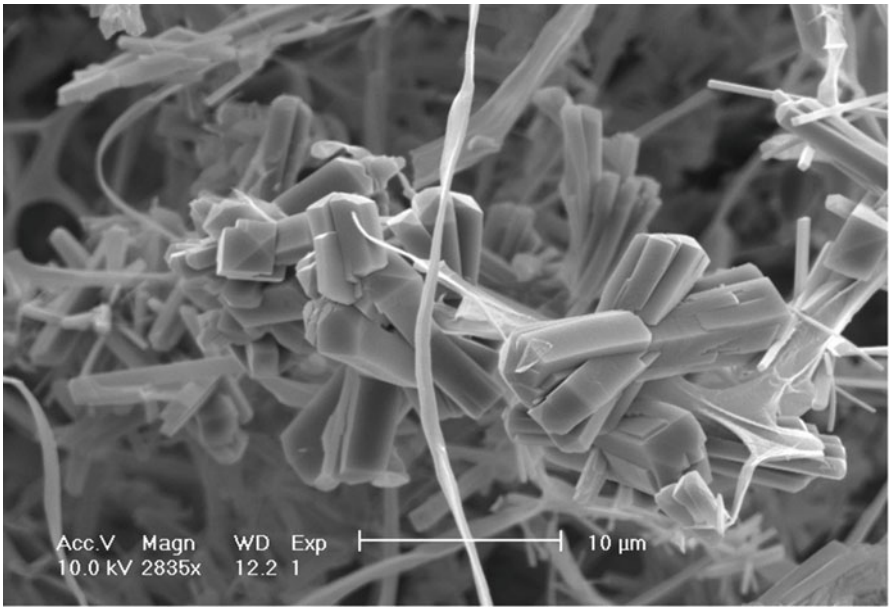

Fig. 9.3 SEM photo of Ca-oxalate crystals (dihydrate, weddellite) on fungal hyphae

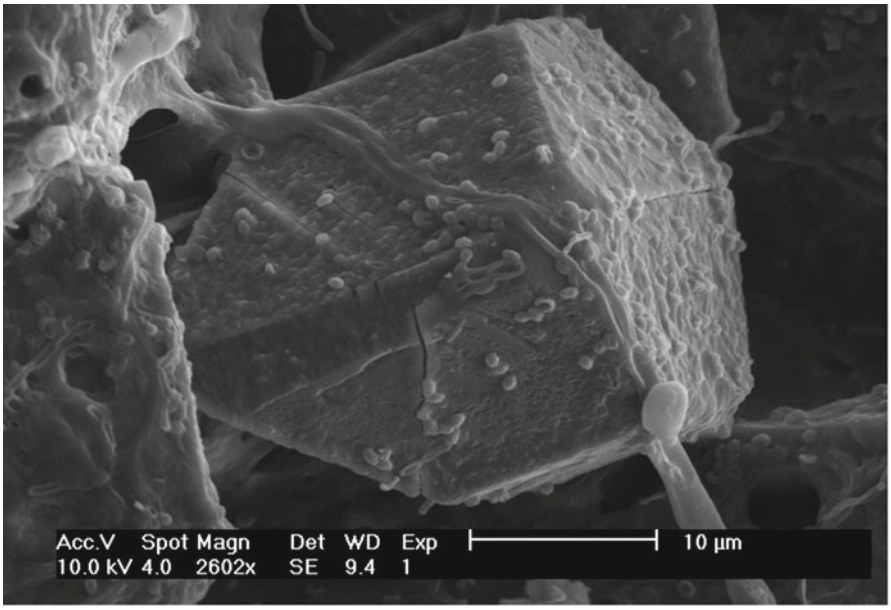

Fig. 9.4 SEM photo of Ca-oxalate crystal with adhering mycelium and bacterial cells

In pure cultures of oxalotrophic bacteria in Petri dishes, it was shown that the bacterial oxidation of Ca-oxalate was accompanied by alkalinisation and precipitation of $CaCO_3$ (Braissant et al. 2002). However, when tested in microcosms containing an acidic, ferralitic soil as the substrate, a distinct alkalinisation was only observed in soils amended with Ca-oxalate and grass straw, when fungal strains were added simultaneously to strains of oxalotrophic bacteria (G. Martin and M. Guggiari, pers. comm.). The implication of fungi in these assays has so far not been explained.

Otherwise, fungi, either saprophytic or mycorrhizal, may be agents of the weathering of calcium-containing minerals and of calcium translocation to the tree roots. Therefore, the importance of the fungal component in the oxalate-carbonate pathway has to be studied in depth.

9.8 Quantitative Estimation of the Biomineralizing Activity

From studies on Iroko trees in Ivory Coast (Braissant et al. 2004), calculations using titrations in soil and tree webs, showed that a full-grown 80 year-old tree traps 1,500 kg C as $CaCO_3$. At the present rate of atmospheric CO_2 increase (2 ppm/year, that is, 1 mg/m^3.yr C at 100 kPa), one such tree would stabilize the CO_2 concentration in 18,750,000 m^3 air at 100 kPa, that is, in the whole air column above a ca 2,400 m^2 surface.

9.9 Extent of the Phenomenon

By no means is the operation of the oxalate-carbonate pathway limited to the Iroko tree species. A systematic survey of its occurrence under different tree species, in different regions of the world and under different climatic conditions, has still to be performed. However, after a limited number of investigations, several other plant species from different families were shown to induce this phenomenon:

In Africa (Burkina Faso, K. Ferro, pers. com.):
Bombax costatum, Malvaceae
Afzelia africana, Fabales-Caesalpinaceae

In Bolivia (Alto Beni, Amazona, M. Mota, pers. com.):
Pentaplaris davidsmithii, Tiliaceae
Ceiba speciosa, Malvaceae
Terminalia amazonica, Combretaceae
Myroxolon balsamum, Fabales-Caesalpinaceae

In India (Madhya Pradesh and Uttarakhand, S. Gupta and A.K.Sharma, pers. com.):
Terminalia bellirica, Combretaceae
Aegle marmelos, Rutaceae
Soymida febrifuga, Meliaceae

Therefore, this phenomenon could be much more widespread than initially suspected. Even if no quantitative estimation of its global importance is yet possible, it could be at least a partial explanation to the "lack of carbon" in the carbon cycle of tropical forest, as pointed out by Gifford (1994).

9.10 Prospects for Applications

Even if the precise conditions (climate, soil, landscape characteristics, tree species, time span) for obtaining an optimal biomineralizing activity are still to be investigated, the existing estimations are encouraging enough to look forward to potential applications:

1. The observed widespread occurrence of this phenomenon in tropical forests gives a new importance to their conservation, as an efficient long-term CO_2 trapping system.
2. Encouraging the planting of biomineralizing trees in sustainable reforestation could be done at the governmental level by mobilizing carbon incentive credits.
3. The use of biomineralizing trees in three-level agroforestry practices could combine the benefits of the CO_2 sink with the delay of crops and fruit maturation and the improvement of soil fertility through neutralization and calcium carbonate accumulation.

9.11 Conclusions

Although most research performed so far on biological atmospheric CO_2 capture deals with the accumulation of organic carbon, this latter has a residence time much shorter than calcium carbonate in soils. Therefore, $CaCO_3$ biomineralization represents a much longer-term sink for atmospheric carbon. This phenomenon may explain at least in part the "missing carbon" in the global cycles (Beeby and Brennan 1997; Gifford 1994; Gobat et al. 2004, 2010). Since it appears to be widespread in the acidic soils of the whole tropical belt, it gives the tropical forest a supplementary value as an efficient CO_2 sink. Moreover, application of such biomineralizing trees in tropical agroforestry might improve soil fertility through pH neutralization. Together with reforestation using such trees, such initiatives could be supported by carbon incentive credits.

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Chapter 10
Bacterial Degradation of Aromatic Xenobiotic Compounds: An Overview on Metabolic Pathways and Molecular Approaches

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Abstract Environmental contamination due to xenobiotic compounds is mainly due to their large-scale manufacturing, processing and handling. Owing to long-term persistence, and acute toxic and teratogenic effects of these compounds, remediation of contaminated environment is necessary. Due to its capability to degrade toxic xenobiotic compounds in a safe and cost-effective manner, interest in bioremediation using microorganisms, particularly bacteria and fungi, has increased in the past two decades. In order to survive and grow in toxic environments, bacteria have evolved a complex range of mechanisms at the cellular and molecular levels, including catabolic enzymes, membranes, protein synthesis machinery, responsible genes etc. However, there are several factors which may limit the biodegradation of xenobiotic compounds. For the development of successful and improved bioremediation processes, understanding of the biochemical and molecular aspects of xenobiotics biodegradation is required. The present chapter aims to provide an overview of the metabolic pathways and genetic adaptation in bacteria for degradation of aromatic xenobiotics. Also, recent advances made based on these aspects to overcome certain limitations in bacterial aromatics metabolism are presented.

Keywords Xenobiotic compounds • Biodegradation • Catabolic pathways • Genetic adaptation • Aromatic hydrocarbons • Bacterial metabolism

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10.1 Introduction

Industrial activity over the past century has resulted in the release of enormous quantities of xenobiotic compounds into the ecosphere ("xenobiotic" in a broad sense, as defined by Leisinger (1983), "guest" chemicals not natural to the environment). Xenobiotics from several industries like pharmaceuticals, paper and pulp, petroleum refinery, dyes, pesticide are highly recalcitrant and bear serious implications as environmental pollutants. Microbial populations have demonstrated considerable adaptability to a wide range of such recalcitrant toxic xenobiotics, resulting in the development of a microbial consortium capable of degrading many of the toxic residues. The ability to degrade a variety of environmental pollutants including halogenated aromatic compounds and polycyclic aromatic hydrocarbons has been demonstrated by both bacteria and fungi. The metabolic pathways followed by these microorganisms are specific for a given target pollutant, which may produce in addition to biomass, transient or stable metabolites or, in the case of mineralization, inorganic end products. Although several factors may limit biodegradation of xenobiotics, incomplete transformation of more recalcitrant chemicals by microbes is often due to the lack of degradative pathway/enzymes for carrying out mineralization of these xenobiotics. The key enzymes involved in many of the early stages in aerobic bacterial metabolism of aromatic xenobiotic compounds are mono- and/or dioxygenases. Degradation of many of these compounds may also take place under reducing or denitrifying conditions. For example, while chlorinated aromatic compounds usually undergo reductive chlorination reactions, nitroaromatic compounds are subjected to reductive denitration reactions for their degradation. There is no ample evidence that microbes which do not the have the requisite metabolic pathways for degrading certain novel xenobiotics evolve with time in such a manner as to be capable of developing suitable catabolic systems. Such a unique adaptation of microbial system depends on the diversity of the bacterial genomes, and Arber (2000) summarizes three qualitatively different attributes which contribute to the overall production of genetic variants: (1) small local changes, such as in the nucleotide sequence of the genome, (2) intragenomic reshuffling of segments of genomic sequences, and (3) the acquisition of DNA sequences from another organism. The adaptation of microorganisms to environmental conditions is driven by the production of genetic variants and subsequent selection of robust strain(s).

Extensive research work carried out over the last few years have suggested that phylogenetically unrelated bacterial strains isolated from geographically distinct area, reveal considerable degree of commonality in the metabolic pathways and enzymes involved in the degradation of xenobiotics. Such similarities in the biochemical features can be probably attributed to the similarity in the levels of gene organizations and nucleotide sequences. In this context, it is construed that intergenomic gene acquisition by horizontal (lateral) gene transfer, may play a major role. With regard to xenobiotics degradation, transferable plasmids have assumed importance, as growing evidence has suggested the presence of xenobiotics-degradative mobile genes on such plasmids (Nojiri et al. 2004). Catabolic plasmids are commonly rather large (>50 kb),

and genetic analyses have revealed that such plasmids contain complete sets of catabolic genes for the conversion of xenobiotic aromatics to biotic compounds. Besides the acquisition of entire catabolic pathways, recombination events may also lead to the creation of mosaics of catabolic operons which can give rise to totally novel catabolic routes.

The present chapter aims to provide an overview on the metabolic pathways and the role of catabolic plasmids and genes involved in the degradation of aromatic xenobiotics by bacterial species since knowledge of these aspects can provide us with necessary information to test, design and engineer suitable biocatalysts with desired capabilities for achieving successful bioremediation of contaminated environment. Also, some novel strategies based on these aspects to overcome certain limitations in bacterial aromatics metabolism are discussed in a subsequent section.

10.2 Xenobiotics Biodegradation Mechanisms and Pathway

Compounds containing the benzene ring constitute the second largest group of naturally occurring substances (after sugar residues). Aromatic compounds display resistance to environmental chemical degradation, owing to the inherent thermodynamic stability of the benzene ring. Hence, such aromatic compounds are believed to be hazardous to the biosphere. However, microorganisms possess a wide range of catabolic biodegradation pathways and, thus, use these hazardous xenobiotics as the sole source of carbon and energy. In general, the microorganisms degrade xenobiotics either aerobically or anaerobically and in rare cases a combination of both. A representative aerobic biodegradation pathway exhibited by microorganisms is depicted in Fig. 10.1. In the case of aerobic biodegradation, the original substrate undergoes mostly oxygenation reactions and is converted to dihydroaromatic derivatives such as catechol, protocatechuate, gentisate, homoprotocatechuate, homogentisate, hydroquinone and hydroxyquinone. The key enzymes catalyzing the reactions are monooxygenases and/or hydroxylating dioxygenases. These intermediates may act as substrates for enzymes that cleave the aromatic ring in reactions involving molecular oxygen. The aromatic ring may be opened by cleavage of the bond between two neighbouring carbon atoms carrying hydroxyls. In this case, the process is termed *ortho* – cleavage, and the enzymes involved are intradiol dioxygenases. On the other hand, if other bonds taking a meta-position are cleaved in order to open the aromatic ring, the term used is meta-cleavage with the enzymes extradiol dioxygenases involved in the process. Central metabolic pathways involve series of reactions that culminate in the formation of intermediates of the Krebs cycle.

Anaerobic catabolism of aromatic compounds involving peripheral metabolic pathways most often lead to the formation of benzoyl-CoA and is subsequently acted upon by a specific multiple-subunit ATP-dependent reductase. In certain microorganisms, mixed aerobic–anaerobic metabolic pathways have been characterized,

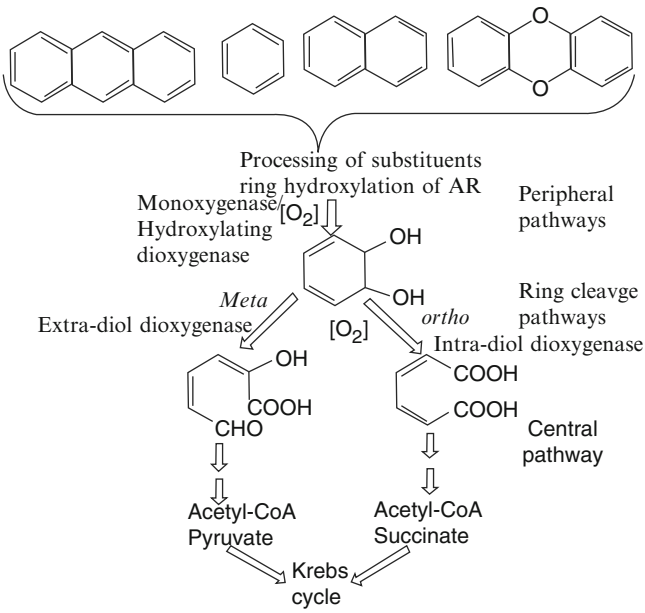

Fig. 10.1 Schematic representation of the principal metabolic pathways involved in aerobic biodegradation of aromatic compounds by microorganisms (Khomenkov et al. 2008)

such as the pathway of phenyl-acetate utilization, which involves the formation of CoA derivatives, a hallmark of anaerobic bioremediation, and aromatic ring oxygenation, which is typical of aerobic biodegradation (Luego et al. 2001).

10.2.1 Simple Aromatic Hydrocarbons

Generally, the aerobic degradation of simple aromatic compounds follows different metabolic pathways based on the enzyme system present in the microorganisms. The biodegradation of phenol, for example, is initiated by the formation of catechol, which later undergoes ring cleavage either via meta-fission (Hermann et al. 1995) or ortho-fission (Nilotpala and Ingle 2003) to intermediates of the central metabolism. On the other hand, five different biochemical pathways have been characterized for BTEX (Benzene, toluene, ethylene, xylene) degradation by aerobic bacteria. Toluene degradation follows five different schemes depending upon the bacteria involved. For example, while *Pseudomonas putida* mt-2 has been shown to transform the compound to catechol via benzyl alcohol formation, *Pseudomonas mendocina* KR1 forms protocatechuate via *p*-cresol formation (Gulensory and Alvarez 1999). Further breakdown of the intermediates catechol and protocatechuate are carried out by *meta* ring and *ortho* ring fission mechanisms, respectively. Bacteria like *P. Putida* F1, *Burkholderia cepacia* G4 and *B. pickettii* PK01 degrade toluene via a different intermediate, methyl catechol, followed by meta ring-fission of the

compound (Gulensory and Alvarez 1999). Based on the first step of formation of the catechols, the pathways can be classified into ring hydroxylation pathway and alkyl substituent oxidation pathway. In ring hydroxylation pathways [toluene dioxygenation (TOD), toluene o-monooxygenation (TOM), toluene monooxygenation (TBU) and toluene p-monooxygenation (TMO)], dioxygenases attack the aromatic rings and produce dihydroxyl compounds, while monooxygenases attack the aromatic rings and produce arene oxides as intermediates, which are highly unstable and further converted to phenols (Mitchell et al. 2002). Monooxygenases are capable of performing subsequent monooxygenation on these phenols and convert them to dihydroxyl compounds, such as catechols (Tao et al. 2004). The alkyl substituent oxidation pathway (TOL) involves the oxidation of the alkyl substituent to produce dihydroxyl compounds. The dihydroxyl compounds produced subsequently undergo ring fission to produce intermediates of the Kreb's cycle.

10.2.2 Polycyclic Aromatic Hydrocarbons (PAHs)

The catabolic pathways for PAHs, especially those with two and three rings, have been reported (Bamforth and Singleton 2005; Cerniglia 1992). Most of the PAHs-degrading bacteria oxidize naphthalene using dioxygenases. A few bacteria, such as *Mycobacterium* sp. are also capable of oxidizing the PAHs aromatic ring via cytochrome P450 monooxygenase enzyme to form trans-dihydrodiols rather than cis-dihydrodiol (Kelley et al. 1990). The initial monooxygenation of fluoranthene has been reported in *Sphingomonas* sp. LB126. This strain was able to co-oxidize anthracene, phenanthrene and fluoranthene without the accumulation of dead-end intermediates (van Herwijnen et al. 2003b). The main pathway for bacterial degradation of anthracene proceeds via 3-hydroxy-2-naphthoic acid, 2,3-dihydroxynaphthalene, and further through a pathway similar to the naphthalene degradation pathway. Species from the genera *Pseudomonas, Sphingobium, Nocardia, Rhodococcus* and *Mycobacterium* are known to perform through this pathway (Dean-Ross et al. 2001; Cerniglia 1992). With detection of 3-hydroxy-2-naphthoic and o-phthalic acids, a new catabolic pathway for anthracene by *Mycobacterium* sp. strain LB501T has been proposed (van Herwijnen et al. 2003a). This diverges from the known pathway after the formation of 3-hydroxy-2-naphthoic acid.

The typical biphenyl degradation pathway is conserved among polychlorinated biphenyls (PCB)/biphenyl degraders, where the conversion of biphenyl to a tricarboxylic acid cycle intermediate and benzoate is catalyzed by a series of enzymes: BphA, BphB, BphC, BphD, BphE, BphF, and BphG (Yoshiyuki et al. 2004). It has been reported that most PCB degraders could only transform the compound into chlorinated benzoates and grew at the expense of 2-hydroxypenta-2,4-dienoate, while chlorinated benzoates could be degraded by various bacteria via chlorocatechol, via hydrolytic dehalogenation of 4-chlorobenzoate to give 4-hydroxybenzoate, via 4,5-dioxygenation of 3-chlorobenzoate and 3,4-dichlorobenzoate to give 5-chloroprotocatechuate, and probably via the gentisate pathway (Pieper 2005).

10.3 Pathway and Regulation of the Styrene Catabolic Operon

Generally, the aerobic degradation of simple aromatic compounds follows different metabolic pathways based on the enzyme system present in the microorganisms. In order to understand more clearly the molecular mechanism involved in aromatic xenobiotic biodegradation by bacteria, a simple aromatic compound like styrene is discussed more elaborately. A schematic representation of styrene degradation metabolic pathways and associated genes is depicted in Fig. 10.2.

The first two genes of the operon are *styA* and *styB*, encoding the two subunits of styrene monooxygenase (SMO), the catalyst responsible for the transformation of styrene to styrene epoxide followed by styC, which encodes for styrene oxide isomerase (SOI) involved in transformation of styrene epoxide to phenylacetaldehyde. Similarly *styD* encodes the phenylacetaldehyde dehydrogenase (PAALDH) activity necessary for the oxidation of phenylacetaldehyde to phenylacetic acid through the action of either an NAD + or phenazine methosulfate-dependent phenylacetaldehyde dehydrogenase (Santos et al. 2000). The conversion of styrene to phenylacetic acid is referred to as the upper pathway of styrene metabolism (O'Connor et al. 1995; Velasco et al. 1998). The presence of an additional gene *styE* located further downstream of *styD* in *P. putida* CA-3 was recently reported where StyE functions in transporting styrene across the outer cell membrane (Mooney et al. 2006). The metabolic steps after phenylacetic acid production that are involved in initial conversion to phenylacetyl – CoA coded by *styE* (PACoA ligase) are referred to as the lower pathway of styrene metabolism (Ferrandez et al. 1998; Mohamed et al. 2002). It is believed that phenylacetyl-CoA then undergoes numerous aerobic enzymatic reactions before it enters the tricarboxylic acid (TCA) cycle as acetyl-CoA (Mohamed et al. 2002). Two styrene catabolon genes, namely, *styS* and *styR*, were also identified and they are believed to be

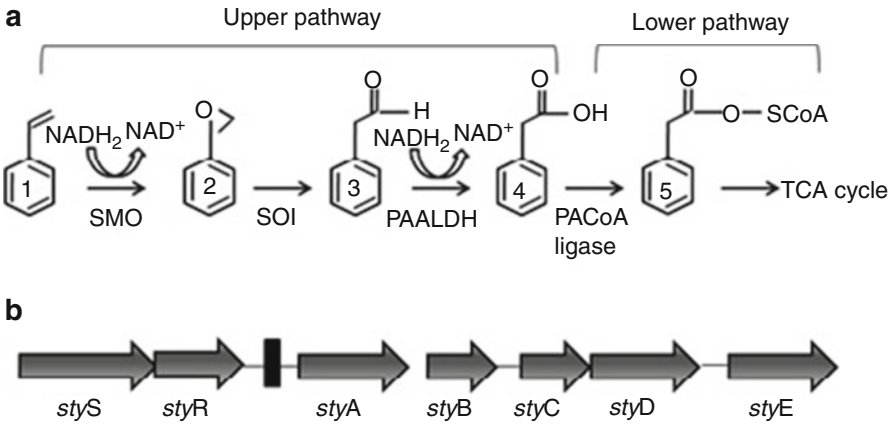

Fig. 10.2 Styrene biodegradation pathway (**a**) and operon organization (**b**)

involved in regulating the styrene-degradation pathway (Velasco et al. 1998). The exact mechanism by which StyS detects the presence of styrene still remains to be elucidated. However, it has been suggested that the PAS sensing domain containing the heme group senses the presence of styrene as a result of the altered redox potential of the cell (Santos et al. 2000; Taylor and Zhulin 1999), while it was proposed that the second PAS domain functions in detecting the presence of extracellular styrene. Complementation studies performed in *E. coli* with elements of the *sty* operon from both *Pseudomonas* sp. strain Y2 and *Pseudomonas* sp. strain VLB120, demonstrated that the StyS and StyR proteins were required for the styrene-dependent induction of the *sty* upper pathway genes (Velasco et al. 1998; Panke et al. 1998). It has also been reported in *P. putida* CA-3 that transcription of the styrene upper pathway genes is entirely dependent upon expression of *stySR* (O'Leary et al. 2001). The activation of the pathway is dependent on the presence of styrene but can be repressed in the presence of various other carbon sources, for example, phenylacetic acid or citrate (Velasco et al. 1998; O'Leary et al. 2001). Furthermore, addition of the lower pathway substrate, PAA (phenyl acetic acid) into styrene-growing cultures caused a complete loss of detectable upper pathway activity and this was linked to transcriptional repression of *stySR*. Therefore it is concluded that *stySR* have no role in the regulation of the lower pathway. The expression of the styrene-degradative catabolon was reported to be subject to catabolite repression with this effect being exerted via the expression of the *stySR* genes (O'Connor et al. 1996; O'Leary et al. 2001; O'Connor et al. 1995). *StySR* transcripts were not detected when batch cultures were grown on styrene in the presence of phenylacetic acid, citrate, and glutamate and, therefore, the transcription of the upper pathway genes did not occur (O'Leary et al. 2001). However, Santos et al. (2000) have reported that *stySR* expression was constitutive in *P. fluorescens* ST regardless of the carbon source present in the environment, but that the transcription of *styA* required the presence of styrene.

10.4 Molecular Basis of Genetic Adaptation of Microorganisms for Aromatic Xenobiotics Utilization

The high degree of consensus amino acid sequence prevalent amongst the enzymes involved in utilization of aromatic xenobiotics and the distinctive organization of catabolic gene clusters suggest that functional clusters of such genes are organized as modules within mobile elements. Indeed, mobility has been observed in case of genes involved in the modified pathway of *ortho*-cleavage, *meta*-cleavage, three and two-component dioxygenases of the aromatic ring and dihydrodiol dehydrogenases. A comparative analysis of the genes relevant to biodegeradation in diverse bacterial species has revealed that genetic recombination processes constitute a major mechanistic element in the adaptation of these microbes to

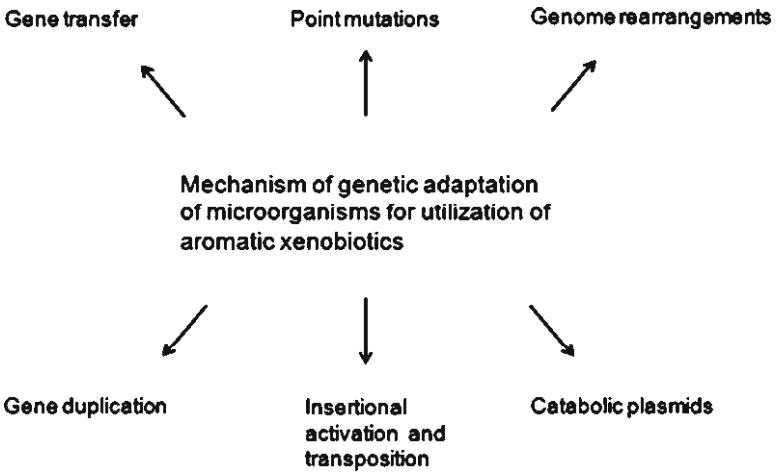

Fig. 10.3 Schematic showing various mechanisms of genetic adaptation of microorganisms for utilization of aromatic xenobiotic compounds

aromatic substrates. Some of the salient genetic mechanisms of such adaptation are depicted in Fig. 10.3, and a brief description of each mechanism is made in the following section.

10.4.1 Gene Transfer

Literature survey provides a great deal of evidence that majority of genes involved in xenobiotic utilization evolved through horizontal transfer among taxonomically distant microorganisms. Horizontal gene transfer in microbial communities is attributable to several mechanisms, such as conjugation, transduction (Zeph et al. 1988; Saye et al. 1990), and natural transformation. Burlage et al. (1990) have shown that pJP4, pAC25, pSS50, and pBRC60, catabolic plasmids carrying genes for chloroaromatic degradation, have a strongly homologous plasmid backbone that determines replication and transfer functions. These observations suggest that a few common self-transmissible ancestor replicons may have been involved in the acquisition and spread of different catabolic modules. The importance of gene transfer for adaptation of host cells to new compounds has been illustrated in many studies on experimental evolution of novel metabolic activities (Mokross et al. 1990; Bruhn et al. 1988; Oltmanns et al. 1988). Such studies could identify biochemical blockades in natural pathways which prevented the degradation of novel substrates and could overcome these barriers by transferring appropriate genes. It has also been proposed that the potential for substrate degradation may be enhanced if enzymes with limited substrate specificity are replaced by their less specific counterparts, or by utilizing additional enzymes capable of channeling the substrates to the existing catabolic pathways.

10.4.2 Point Mutations

Introduction of point mutations is a powerful technique in the regime of protein engineering of catalytic enzymes and this strategy has been extensively used to alter the substrate specificity of enzymes. Several examples have illustrated that single-site mutations can alter substrate specificities of enzymes or effector specificities. The substrate specificity of catechol-2,3-dioxygenase, encoded by the pTOL-plasmid pWW0, was enhanced by replacing a single amino acid residue (Boe 1990). The specificity of the enzymatically inactive regulatory protein XylS was also changed by mutagenesis, which conferred the ability to recognize as effectors molecules such compounds as 4-ethylbenzoate, salicylate, and dichlorobenzoates (3,5-, 2,5-, and 2,6-) (Khomenkov et al. 2008). Besides, XylS mutants were also obtained, capable of activating the genes of the pathway of *meta*-cleavage in the absence of any effectors (Ramos et al. 1986).

10.4.3 Genome Rearrangements

The phenomenon of genome rearrangement has been frequently observed in microorganisms involved in the biodegradation of aromatic hydrocarbons. For example, groups of genes encoding pathways of *ortho*-cleavage in *A. calcoaceticus* and *P. putida* were seen to be distinct from each other and similar genes of other organisms, probably as a consequence of genomic DNA rearrangements in the corresponding loci (Perkins et al. 1990; Ghosal and You 1989). The genes of three-component dioxygenases *todCICIIBAD* have been shown to be inserted between *todF* and *todJ* within the operon of the pathway of *meta*-cleavage, resulting in a new pathway of toluene degradation (Oltmanns et al. 1988). There are also reports which indicate that DNA elements of three-component of dioxygenases are inserted into the center of separate catabolic operon (Harayama et al. 1991; Markus et al. 1986).

10.4.4 Gene Duplication

Gene duplication plays an important evolutionary role in microorganisms. As a consequence of gene duplication it is construed that the presence of an additional copy of the gene reduces negative selection pressure, which in turn may acquisition of mutations. Literature report provides sufficient evidence of duplication events in genes concerned with biodegradation of xenobiotic compounds. For example, it has been shown that in case of the TOL-plasmids, the operons of upper and lower pathways (e.g., xylS and xylR) can manifest various configurations such as inversions duplications, or even deletions of multiple copies (Burlage et al. 1989; Meulien et al. 1981; Sinclair et al. 1986; Nakai et al. 1990). It has also

been shown that a 1.5-kbp direct repeat within TOL-plasmids was subjected to multiple recombination events, which resulted in insertion of catabolic TOL operons from the chromosome (Schreiner et al. 1991; Kaphammer et al. 1990). In the context of 2,4-dichlorophenoxyacetate degradation, it has been suggested that the genes involved in degradation of the compound, which are resident on the plasmid pJP4, underwent several rounds of duplication events (Paul et al. 1990; Suzuki et al. 1991).

10.4.5 Insertional Activation and Transposition

Gene expression levels can be modulated via polar effects and in this context insertion sequence (IS) elements represent one of the major tools to mediate gene activation. Activation of genes by insertion elements has been observed in case of operons involved in degradation of aromatic compounds. For example it has been reported that in *E.coli*, genes belonging to the bgl operon were activated following insertion of IS1 and IS5 sequences (Trevors et al. 1987; Rochelle et al. 1989). With regard to the catabolic operon *TOL*, it is suggested that the operon is part of a large mobile element *Tn4651*, which belongs to the family of type *Tn3* transposons (Zylstra et al. 1988; Haugland et al. 1990). In case of *P. cepacia* 249, the high adaptive potential of the strain has been attributed to the presence of high copies of multiple insertional elements (Sangodkar et al. 1988). In case of *P. putida* PP3, the dehalogenase genes are carried on IS elements, whereas in *Alcaligenes* sp. BR60, genes involved in utilization of 3-chlorobenzoate are resident on the transposon *Tn5271* (Slightom et al. 1986; Aronson et al. 1989).

10.4.6 Catabolic Plasmids

The first plasmids involved in the degradation of organic xenobiotics were described about three decades ago and since then a number of examples of such catabolic plasmids have been reported in the literature. Catabolic plasmids are commonly rather large in size (>50 kb) and genetic analyses have indicated that such plasmids possess complete sets of transfer and catabolic genes. The plasmid-encoded catabolic pathways confer an additional advantage of facilitating horizontal transfer of the respective catabolic genes in a microbial population, resulting in a rapid adaptation of microorganisms to new aromatic pollutants present in a particular ecosystem. Examples of catabolic plasmids include pVI150 in *Pseudomonas sp. CF600*, pL6.5 in *P. fluorescens*, pDTG1 in *P. putida NCBI 9816–4,* pJP4 in *R. eutropha JMP134,* SAL1 in *P. putida R1,* pSAH in *Alcaligenes sp. O*-1, pPS12-1 in *Burkholderia sp. PS12,* which are involved in the biodegradation of phenol, toluene, naphthalene, 3-chlorobenzoate, salicylate, 2-aminobenzoate sulfonate and tetrachlorobenzene respectively (Dennis 1992).

10.5 Novel Methods to Improve Aromatic Xenobiotics Biodegradation

For successful biodegradation and therefore bioremediation of aromatic xenobiotic compounds, a number of factors that may limit the process need to be overcome. These factors can include: (1) inadequate numbers of organisms capable of degrading more recalcitrant compounds, (2) reduced activities of responsible enzymes, (3) low bioavailability, (4) depletion of growth substrates, (5) accumulation of inhibitory products. In addition to these factors, limitations in oxygen and other nutrients might limit biodegradation aromatic xenobiotics, particularly during *in situ* treatment operations. If improvements in bacterial aromatics metabolism are to be made, then strategies to achieve the same need to be evaluated first based on understanding of the factor(s) limiting biodegradation. The following subsections discuss some novel strategies that have been developed to overcome some of the above limitations, and a schematic showing these strategies is presented in Fig. 10.4.

10.5.1 Bioaugmentation

If the performance of on-going biological processes in bioreactors or polluted sites can be improved by application of indigenous, wild type or genetically engineered microorganisms (GEM), the process can be termed as bioaugmentation. For engineering remediation genes, two main approaches can be followed: gene introduction and gene alteration. In the first case, specific remediation genes are introduced into plasmids or the chromosome of the target microorganism. The most straight forward way is to introduce a plasmid containing the desired gene. This transfer can be accomplished with naturally occurring plasmids, if they are transmissible, or by mating of a donor with a target microorganism. The above-mentioned process does not involve any recombinant DNA techniques and occurs commonly in nature.

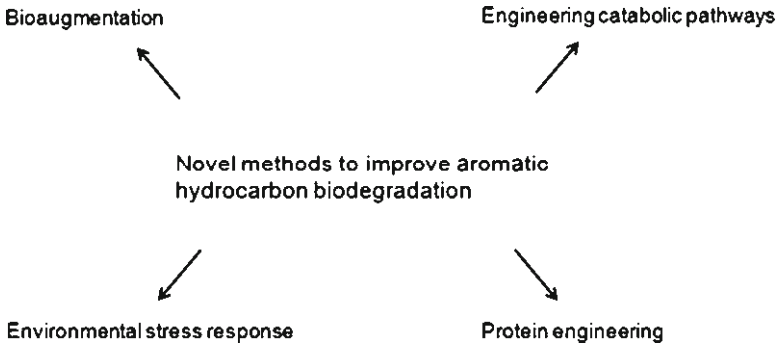

Fig. 10.4 Schematic showing some novel methods to overcome certain limitations with bacterial metabolism of aromatic xenobiotic compounds

When an appropriate naturally occurring plasmid is not available, it may be necessary to clone the gene into a broad-host-range plasmid, which is then added to the donor microorganism either through conjugation or transformation. It may be desirable to incorporate the gene into the host chromosome to reduce the potential for gene transfer to other microorganisms. The mini-Tn5 transposon system is commonly used to insert genes into gram-negative bacteria. The original mini-Tn5 transposon system is constructed as a plasmid with a selectable marker, such as antibiotic resistance, a suicide function, and a multiple cloning site for insertion of foreign DNA via recombinant DNA techniques. Once the recombinant plasmid is added to the target organism, the transposon containing the added gene incorporates into the chromosome of some of the target bacteria. These GEMs are then selected based on the incorporated phenotypic trait. The disadvantages of the mini-Tn5 system are that it incorporates randomly into the host chromosome, possibly inactivating vital genes, and that it contains antibiotic-resistant genes that may ultimately hinder the release of the constructed GEM.

In the second option, i.e., gene alteration, it is possible to alter selected genes so that they show optimal activity under different environmental conditions. Gene alteration is useful for bioremediation processes. Traditionally, the gene of interest is first cloned into a laboratory organism, such as *Escherichia coli*, that is easily manipulated and cultivated in the laboratory. The factors that can be altered to increase gene expression include the transcriptional promoter and terminator sequences, the number of copies of the gene in the host organism and the stability of the cloned gene protein. The gene is then altered and ultimately reintroduced into the desired microorganisms. For bioaugmentation, should satisfy a minimum of the following three criteria. First, they must be catabolically active to degrade pollutants in the complex microbial communities of biotreatment systems. Second, they must be competitive, and hence persistent after being introduced into biotreatment systems. Third, they should be compatible with indigenous microbial communities so that they will not adversely affect the indigenous microbial communities. Several cases of bioaugmentation using GEMs in conventional bioreactors have been reported while the target chemicals were focused on aromatic compounds. Liu and Huang (2008) constructed an atrazine-degrading GEM by cloning atrazine chlorohydrolase gene ADP of *Pseudomonas* sp. into the plasmid vector pACYC184 and then transformed into *Escherichia coli DH5α*. The GEM bioaugmented in membrane bioreactor system (MBR) showed atrazine removal efficiency as high as 94% for an hydraulic retention time (HRT) of only 8 h and average atrazine loading rate of 48.2 mg l^{-1} day^{-1}. Compared to this value, wild-type atrazine-degrading strain or mixed microbial consortium could achieve maximum removal efficiency in the range 40–90% for high HRT values of 5–7 d in the bioreactor. Despite its many advantages, GEMs suffer from certain limitations, such as the transfer of undesirable genes. In addition, because of problems associated with survivability, high degradative activity and stability of inoculated bacteria, bioaugmentation seems unreliable in some cases. Further, undesirable antibiotic resistance borne by some of these bacteria may pose threats to human

health and the world's ecosystems. Ecological risk is another reason since GEMs leak into the environment from bioreactors seems unavoidable. While the approach on chromosomal integration through mini-Tn5 transposon and homologous recombination can be used to overcome the instability problem with strains carrying recombinant plasmids, nonantibiotic resistance markers such as resistance to mercuric salts and organomercuric compounds can be used to avoid the problem with undesirebale antibiotic resistance markers. Researchers have investigated the use of naturally occurring horizontal gene transfer to introduce remediation genes into a contaminated site. Recent advances in genome sequencing are revealing the large role that horizontal gene transfer has played in microbial development and adaptation in the environment, which may occur via uptake of naked DNA (transformation), mediation by bacteriophage (transduction), or physical contact and exchange of genetic material such as plasmids or conjugative transposons between microorganisms (conjugation). The potential advantage of introducing genes in a mobile form such as, with a transmissible plasmid, over the traditional cell bioaugmentation approaches is the introduction of remediation genes into indigenous microorganisms that are already adapted to survive in the environment. The challenge is to generate vigorous microorganisms that, when applied to the contaminated site, dissipate the target contaminant with high efficiency and limited risks. The risks associated with uncontrolled growth and proliferation of GEMs and horizontal gene transfer may be minimized through rapid death after dissipation of the aromatic hydrocarbon. In order to enhance the capability of microorganisms to degrade aromatic xenobiotics strategies such as cloning of complete metabolic pathway, improvement in genetic stability of catalytic activities, increasing the substrate range of catabolic enzymes, improving enzyme stability, altering the kinetics of enzymatic degradation, preventing uncontrolled proliferation of introduced strains etc. Some more emerging technologies have been developed to improve bioaugmentation with GEMs. One of them is to encapsulate bacterial cells in a carrier before introduction into contaminated sites. This method would be helpful to maintain a larger survival population size. Recently, Sarma et al. (2011) reported encapsulation of *Mycobacterium frederiksbergense* in calcium alginate beads for biodegradation of highly hydrophobic pollutants such as pyrene in a two-phase partitioning bioreactor (TPPB) system. In a similar study by Mahanty et al. (2008), the polycyclic aromatic compound was reported to be efficiently degraded by the mycobacterium in a TPBB system with silicone oil as the non-aqueous phase liquid. However, economics of these methods still needs to be worked out in detail for better application potential. In addition, the ecological risk of GEMs needs to be evaluated carefully. Another focus could be on transfer of degradation genes from GEMs to indigenous bacteria if degradation genes were in the mobile forms such as transmissible plasmids. This method would accelerate indigenous degrading-bacteria evolution and make bioaugmentation more effective. However, the horizontal transfer frequencies of many degradation genes can be low and inefficient. The dissemination of foreign genes in microorganism community would also cause more ecological risk.

10.5.2 Engineering Catabolic Pathways

To enhance the ability of the microbes to degrade xenobiotics, strain improvement or genetic engineering of microbes has been reported in the literature. Some recent examples are: (1) cloning of the *ohb* operon from *Pseudomonas aeruginosa* and the *fcb* operon from *Arthrobacter globiformis* into *Comamonas testosteroni* strain VP44 to render its capability to completely mineralize monochlorobiphenyls (Hrywna et al. 1999); (2) constructing a hybrid pathway in *Pseudomonas* strains for biodegradation of 2-chlorotoluene using toluene dioxygenase to produce 2-chlorobenzyl alcohol, the TOL upper pathway to convert 2-chlorobenzyl alcohol to 2-chlorobenzoate, and the native or modified ortho-cleavage pathways to mineralize 2-chlorobenzoate (Haro and de Lorenzo 2001); (3) introducing the *opd* gene from *Flavobacterium* sp. ATCC 27551 and the p-nitrophenol degradation pathway genes from *Pseudomonas* sp. ENV2030 into *P. putida* KT2440 to construct a parathion-degrading pathway (Walker and Keasling 2002); (4) introducing the genes encoding the 2,4-dinitrotoluene (2,4-DNT) degradation pathway from *Burkholderia sp*. strain DNT into *Pseudomonas fluorescens* ATCC 17400, which could completely degrade 2,4-DNT (Monti et al. 2005); (5) using *E. coli* as a host for cloning and expressing the genes encoding a novel partial reductive pathway for 4-chloronitrobenzoate and nitrobenzene from *Comamonas* sp. strain CNB-1 (Wu et al. 2006). A hybrid strain, *P. putida* TB101, was constructed by the introduction of the TOL plasmid pWWO. Lee et al. (1995) performed genetic and biochemical analysis of the TOD and TOL pathways and assembled a hybrid route by combinations of appropriate sections of the two pathways. A hybrid strain of *Pseudomonas putida* was constructed by cloning of the relevant genes which led to complete mineralization of benzene, toluene and p-xylene mixture by the hybrid strain. Chen et al. (2004) reported cloning of catechol 2,3-dioxygenase (C23O) gene, responsible for the conversion of catechol to 2-hydroxymuconic semialdehyde due to cleavage of aromatic C–C bond at the *meta* -position of dihydroxylated aromatic substrates, from *Pseudomonas aeruginosa* ZD 4–3 in the *E. coli* expression system. The recombinant organism showed ability to degrade both single and bicyclic compounds via a *meta*-cleavage pathway with a higher enzyme activity of eightfold.

10.5.3 Environmental Stress Responses

Exposure of microorganisms to harsh physico-chemical parameters such as extreme temperatures, caustic chemicals, and other stresses can be thought to bring out a myriad of physiological responses such as changes in the membrane properties, decrease in metabolic rates, denaturation of vital proteins etc. Bacterial strains which are exposed to changing environmental conditions are likely to respond by effecting changes in the fatty acid composition of membrane lipids so as to protect themselves from substrate toxicity and membrane damage. This phenomenon has

been demonstrated in case of *Pseudomonas putida* P8 (Heipieper et al. 1992). In another study it has been suggested that the bioavailability of PAHs increased at high temperature due to increase in solubility, diffusion, and reaction rates (Tabak et al. 2003). Similarly, at high temperature of 60–70°C, efficient conversion of PAH with three to five rings by pure and mixed cultures of extreme thermophilic bacteria *Bacillus* sp. and *Thermus brockii* has been demonstrated (Feitkenhauer et al. 2003). Proteomics approaches have been used to gain insights into the responses of microorganisms to temperature, and other stresses. Several studies have shown up-regulation of known stress-response proteins in conjunction with proteins involved in other detoxification or adaptation mechanisms. Other stress related adaptive responses have also been documented. For example in case of *Pseudomonas putida* DOT-T1E, a toluene-tolerant strain, tolerance to 4HBA involved increased rigidity of the cell membrane as a consequence of decrease in the cis/trans ratio of unsaturated fatty acids (Ramos-González et al. 2001).

10.5.4 Protein Engineering

In addition to engineering catabolic pathways, modification of certain genes encoding biodegradative enzymes is an effective approach to enhance bioremediation properties. DNA shuffling is a common and powerful method for protein mutagenesis. Using DNA shuffling, Rui et al. 2005 produced a mutant of toluene orthomonooxygenase (TOM) of *Burkholderia cepacia* G4 containing the mutation TomA3 A113V. In a separate study Suyama et al. (1996) created hybrid *Pseudomonas* strains in which the bphA1 gene (coding for a large subunit of biphenyl dioxygenase) was replaced with the todC1 gene (coding for a large subunit of toluene dioxygenase of *Pseudomonas putida* F1) within chromosomal biphenyl-catabolic bph gene clusters. The hybrid strains were observed to grow on a wide range of aromatic hydrocarbons, and degrade chloroethenes such as trichloroethylene and cis-1,2-dichloroethylene. Various successful modifications of some catabolic enzymes involved in biodegradation of aromatic compounds are summarized in Table 10.1. In addition, recombinant DNA technology and *in vitro* mutagenesis has been employed to produce hybrid genes encoding fusion proteins to enhance catabolic enzyme expression. For example, Meyer et al. (2002) demonstrated the use of error-prone PCR to enhance the substrate reactivity of the flavoenzyme 2-hydroxybiphenyl 3-monooxygenase.

10.6 Conclusions and Future Perspectives

Exploration of the metabolic pathways and responsible genes and enzymes involved in bacterial systems for biodegradation of various aromatic xenobiotic compounds has considerably improved our understanding of the mechanisms by which microbes

Table 10.1 Modified catabolic enzymes for biodegradation of aromatic compounds

Enzyme/source	Approaches	Remarks	Reference
Catechol-2,3- dioxygenase encoded by the pTOL-plasmid pWW0	Single residue amino acid replacements	Substrate specificity of catechol-2, 3- dioxygenase broadened to include 4-ethylcatechol	Boe (1990)
Biphenyl dioxygenase/*Burkholderia xenovorans LB400*	Directed evolution via random mutagenesis of region III	2,2′-dichlorobiphenyl is converted to 3,4-dihydro-3,4-dihydroxy-2,2′-dichlorobiphenyl	Barriault and Sylvestre (2004)
2,4-dinitrotoluene dioxygenase/*B. cepacia R34*	Directed evolution via random mutagenesis of V350 and expression in E. coli TG1	Increased activity with o-nitrophenol (47-fold), m-nitrophenol (34-fold), and o-methoxyphenol (174-fold)	Keenan et al. (2004)
Toluene 4-monooxygenase of *Pseudomonas mendocina* KR1	Variant A107T/E214A	Favored hydroxylation of naphthalene in the C2 position	Tao et al. (2004)
Tetrachlorobenzene dioxygenase/*Ralstonia* sp. PS12	Rational design via site-directed mutagenesis based on naphthalene dioxygenase structure	Increased product formation rate with dichlorotoluenes; changed oxidation of trichlorotoluenes	Pollmann et al. (2003)
Toluene orthomonooxygenase/ *B. cepacia G4*	Directed evolution via DNA shuffling	Faster oxidation of phenanthrene, fluorene and anthracene when expressed in E. coli	Canada et al. (2002)
TOM-Green/*B. cepacia G4*	Directed evolution via random mutagenesis of A106	Faster degradation of toluene	Rui et al. (2004)
Catechol 2,3-dioxygenase/*P. putida UCC2*	Directed evolution via cassette PCR	Increased resistance to 4-methylcatechol; growth on p-toluate	Okuta et al. (2004)
Pseudomonas stutzeri OX1 toluene o-xylene monooxygenase	TouA DNA shuffling variantE214G/ D312N/M399V	Oxidized nitrobenzene sixfold faster than wild-type ToMO and produced m- and p-nitrophenol as well as 4-nitrocatechol, whereas wild-type ToMO produced only m- and p-nitrophenol.	Vardar and Wood (2005)

2-Hydroxybiphenyl 3-monooxygenase/ *P. azelaica HBP*	In vitro manganese mutagenesis	Increased activity with 2-tert-butylphenol as substrate (fivefold)	Meyer et al. (2002)
Burkholderia sp. strain DNT monooxygenase (DntB M22L/L380I)	Using error-prone PCR	Create a variant of 4-Methyl-5-nitro-catechol (4M5NC) monooxygenase (DntB M22L/L380I). which accepts the two new substrates 4-nitrophenol (4NP) and 3-methyl –4-nitrophenol (3M4NP) with compare to 2,4-dinitrotoluene	Leungsakul et al. (2006)
P. pseudoalcaligenes KF707 dioxygenase	Mutation of Thr376 (KF707) to Asn376 (LB400)	Expansion of the range of biotransformable poly chlorinated biphenyl congeners	Kimura et al. (1997)

have evolved to degrade such compounds in the environment. This has also led to the opportunities in strain and process improvement for better biodegradation of xenobiotics. In addition to overcoming the problems of pollutant bioavailability and accumulation of inhibitory intermediates, suitable strategies need to be developed to allow more recalcitrant compounds to serve as growth substrates for bacteria. Further, because complex microbial communities rather than only bacteria are important for effective bioremediation of contaminated environment, more research is required to elucidate the interactive and symbiotic roles played by individual species in these communities. It is anticipated that current research on biochemical and molecular aspects of bacterial degradation of aromatic xenobiotic compounds will lay the necessary foundation for successful development of bioremediation processes in the future.

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Chapter 11
Molecular Analyses of Microbial Activities Involved in Bioremediation

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Abstract Microorganisms play essential roles in nutrient cycles, interact with all living organisms and form bedrock of sustainable ecosystems. For implementation of bioremediation strategies it is essential that we understand how the environment (oxygen, water, nutrients, temperature and pH) modulates microbial activities. Microbial activities like oxidation, reduction, binding, immobilization, volatization, or transformation are carried out by enzymes such as oxidases, reductases, oxygenases and many more. Only few enzymes by their specific function are involved in bioremediation. However, there are many enzymes which by their specific role are involved in cellular metabolic functions but under stress conditions induced by anthropogens such as hydrocarbons, dyes, aromatic and xenobiotic compounds they perform alternate functions in metabolic pathways involved in biodegradation.

The complete genome sequencing has become a very regular phenomenon and there is a significant augmentation in microbial genome databases. Consequently, it is possible to hypothesize the role of genes involved in bioremediation. However, to ascertain how many of them are actually involved in bioremediation we need transcriptome and proteome profiles. Cellular expression of proteins and metabolites varies with the stress and characterizing the differentially expressed molecules will provide the missing links in the degradation pathways.

Innovative breakthroughs in technologies of sequencing, fingerprinting techniques, microarray and mass spectrometry along with bioinformatics tools have led

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a paradigm shift in characterizing microbial activities at molecular level. At the same time the use of molecular techniques has led to realization that microbial diversity is several folds higher than ever anticipated. Any one particular microorganism is incapable of processing all the metabolic reactions to degrade recalcitrant compounds, however a group of organisms form a community and collectively process all the reactions for bioremediation. Emerging fields like metagenomics, metatranscriptomics, metaproteomics and metabolomics have solved and are solving the complex biodegradation pathways. In future, applications of techniques such as lateral gene transfers, genetic engineering and protein engineering by rational or irrational experimental designs for direct or indirect evolution will develop recombinant strains with novel capabilities.

Keywords Molecular analyses • Bioremediation • Xenobiotics • Metagenomics • Metatranscriptomics • Metaproteomics • Metametabolomics • Biodegradation pathways

11.1 Introduction

Microorganisms, the only living creatures to be omnipresent in environment, have essential functions in all ecological cycles. The environmental factors like moisture, humidity, temperature, oxygen, nutrients and pH are important regulators in microbial activities. As a result microbes can be functionally active or dormant depending on the conditions. Genes and corresponding encoded enzymes are necessary for action but environment plays an important role in their regulation. Yang et al. (2011) studied effects of soil organic matter on the microbial polycyclic aromatic hydrocarbons (PAHs) degradation potentials. The microbial activities were relatively lower in the soils with the lowest and highest organic matter content, which were likely due to the nutrition limit and PAH sequestration, respectively. The microbial activities developed in humic acid were much higher than those developed in humin, which was demonstrated to be able to sequester organic pollutants stronger. The results suggested that the nutrition support and sequestration were the two major mechanisms, that influenced the development of microbial PAHs degradation potentials (Yang et al. 2011). The variation in environmental factors from location to location and even from one niche to another niche within a location has lead to a huge diversity in microorganisms and their capabilities. Nutrient requirement, add another dimension in bioremediation, in a sense that many microorganisms are able to use xenobiotic compounds as sole carbon and/or nitrogen source. However, many times they cannot survive solely on xenobiotic compound and require additional carbon and/or nitrogen source for growth and then by co-metabolism they can transform or degrade the pollutants.

Microorganisms are the fastest evolving and adapting living creatures, which make them most suitable for coping with all the changes in environment. Ben Said et al. (2008) characterized polycyclic aromatic hydrocarbon (PAH)-degrading

bacteria from sediments of the Bizerte lagoon in presence of multiple contaminants such as organic pollutants, heavy metals and antibiotics. This study highlights the need of bacterial strains to be adapted to multiple contaminants to become a promising potential for bioremediation processes. Microorganisms have sets of catabolic genes, capable of processing various metabolic pathways, which are integrated in such a manner that xenobiotic compounds are converted to intermediates which can enter central metabolism (such as kreb's cycle, glycolysis and others). The immense potential of microorganisms does not depend solely on the wealth of catabolic enzymes that they possess, but also upon their capacity for adaptive change (Houghton and Shanley 1994). Any one particular microorganism is incapable of processing all the metabolic reactions to degrade environmental pollutants, however a group of diverse organisms form a community known as mixed culture or consortium and show a greater potential by collectively processing all the metabolic reactions for complete biodegradation. Another approach is to develop a recombinant strain, harbouring diverse metabolic pathways, using novel molecular and omic technologies.

In the last decade, the focus on enzymes for environmental bioremediation gained considerable importance and thus various new approaches have been applied for detailed studies on some classes of enzymes. Several enzymes are endowed with promiscuous activities. The term 'catalytic promiscuity' describes the capability of an enzyme to catalyse different chemical reactions, called secondary activities, at the responsible active site. Furthermore, from a basic point of view, studies of catalytic promiscuity offer clues to understand natural evolution of enzymes and to translate this into *in vitro* adaptation of enzymes to specific human needs (Mandrich et al. 2010). Many of the enzymes active in degradation pathways are linked from their protein phylogeny and not strictly linked to the taxonomical affiliation of the bacteria (Perez-Pantoja et al. 2009), indicating that the genes encoding those catabolic enzymes are involved in very dynamic events. Therefore, the microbial community structure of a particular environmental sample, expressed as the taxonomical composition, indicates only the fitness of bacterial phylogenetic groups. Consequently, the catabolic gene potential has to be analysed independently as any presumption based only on taxonomy of the genomes present will result in circumstantial and unresolved associations (Vilchez-Vargas et al. 2010). To characterize the catabolic potential for biodegradation it is necessary to take into consideration the broad diversity of catabolic routes evolved by microorganisms and also the diversity of enzymes of a given gene family or even between gene families. The current capabilities to assess and predict catabolic potential of environmental sites include gene fingerprinting, catabolome arrays, metagenomics and complementary 'omics' technologies (Vilchez-Vargas et al. 2010).

Over the last few years, the scientific literature has revealed the progressive emergence of genomic high-throughput technologies in environmental microbiology and biotechnology and described their possible or already demonstrated applications to assess biotreatment of contaminated environments (Stenuit et al. 2008). The standard molecular biology approaches together with new high-throughput technologies (like genome sequencing, transcriptomics, proteomics and metagenomics), have

provided immense information regarding the genetics, regulation, ecophysiology, and evolution of anaerobic aromatic degradation pathways. These studies revealed that the catabolism of anaerobic aromatic compounds is more diverse and widespread than previously thought and involves complex metabolic and stress processes (Carmona et al. 2009). Systems biology approaches to biodegradation, where the system is analysed as a whole and the interactions amongst the involved parts are the main subject of study, have provided the global analysis of the biodegradation metabolic network in the form of structure, behaviour and evolution (Trigo et al. 2009). Advances have been made for enhanced inorganic chemical bioremediation and organic chemical biodegradation using various pathway-engineering approaches that harness molecular, genetic, microbiology, and protein engineering tools. They rely on identification of novel metal-sequestering peptides, rational and irrational pathway engineering, and enzyme design (Singh et al. 2008). Various experimental and conceptual approaches have been developed for metabolomics to be applied in bioremediation research, such as strategies for elucidation of biodegradation pathways using isotope distribution analysis and molecular connectivity analysis, the assessment of mineralization process using metabolic footprinting analysis, and the improvement of the biodegradation process via metabolic engineering. The use of metabolomics tools can significantly extend and enhance the power of existing bioremediation approaches by providing a better overview of the biodegradation process (Villas-Boas and Bruheim 2007). The environmental application of high-throughput technologies-metagenomics, metatranscriptomics, metaproteomics and metametabolomics harbours the most promising opportunities to understand the biodegradation process, and at the same time poses tremendous challenges from the data management and data mining point of view.

Microbial bioremediation strategies can be applied either *ex situ* (off-site separate specific treatment facility) or *in situ* (on-site bioremediation of the contaminants) in order to restore a contaminated environment. *Ex situ* treatment facilities generally involves a bioreactor or an effluent treatment plant for degradation of contaminants and *in situ* treatment generally involves natural attenuation or intentional biostimulation of indigenous microbial communities or bioaugmentation (Desai et al. 2010). Ideally bioremediation strategies should be designed based on the knowledge of indigenous microbial community and their metabolic capabilities under environmental stress (Lovley 2003). However, in reality much of the information is not available and whatever is available is not assembled and linked with other information. But it is indeed difficult to compile all (successful and failed) bioremediation strategies and compare them as many different factors (such as aerobic/anaerobic, soil texture, moisture, pH, nutrients, season and many other parameters along with indigenous microbial population) influence bioremediation processes. Moreover, a success or a failure of a strategy in one case/application does not imply that it will be the same way in other cases/applications. Hence, the utmost priority is to analyse the capabilities of indigenous microbial populations and based on that information necessary modifications may be made to fasten the bioremediation process.

In this chapter we describe the molecular profiles of microbial activities and current scenario and future applications of omic technologies in bioremediation

strategies. A detailed description and analyses of microbial enzymes and plasmid profiles, involved in biodegradation has been presented. Metagenomics, metatranscriptomics, metaproteomics and metametabolomics are the approaches to move forward in designing novel and fast bioremediation processes. These technologies provide deep insight into molecular aspects of the biodegradation pathways, however there is a need to integrate these technologies to obtain a complete factual picture.

11.2 Tracking Gene Resources, Expression Analysis and Quantification of Functional Genes in Indigenous Microbial Population Actively Involved in Environmental Bioremediation

The molecular and omic technologies have enabled to analyse the biodegradation and bioremediation at molecular level and thus have lead to a paradigm shift in environmental restoration. The detailed genomic/metagenomic, transcriptomic/metatranscriptomic, proteomic/metaproteomic, metabolomic/metametabolomic, bioinformatic and other high-throughput analyses of environmentally relevant microorganisms/environmental sites provide exceptional and novel insights into key biodegradative pathways and about the ability of organisms to adapt to changes in environmental conditions. Figure 11.1 schematically represents how integration of molecular analyses can facilitate in understanding bioremediation strategies.

11.2.1 'Meta' Genomics and 'Meta' Transcriptomics

Metagenomics is to access the total genomic potential of an environmental sample either directly or after enrichment of indigenous microbial communities. The term 'metagenomics' (also known as community genomics, ecogenomics, or environmental genomics) has had the greatest impact within the last few years as it has enabled to explore the uncultivable microorganisms and understand their role in various metabolic pathways. The 'meta' genomics furnishes the details about which genes are present, however it does not inform about which genes are transcribed and functional. Consequently, metatranscriptomics, metaproteomics and metametabolomics analyses become essential to obtain real idea about which genes are playing a functional role. Metatranscriptomics is analyses of transcripts of the entire interacting community to gain insights into the up- or down-regulation of genes under stress conditions in environmental microbial communities. The first regulatory point for successful synthesis of a protein from a gene is regulation of gene expression, one of the key processes necessary for adapting (to sustain) in affected environmental conditions. Often increased mRNA concentration can be at least qualitatively associated with higher rates of contaminant degradation (Schneegurt and Kulpa

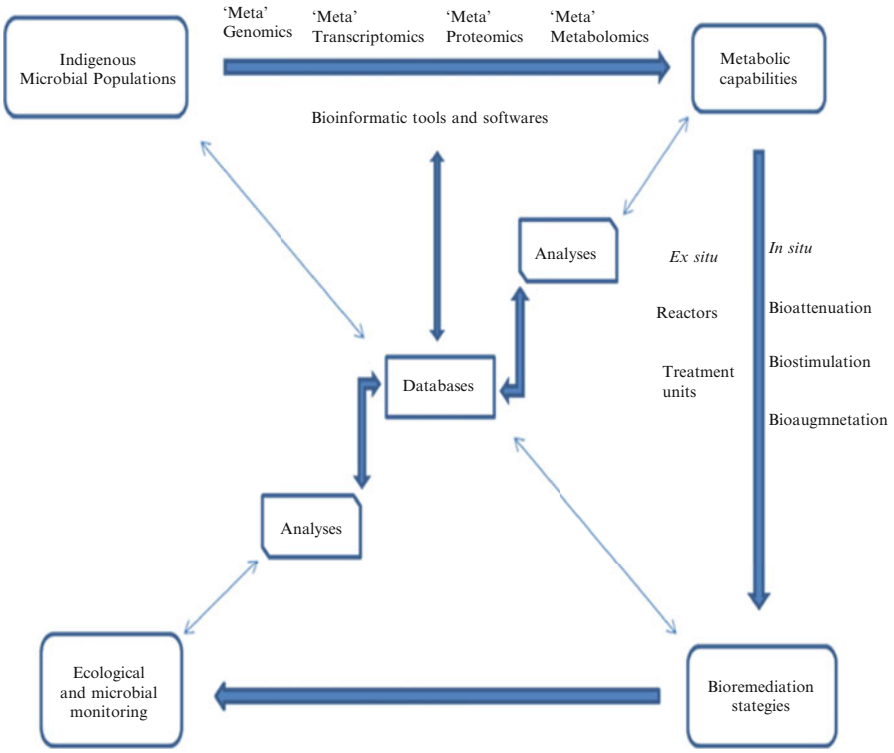

Fig. 11.1 A schematic representation of integration of molecular analyses in understanding bioremediation strategies

1998; Lovley 2003). The biggest concern while studying RNA is that it is much less stable than other molecules and therefore samples have to be properly obtained, processed and stored. Moreover, these steps can introduce bias and hence proper precautions are necessary to acquire authentic and accurate facts.

A significant achievement in the field of microbial ecology was the finding of the 16S rRNA gene and its peculiarity. The gene consists of highly conserved sequences that are present in all microorganisms as well as variable sequences unique to each and is considered as a "gold standard" for characterizing phylogenetic affiliations of micro-organisms (Lane et al. 1985; Amann et al. 1995; Lovley 2003). Nucleic acid based molecular techniques such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), terminal restriction fragment length polymorphism (T-RFLP), amplified ribosomal DNA restriction analysis (ARDRA), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), automated rRNA intergenic spacer analysis (ARISA) and single strand conformation polymorphism (SSCP) are used in conjunction with fluorescence *in situ* hybridization, PCR technologies, sequencing techniques and microarrays. These combined studies, enable us to monitor the changes in bacterial

community and provide insights on up/down regulations of genes, microenvironment of the sites and impact of nutrients on bioremediation. These facts in turn assist in process optimization, validation and understanding the impact of designed bioremediation strategies on the ecosystem. Fluorescence in situ hybridization in conjunction with microautoradiography establishes both phylogenetic as well as functional links of the species involved in the process of bioremediation (Rogers et al. 2007). Recent advances in molecular techniques, including high-throughput approaches such as microarrays and metagenomics, have opened up new perspectives and pointed towards new opportunities in pollution abatement and environmental management. The current potential of microarrays and metagenomics is capable to investigate the genetic diversity of environmentally relevant micro-organisms and identify new functional genes involved in the catabolism of xenobiotics (Eyers et al. 2004). The computational-based annotation and comparative genomic analyses of DNA sequences have provided information regarding gene function, genome structures, biological pathways, metabolic and regulatory networks, and evolution of microbial genomes, which has greatly enhanced our understanding of microbial metabolism (Schoolnik 2001; Ward and Fraser 2005; Sharan and Ideker 2006; Cardenas and Tiedje 2008; Rocha 2008; Zhang et al. 2010).

There are numerous reports on sequencing of complete genome or functionally essential genes involved in metabolism of biodegradation (Nelson et al. 2002; Kim et al. 2008; Mattes et al. 2008; Desai et al. 2010). The genome sequence of *Pseudomonas* sp. has revealed details about oxygenases, oxidoreductases, ferredoxins and cytochromes, dehydrogenases, sulfur metabolism proteins and many others. Moreover many operons coding for the metabolism of a large number of aromatic compounds and gene clusters encoding for enzymes which are predicted to be involved in the metabolism of non-natural substrates were found (Nelson et al. 2002). Desai et al. (2009) tracked the influence of long-term chromium pollution on soil microbial communities by analysing 16S rRNA gene clone libraries and observed community shifts from *Proteobacteria* to *Firmicutes* at chromium polluted sites. Lee et al. (2010) characterized reductive dechlorinating activities and population dynamics in tidal flat sediments, where tetrachloroethene (PCE) and trichloroethene (TCE) are common groundwater contaminants, using latest titanium pyrosequencing besides 16S rRNA gene clone libraries and dechlorinator-targeted quantitative real-time PCR (qPCR). Nowadays, metagenomic sequencing is generating a wealth of data and as a result there is a continuous augmentation of information in databases. Novel bioinformatic tools and approaches have made it possible to analyse and compare the data at a large-'meta' scale. Many novel and valuable information can be gathered. More and more sequences are phylogenetically affiliated and the sequences are being annotated for more and more number of genes. Besides functional analysis, many research groups are also focussing on phylogenetically linking the functional genes. However, 16S rRNA remains the most valuable phylogenetic marker, but these kind of approaches of phylogenetic affiliation of functional genes will be of immense value in characterization of bacterial communities. One such tool is WebCARMA (a redefined version of CARMA) as a web application for the taxonomic and functional classification of unassembled

(ultra-) short reads from metagenomic communities (Gerlach et al. 2009). DNA stable isotope probing (DNA-SIP) is another technique used in conjunction with metagenomics to establish links between microbial identity and particular metabolic functions. The combination of DNA-SIP and metagenomics not only permits the detection of rare low-abundance species but also facilitates the detection of novel enzymes and bioactive compounds (Chen and Murrell 2010).

The functional screening of metagenomic libraries have led to the discovery of novel genes encoding polyphenol oxidase (Beloqui et al. 2006), ester and glycosyl hydrolase (Ferrer et al. 2005) and also given indications on the diversity of extradiol dioxygenases in coke plant wastewater (Suenaga et al. 2007). Brennerova et al. (2009) revealed the diversity and abundance of meta-cleavage pathways in microbial communities from soil highly contaminated with jet fuel (aliphatic and aromatic hydrocarbons) under air-sparging bioremediation using metagenomics. Moreover, the extradiol dioxygenase diversity was assessed by functional screening of a fosmid library in *Escherichia coli* with catechol as substrate.

Ní Chadhain et al. (2006) used a combination of techniques to study degradation of polycyclic aromatic hydrocarbons (PAHs) by bacteria. They designed PCR primers targeting the gene fragment encoding the Rieske iron sulfur center common to all PAH dioxygenase enzymes to track dioxygenase gene population shifts in soil enrichment cultures following exposure to naphthalene, phenanthrene, or pyrene. PAH degradation was monitored by gas chromatograph with flame ionization detection. Molecular monitoring of the enrichment cultures before and after PAH degradation using denaturing gradient gel electrophoresis and 16S rRNA gene libraries provided information about specific phylotypes of bacteria that were associated with the degradation of each PAH (Ní Chadhain et al. 2006). Jorgensen (2008) in his review has shown that Real-time PCR in actual bioremediation studies have been concerned with naphthalene (Debruyn et al. 2007), nitro aromatics (Indest et al. 2007), arsenic (Sun et al. 2004) and anaerobic degradation of hydrocarbons (Beller et al. 2002). Baek et al. (2009) evaluated the activity and abundance of the crude oil degrading bacterium *Nocardia* sp. H17-1 during bioremediation of oil-contaminated soil, by quantifying the genes encoding 16S rRNA, alkane monooxygenase and catechol 2,3-dioxygenase using real-time PCR. When RT-PCR was applied in conjunction with DGGE to study a petroleum-hydrocarbon contaminated site, the genes of phenol hydroxylase (PHE), ring-hydroxylating toluene monooxygenase (RMO), naphthalene dioxygenase (NAH), toluene monooxygenase (TOL), toluene dioxygenase (TOD), and biphenyl dioxygenase (BPH4) were quantified by RT-PCR and variations in microbial community were analysed by DGGE. Knowledge and comprehension obtained from this study was helpful in evaluating the occurrence and effectiveness of intrinsic bioremediation of petroleum-hydrocarbon contaminated groundwater (Kao et al. 2010). Bordenave et al. (2009) applied RT-PCR for analysis of differentially expressed cDNA involved in microbial mat response after heavy fuel contamination.

The development of microbial ecological DNA microarrays has enabled researchers to simultaneously analyze thousands of phylogenetic links and functional genes

in order to characterize microbial communities involved in bioremediation (He et al. 2007). Microarrays, developed based on sequence analysis, have been used to monitor genes involved in 2,4-dichlorophenoxyacetic acid (2,4-D) degradation in environmental samples (Dennis et al. 2003). Loy et al. (2005) used a 16S rRNA gene-targeted oligonucleotide microarray (RHC-PhyloChip) for cultivation-independent diversity analysis of betaproteobacterial order '*Rhodocyclales*' in activated sludge from an industrial wastewater treatment plant. Functional gene arrays (FGA) are constructed using PCR amplified products or oligonucleotides derived from functional genes and are used to identify genes encoding key enzymes involved in various ecological and environmental processes such as carbon fixation, nitrification, denitrification, sulphate reduction and contaminant degradation (Rhee et al. 2004). He et al. (2007) developed GeoChip, a comprehensive microarray containing 24,243 oligonucleotide (50 mer) probes and covering more than 10,000 genes in more than 150 functional groups and in 2010 they (He et al. 2010b) have developed a new generation gene array, GeoChip 3.0 with around 28,000 probes covering approximately 57,000 gene variants from 292 functional gene families involved in nitrogen, carbon, sulphur and phosphorous cycling, metal reduction and resistance, and organic contaminant degradation. GeoChip 3.0 is a high-throughput powerful tool for tracking the dynamics of microbial community, functional structure and linking microbial communities to ecosystem processes and functioning in *in situ* bioremediation study. Consequently these techniques can be used in bioremediation studies to identify microbial populations in ecosystems, assess shifts in microbial populations due to xenobiotic compounds, measure changes in expression levels (gene expression profiling), co-relate and compare genomes, identify genes encoding enzymes, detect mutations and single nucleotide polymorphisms (SNPs). He et al. (2010a) carried out metatranscriptomic analysis of gene expression and regulation of *Candidatus Accumulibacter* enriched lab-scale sludge during enhanced biological phosphorus removal (EBPR) using medium density oligonucleotide microarrays. They analysed both aerobic and anaerobic phases and detected the expression of a number of genes involved in the carbon and phosphate metabolisms, as proposed by EBPR as well as novel genes discovered through metagenomic analysis.

Maphosa et al. (2010a) in their review have discussed 'Ecogenomics' – the application of genomics to ecological and environmental sciences – defining phylogenetic and functional biodiversity at the DNA, RNA and protein levels. They have described the potential of ecogenomics approaches in developing high-throughput methods for detecting and monitoring organohalide respirers, and for providing improvements to selection, specificity and sensitivity of target biomarkers and their application to evaluate bioremediation strategies. This knowledge helps to elucidate functions and interactions of organisms at the ecosystem level in relation to ecological and evolutionary processes. Mateos et al. (2006) have strains of *Corynebacterium glutamicum* that are resistant to arsenite (up to 60 mM) and using genetic manipulation tools they are attempting to obtain *C. glutamicum* mutant strains which are able to remove arsenic from contaminated water.

11.2.2 'Meta' Proteomics and 'Meta' Metabolomics

Metaproteomics is analysis of protein expression profile of the whole community. The cellular expression of proteins in an organism varies with environmental conditions. The changes in physiological response may occur due to the organism's adaptive responses to different external stimuli such as presence of toxic chemicals in the environment. At present, amongst all the omic technologies, metaproteomic analyses still faces lots of practical challenges. The main one being that still there is no common extraction procedure for all different (hydrophilic/hydrophobic/etc.) kinds of proteins. Moreover, with current technologies we can resolve only a minute fraction of proteins present in complex environmental samples. Metametabolomics is analyses of repertoire of metabolites of entire interacting community. The real time flux analysis of cellular molecules/metabolites within a cell/community over a time period is known as fluxomics (Wiechert et al. 2007).

Kim et al. (2009) in their review have given a summary on proteomic approaches used to study bacterial degradation of aromatic hydrocarbons. Roma-Rodrigues et al. (2010) applied two dimensional gel electrophoresis (2DE) to quantitatively compare the membrane proteins of *Pseudomonas putida* KT2440, before and after phenol exposure. This helped in understanding the *Pseudomonas* adaptation to solvents and possible impact in biodegradation, bioremediation and biocatalysis. Chuang et al. (2010) applied targeted shotgun mass spectrometry-based proteomic methods to ethene-enriched groundwater microcosms from a vinyl chloride-contaminated site and identified polypeptides from the enzymes alkene monooxygenase (EtnC) and epoxyalkane: CoM transferase (EtnE), both of which are expressed by aerobic etheneotrophs and vinyl chloride-assimilating bacteria. Wilmes and Bond (2004) successfully carried out extraction and purification of the entire proteome from a laboratory-scale activated sludge system for enhanced biological phosphorus removal, separation by two-dimensional polyacrylamide gel electrophoresis and mapping of the metaproteome. Highly expressed protein spots were excised and identified using quadrupole time-of-flight mass spectrometry and *de novo* peptide sequencing. Wilmes et al. (2008) applied 2-DE along with MALDI-TOF MS and Q-TOF MS/MS to identify highly expressed proteins in a mixed culture activated sludge system and this study provided direct evidence linking the metabolic activities of '*Accumulibacter*' to the chemical transformations observed in EBPR. Singh and Chandel (2009) applied two-dimensional gel electrophoresis (2DE) in conjunction with mass spectrometry (MS) and identified a set of catabolic enzymes and heat shock molecular chaperones associated with the regulatory network that was found to be overexpressed under phenol-stressed conditions. Benndorf et al. (2007) applied 2-DE along with LC-ESI/MS for functional analysis of metaproteomes of soil enriched with 2,4-dichlorophenoxy acetic acid (2,4-D) and groundwater from the aquifer of a chlorobenzene-contaminated site. Ram et al. (2005) carried out an extensive metaproteomic study using genomics and mass spectrometry based methods. They evaluated gene expression, identified key activities and examined partitioning of metabolic functions in a natural acid mine

drainage (AMD) microbial biofilm community. D'Souza-Ticlo et al. (2009) purified and characterized a thermostable metal-tolerant laccase having bioremediation potential. The protein Lac IId was purified by 2DPAGE and analysed by N-terminal sequencing and internal peptide sequencing.

Pseudomonas putida is a model organism for bioremediation because of its remarkable metabolic versatility, extensive biodegradative functions, and ubiquity in contaminated soil environments. Thompson et al. (2010) characterized proteome profile of aerobically grown Cr(VI)-stressed *Pseudomonas putida* F1 in two dissimilar nutritional environments: rich (LB) media and minimal (M9L) media containing lactate as the sole carbon source. Comparative analysis indicated that the core molecular response to chromate, irrespective of the nutritional conditions tested, comprised seven up-regulated proteins belonging to six different functional categories including transcription, inorganic ion transport/metabolism, and amino acid transport/metabolism. These proteins might potentially serve as indicators of chromate stress in natural microbial communities. However, TonB-dependent siderophore receptors involved in ferric iron acquisition and amino acid adenylation domains characterized up-regulated systems under LB-Cr(VI) conditions, while DNA repair proteins and systems scavenging sulfur from alternative sources (e.g., aliphatic sulfonates) tended to predominate the up-regulated proteome profile obtained under M9L-Cr(VI) conditions (Thompson et al. 2010).

Trautwein et al. (2008) applied 2D-DiGE to quantify the proteomic response of the denitrifying bacterium *Aromatoleum aromaticum* Strain EbN1 to solvent stress. They determined up-regulation of PHB granule-associated phasins, cytochrome *cd1* nitrite reductase of denitrification, and several proteins involved in oxidative (e.g., SodB) and general (e.g., ClpB) stress responses. Wu et al. (2010) performed systematic analyses at the transcriptomic [SDS-PAGE] and proteomic [Isotope-Coded Affinity Tag (ICAT)] levels to investigate the expression changes due to high Mn in environment. These studies revealed that under conditions of increased Mn concentration, there was a change in regulation of proteins involved in virulence (e.g., pilin, a key adhesin), oxidative stress defence (e.g., superoxide dismutase), cellular metabolism, protein synthesis, RNA processing and cell division. Mn regulation of inorganic pyrophosphatase (Ppa) indicated the potential involvement of phosphate metabolism in the Mn-dependent oxidative stress defence. A detailed analysis of the role of Ppa and polyphosphate kinase (Ppk) in the gonococcal oxidative stress response revealed that ppk and ppa mutant strains showed increased resistance to oxidative stress. Kim et al. (2006) carried out quantitative proteomic analysis of aromatic pathways in *Pseudomonas putida* KT 2440 using 2-DE/MS and cleavable isotope-coded affinity tag (ICAT) to determine whether proteins involved in aromatic compound degradation pathways were altered as predicted by genomic analysis carried out by Jiménez et al. (2002). The lack of complete knowledge restricts progress in the site-specific mineralization process. In the postgenomic era the emphasis is on 2D PAGE and MS to obtain the incomplete biological information regarding the regulation of growth and metabolism in microbial communities (Singh 2006). Moreover, Singh (2006) in his review emphasizes the need of combining proteomic and metabolomic studies. MS can be used in bioremediation studies for identification, purification, characterization

and quantification of key enzymes and metabolites involved in biodegradation pathways as it can be used in conjunction with almost all other techniques. It bestows with complete facts and scenario in proteome profiling, metabolome profiling and real time metabolite profiling known as fluxomics which creates a system-wide approach for studying site-specific microorganisms during active mineralization processes and aid in implementation of cell-free bioremediation.

Keum et al. (2008) evaluated metabolic profiles of *Sinorhizobium* sp. C4 using gas chromatography and mass spectrometry during degradation of phenanthrene in comparison to natural carbon sources. Several intermediates, in tricarboxylic acid cycles and glycolysis, increased during phenanthrene degradation. Accumulation of trehalose was also evident in the phenanthrene-treated bacterium. Accumulation of sulfur amino acids and nicotinic acid suggested the possible oxidative stress conditions during phenanthrene metabolism. Tang et al. (2009) performed a fluxomics analysis on *Shewanella* sp. known to have co-metabolic pathways for bioremediation of toxic metals, radionuclides and halogenated organic compounds. From the metabolic flux analysis of *Shewanella* sp. using GC-MS and statistical, biochemical and genetic algorithms they deduced that *Shewanella* sp. displays a relatively flexible metabolism fluxes when adapting to different carbon sources. Durand et al. (2010) applied *ex situ* Nuclear Magnetic Resonance (NMR) and Liquid Chromatography-NMR (LC-NMR) as complementary tools to LC-Mass Spectrometry (MS) to define the metabolic pathway of mesotrione, an herbicide, by the bacterial strain *Bacillus* sp. 3B6. The complementarities of *ex situ* and LC-NMR identified six metabolites whereas the structures of only four metabolites were suggested by LC-MS. The presence of a new metabolic pathway was evidenced by NMR. The results demonstrate that NMR and LC-NMR spectroscopy provide unambiguous structural information for xenobiotic metabolic profiling. Wharfe et al. (2010) used FT-IR as a metabolite fingerprinting tool for monitoring the phenotypic and biochemical changes in complex bacterial communities capable of degrading phenol in the activated sludge from an industrial bioreactor. This study demonstrates that FT-IR spectroscopy when combined with chemometric analysis is a very powerful high throughput approach for assessing the metabolic capability of complex microbial communities. Wang et al. (2010) applied fourier transform infrared spectroscopy (FT-IR) and gas chromatography-mass spectrometry (GC-MS) for analysis of degradation of lube oil and TDOC (total dissolved organic carbon) by a mixed bacterial consortium. In addition, they analysed that mixed bacterial consortium can degrade benzene and its derivatives and other aromatic ring organic matters more than 97%.

Microbial biosurfactant production is many times linked with biodegradation. Microbial biosurfactants are amphipathic molecules having typical molecular weights of 500–1,500 Da and are made up of peptides, saccharides or lipids or their combinations. In biodegradation process they mediate in solubilisation, mobilisation and/or accession of hydrophobic substrates to microbes or contaminant desorption from soil (Ward 2010). Reddy et al. (2010) studied biodegradation of phenanthrene in conjunction with biosurfactant production. The bacterial strain PDM-3 degraded 93% of phenanthrene in 6 days, as analysed by HPLC. The ability to produce biosurfactant during phenanthrene degradation was detected by the surface tension measurements

of the culture supernatant and the emulsification index. The biosurfactant was identified by its functional groups through FT-IR spectroscopy.

Maphosa et al. (2010b) used a combination of molecular diagnostics with mass-balancing and kinetic modeling to improve insight into organohalide respiring bacteria and metabolite dynamics in an *in situ* dechlorinating bioreactor and showed its utility in monitoring bioremediation. They applied qPCR targeting 16S rRNA and genes encoding chloroethene reductive dehalogenase (tceA, vcrA, bvcA) and found out that *Dehalococcoides* was the dominant dechlorinating microorganism. The presence of all three reductive dehalogenases genes indicated coexistence of several distinct organohalide respiring bacterial populations in the bioreactor and groundwater. They applied molecular monitoring and model simulations to determine degradation performance of an *in situ* dechlorinating bioreactor and its influence on the contamination plume. Mass balancing revealed that main dechlorinating activities were reduction of cis-dichloroethene and vinyl chloride. Sangwan and Wu (2008) have described molecular ecological techniques for direct identification of microbes involved in polylactide biodegradation. The use of molecular ecological techniques aids in identification and investigation of biodegradation mechanisms and for future development of efficient biological treatment or recycling processes.

11.3 Microbial Enzymes Involved in Biodegradation

We will be discussing enzymes involved in bioremediation and proceed according to general classification of enzymes. Moreover, analysis will also involve University of Minnesota Biocatalysis/Biodegradation Database (UM-BBD, http://umbbd.msi.umn.edu/), which was developed in 1995 and is regularly updated. It contains information on 1,246 compounds, 878 enzymes, 1,345 reactions, 275 biotransformation rules and 510 microorganism entries (Gao et al. 2010).

11.3.1 Oxidoreductases

Oxidoreductases catalyse the transfer of electrons from one molecule (reductant) to another molecule (oxidant). In enzyme classification oxidoreductases are classified as EC 1 and are further subdivided into 22 sub classes. From the UM-BBD we can see that enzymes belonging to sub classes other than EC 1.9, 1.15, 1.19 and 1.21 play a role in biodegradation pathways (Table 11.1 – Enzyme sub classes playing role in bioremediation are in **bold**).

Oxidoreductases play a major role in biodegradation pathways. They carry out key essential steps needed in transformation and mineralization of xenobiotic compounds. A biodegradation pathway involves numerous steps and many bifurcations. Many reports are available describing the catalytic steps of oxidoreductases for various compounds. Usually the oxidoreductases are the first enzymes to act on the

Table 11.1 Description of oxidoreductases

Enzyme class	Description
EC 1.1	**Act on the alcohol (CH-OH) group of donors**
EC 1.2	**Act on the aldehyde or oxo group of donors**
EC 1.3	**Act on the CH-CH group of donors**
EC 1.4	**Act on the CH-NH_2 group of donors**
EC 1.5	**Act on the CH-NH group of donors**
EC 1.6	**Act on NADH or NADPH**
EC 1.7	**Act on other nitrogenous compounds as donors**
EC 1.8	**Act on sulphur as donors**
EC 1.9	Act on heme as donors
EC 1.10	**Act on diphenols and related substances as donors**
EC 1.11	**Act on peroxide as an acceptor**
EC 1.12	**Act on hydrogen as donors**
EC 1.13	**Act on single donors with incorporation of molecular oxygen**
EC 1.14	**Act on paired donors with incorporation of molecular oxygen**
EC 1.15	Act on superoxide radicals as acceptors
EC 1.16	**Oxidize metal ions**
EC 1.17	**Act on CH or CH_2 groups**
EC 1.18	**Act on iron-sulphur proteins as donors**
EC 1.19	Act on reduced flavodoxin as a donor
EC 1.20	**Act on phosphorous or arsenic in donors**
EC 1.21	Act on X-H and Y-H to form an X-Y bond
EC 1.97	**Others**

complex compounds and hence initiate biodegradation. They play a role in all the major metabolic reactions of biodegradation pathways. Mainly oxidoreductases are generally involved in cellular metabolism, but when under the pressure of xenobiotic compounds they start utilising them for energy sources and thus in turn are useful for bioremediation. In UM-BBD 878 enzymes are listed that play a role in bioremediation and out of them 543 belong to oxidoreductases, clearly showing the importance of these enzymes in biodegradation. Moreover the database also has separate section of 76 and 109 reactions for naphthalene 1,2 dioxygenase and toluene dioxygenase highlighting the importance of these enzymes. Arora et al. (2009) compiled a database of biodegradative oxygenases (OxDBase) (http://www.imtech.res.in/raghava/oxdbase/), which provides common name and synonym, reaction in which enzyme is involved, family and subfamily, structure and gene link and literature citation in the form of web accessible database. The entries are also linked to several external database including BRENDA, KEGG, ENZYME and UM-BBD providing wide background information. At present (October 2010) the database contains information of over 235 oxygenases including both dioxygenases and monooxygenases. OxDBase is the first database that is dedicated to oxygenases and provides comprehensive information about them.

Oxidoreductases can be oxygenases [monooxygenases and dioxygenases – incorporate molecular oxygen into organic substrates (classified mainly to 1.13 and 1.14)], reductases [catalyze reductions, in most cases reductases act similar to oxidases (classified mainly to 1.1, 1.3, 1.4, 1.6, 1.7, 1.8, 1.11, 1.12, 1.20 and 1.97)],

oxidases [enzymes involved when molecular oxygen acts as an acceptor of hydrogen or electrons (classified mainly to 1.2, 1.3, 1.4, 1.5, 1.7, 1.8, 1.10 and 1.20)], dehydrogenases [enzymes that oxidize a substrate by transferring hydrogen to an acceptor that is either $NAD^+/NADP^+$ or a flavin enzyme (classified mainly to 1.1, 1.2, 1.3, 1.4, 1.5 and 1.17)], peroxidases [catalyzes the reduction of hydrogen peroxide (1.11)], hydroxylases [add hydroxyl groups to its substrates (1.14 and 1.97)], laccasses [act on phenols and similar molecules (1.10)] and others such as hydrogenases (1.12), demethylases (1.13), nitrogenases (1.18), denitritases (1.7), dehalogenases (1.97), dechlorinases (1.97) and others.

Syed et al. (2010) have identified six PAH-responsive P450 genes (Pc-pah1-Pc-pah6) inducible by PAHs of varying ring size, namely naphthalene, phenanthrene, pyrene, and benzo(a)pyrene (BaP). The cDNAs of the six Pc-Pah P450s were cloned and expressed in conjunction with the homologous P450 oxidoreductase (Pc-POR). The six recombinant P450 monooxygenases showed PAH-oxidizing activity though with varying substrate specificity towards PAHs (3–5 rings). All six P450s oxidized pyrene (4-ring) into two monohydroxylated products. Pc-Pah1 and Pc-Pah3 oxidized BaP (5-ring) to 3-hydroxyBaP whereas Pc-Pah4 and Pc-Pah6 oxidized phenanthrene (3-ring) to 3-, 4-, and 9-phenanthrol. Ferrer et al. (2010) in their chapter provide an overview of screening methods to identify laccase encoding genes from environmental resources.

11.3.2 Transferases

Transferases catalyse the transfer of a functional group from one molecule (donor) to another molecule (acceptor). Generally the donor molecules are coenzymes. In enzyme classification transferases are classified as EC 2 and are further subdivided into 9 sub classes. From the UM-BBD we can see that enzymes belonging to sub classes other than EC 2.2 and 2.9 play a role in biodegradation pathways (Table 11.2 – Enzyme sub classes playing role in bioremediation are in **bold**).

The microbial degradation of 2,4,6 trinitrotoluene is governed by cometabolism and therefore depends on the growth substrate(s) available in contaminated environments (Stenuit and Agathos 2010). While the discovery and engineering of microorganisms with novel/improved degradative abilities are very challenging, deciphering the physiological roles of promiscuous enzymes involved in TNT biodegradation, such as type II hydride transferases of the Old Yellow Enzyme family, opens new perspectives for bioremediation (Stenuit and Agathos 2010).

11.3.3 Hydrolases

Hydrolases catalyse the hydrolysis of a chemical bond. In enzyme classification hydrolases are classified as EC 3 and are further subdivided into 13 sub classes. From the UM-BBD we can see that enzymes belonging to sub classes EC 3.1, 3.3, 3.5, 3.7, 3.8, 3.10, 3.13 play a role in biodegradation pathways (Table 11.3 – Enzyme sub classes playing role in bioremediation are in **bold**).

Table 11.2 Description of transferases

Enzyme class	Description
EC 2.1	**Transfer one carbon groups**
EC 2.2	Transfer aldehyde or ketone groups
EC 2.3	**Acyltransferases**
EC 2.4	**Glycosyltransferases**
EC 2.5	**Transfer alkyl or aryl groups other than methyl groups**
EC 2.6	**Transfer nitrogenous groups**
EC 2.7	**Transfer phosphorous containing groups**
EC 2.8	**Transfer sulphur containing groups**
EC 2.9	Transfer selenium containing groups

Table 11.3 Description of hydrolases

Enzyme class	Description
EC 3.1	**Act on ester bonds**
EC 3.2	Act on sugars
EC 3.3	**Act on ether bonds**
EC 3.4	Act on peptide bonds
EC 3.5	**Act on carbon-nitrogen bonds**
EC 3.6	Act on acid anhydrides
EC 3.7	**Act on carbon-carbon bonds**
EC 3.8	**Act on halide bonds**
EC 3.9	Act on phosphorous-nitrogen bonds
EC 3.10	**Act on sulphur-nitrogen bonds**
EC 3.11	Act on carbon-phosphorous bonds
EC 3.12	Act on sulphur-sulphur bonds
EC 3.13	**Act on carbon-sulphur bonds**

Hydrolases are the second most abundant classes of enzymes involved in bioremediation processes. Mandrich et al. (2010) have focused on the enzymes belonging to the amidohydrolase superfamily as many of them are endowed with promiscuous activities. Recently, a new family of microbial lactonases with promiscuous phosphotriesterase activity, dubbed PTE-Like Lactonase (PLL), has been ascribed to the amidohydrolase superfamily. Among members of this family are enzymes found in the archaea *Sulfolobus solfataricus* and *Sulfolobus acidocaldarius*, which show high thermophilicity and thermal resistance. Enzymes showing phosphotriesterase activity are attractive from a biotechnological point of view because they are capable of hydrolysing the organophosphate phosphotriesters (OPs), a class of synthetic compounds employed worldwide both as insecticides and chemical warfare agents. Thermostable enzymes able to hydrolyse OPs are considered good candidates for the set-up of efficient detoxification tools (Mandrich et al. 2010). Merone et al. (2010) using directed evolution strategy improved the promiscuous activity of thermostable Phosphotriesterase-Like Lactonase from *Sulfolobus solfataricus* against deadly nerve gas Cyclosarin. Kalyani et al. (2009) identified that *Pseudomonas aeruginosa* degraded alpha and beta endosulfan up to

50.25% and 69.77%, respectively in 20 days. Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,3,4-benzo-dioxathiepin-3-oxide) is a cyclodiene organochlorine currently used as an insecticide all over the world and its residues are posing a serious environmental threat. Degradation of the insecticide was concomitant with bacterial growth reaching up to an optical density of 2.34 at 600 nm and aryl sulfatase (hydrolase) activity of the broth reaching up to 23.93 microg pNP/mL/h. Further, the increase in aryl sulfatase activity of the broth with the increase in degradation of endosulfan suggests the probable involvement of the enzyme in the transformation of endosulfan to its metabolites.

11.3.4 Lyases

Lyases catalyse the breaking of various chemical bonds by means other than hydrolysis and oxidation and often resulting in formation of a multiple bonds or a new ring structure. In enzyme classification lyases are classified as EC 4 and are further subdivided into seven sub classes. From the UM-BBD we can see that enzymes belonging to sub classes other than EC 4.6 play a role in biodegradation pathways (Table 11.4 – Enzyme sub classes playing role in bioremediation are in **bold**).

Chien et al. (2010) analysed organomercurials removal by heterogeneous merB genes. Organomercury lyase (MerB) is a key enzyme in bacterial detoxification and bioremediation of organomercurials. The merB1 gene from *Bacillus megaterium* MB1 conferred the highest volatilization ability to methylmercury chloride, ethylmercury chloride, thimerosal and p-chloromercuribenzoate, while the merB3 from *B. megaterium* MB1 conferred the fastest mercury volatilization activity to p-chloromercuribenzoate. The substrate specificities among these MerB enzymes show the necessity for selecting the appropriate bacteria strains or MerB enzymes to apply them in bioremediation engineering for cleaning up specific organomercurial contaminations.

11.3.5 Isomerases

Isomerases catalyze the structural rearrangement of isomers. In enzyme classification isomerases are classified as EC 5 and are further subdivided into 6 sub classes. From the UM-BBD we can see that enzymes belonging to sub classes other than EC 5.99 play a role in biodegradation pathways (Table 11.5 – Enzyme sub classes playing role in bioremediation are in **bold**).

Degradation of 4-methylcatechol via intradiol cleavage usually leads to the formation of 4-methylmuconolactone (4-ML) as a dead-end metabolite. Mineralization of 4-ML is initiated by the 4-methylmuconolactone methylisomerase. This reaction produces 3-ML and is followed by a rearrangement of the double bond catalyzed by the methylmuconolactone isomerase (Marín et al. 2010).

Table 11.4 Description of lyases

Enzyme class	Description
EC 4.1	**Cleave carbon-carbon bonds**
EC 4.2	**Cleave carbon-oxygen bonds**
EC 4.3	**Cleave carbon-nitrogen bonds**
EC 4.4	**Cleave carbon-sulphur bonds**
EC 4.5	**Cleave carbon-halide bonds**
EC 4.6	Cleave phosphorous-oxygen bonds
EC 4.99	**Others**

Table 11.5 Description of isomerases

Enzyme class	Description
EC 5.1	**Catalyze racemisation and epimerisation**
EC 5.2	**Catalyze isomerisation of geometric isomers**
EC 5.3	**Catalyze intramolecular transfer of electrons**
EC 5.4	**Catalyze intramolecular transfer of functional group**
EC 5.5	**Catalyze intramolecular breaking of bonds**
EC 5.99	Others

Table 11.6 Description of ligases

Enzyme class	Description
EC 6.1	Form carbon-oxygen bonds
EC 6.2	**Form carbon-sulphur bonds**
EC 6.3	Form carbon-nitrogen bonds
EC 6.4	**Form carbon-carbon bonds**
EC 6.5	Form phosphoric ester bonds
EC 6.6	Form nitrogen-metal bonds

11.3.6 Ligases

Ligases catalyze the joining of two molecules by forming a new chemical bond usually accompanied with hydrolysis. In enzyme classification ligases are classified as EC 6 and are further subdivided into 6 sub classes. From the UM-BBD we can see that enzymes belonging to sub classes EC 6.2 and 6.4 play a role in biodegradation pathways (Table 11.6 – Enzyme sub classes playing role in bioremediation are in **bold**).

Ligases are enzymes, involved in joining of molecules and not breaking of molecules required for biodegradation of xenobiotic compounds. Yet, ligases are occasionally involved in biodegradation pathways. They add some co-factor or group to make the compound susceptible to degradation by other enzymes. One such example is benzoate-CoA ligase adds CoA to benzoate and leads to formation of benzoyl-CoA on which later on reductases, hydratases and others can act for complete biodegradation.

11.4 Molecular Profile of Plasmids in Bioremediation

The increase in the amount of xenobiotic pollutants in the environment has resulted in the evolution of novel pathways by bacteria for the biodegradation of the organic pollutants. Plasmids have been implicated to carry the catabolic genes involved in the biodegradation of many of these complex xenobiotics (Tan 1999). Top and Springael (2003) in their review discuss the role of mobile genetic elements in allowing bacterial communities to adapt to new xenobiotic compounds. According to their analysis construction of novel pathways seems to occur by an assembly process that involves horizontal gene transfer: different appropriate genes or gene modules that encode different parts of the novel pathway are recruited from phylogenetically related or distant hosts into one single host. The catabolic genes are prone to undergo genetic rearrangement and this is due to their association with transposons and insertion sequences (Tan 1999; Nojiri et al. 2004; Springael and Top 2004).

Martinez et al. (2001) provide insight into how plasmids evolved and are structured to encode the catabolism of compounds recently added to the biosphere. They determined complete 108,845-nucleotide sequence of catabolic plasmid pADP-1 from *Pseudomonas* sp. strain ADP. Regions encoding transfer and replication functions of pADP-1 had 80–100% amino acid sequence identity to an IncPb plasmid, pR751, previously isolated from *Enterobacter aerogenes*. pADP-1 contains a functional mercury resistance operon with 99% identity to Tn*5053*. Copies of transposases had 99% amino acid sequence identity to TnpA from IS*1071* and TnpA from *Pseudomonas pseudoalcaligenes*. By functional analyses they identified three new catabolic genes, *atzD*, *atzE*, and *atzF,* which participate in atrazine catabolism. AtzD showed 58% amino acid sequence identity to TrzD, a cyanuric acid amidohydrolase, from *Pseudomonas* sp. strain NRRLB-12227. Crude extracts from *Escherichia coli* expressing AtzD hydrolyzed cyanuric acid to biuret. *E. coli* strains bearing *atzE* and *atzF* were shown to encode a biuret hydrolase and allophanate hydrolase, respectively. These data reveal the complete structure of a catabolic plasmid and show that the atrazine catabolic genes are dispersed on three disparate regions of the plasmid (Martinez et al. 2001). Park and Kim (2000) demonstrated that *Pseudomonas putida* HS12 is able to degrade nitrobenzene due to plasmids pNB1 and pNB2, which carry the catabolic genes. Dennis and Zylstra (2004) characterized the 83 kilobase naphthalene degradation plasmid from *Pseudomonas putida* strain NCIB 9816–4. The distinguishing feature of this plasmid is that naphthalene degrading operons are bordered by numerous defective mobile genetic elements.

Sota et al. (2003) analysed the IncP-1β plasmid pUO1 isolated from *Delftia acidovorans* strain B. On characterization of the residing haloacetate-catabolic transposons, Tn*Had1* and Tn*Had2*, and the *mer* genes for resistance to mercury it was revealed that the *mer* genes were also carried by two Tn*402*/Tn*5053*-like transposons, Tn*4671* and Tn*4672*, and that the pUO1 backbone regions shared 99% identity to those of the archetype IncP-1β plasmid R751 (Sota et al. 2003). Schlüter

et al. (2007) isolated IncP-1β Plasmid pGNB1 from bacterial community residing in the activated sludge compartment of a wastewater treatment plant by using a transformation-based approach. This 60-kb plasmid harbouring triphenylmethane reductase gene *tmr* enables its host bacterium to decolorize triphenylmethane dyes crystal violet, malachite green and basic fuchsin.

Jencova et al. (2008) characterized the plasmid pA81 harboured by the bacterium *Achromobacter xylosoxidans* A8. The genes encoding (halo)aromatic degradation were clustered in type I transposon and heavy metal resistance genes were clustered in type II transposon. Moura et al. (2010) investigated the mobile gene pool present in wastewater environments using DGGE, PCR and Southern hybridization techniques. From their observations, they inferred that wastewater environments promote the development of bacterial communities that support and bring together different types of molecular elements that, in association, play a major role in bacterial adaptation and evolution.

Many times it is the plasmid, which harbours the catabolic genes for biodegradation of xenobiotic contaminants. Moreover, the catabolic genes are generally present associated with transposons and insertion elements, clearly indicating the role of these mobile genetic elements in mobility of such catabolic genes and thus leading to divergent evolution.

11.5 Elucidating the Metabolic Pathways Involved in Biodegradation

Hundreds of xenobiotic compounds, hundreds of enzymes, hundreds of reactions, hundreds of interlinked pathways and add to this environmental conditions (aerobic or anaerobic, nutrient availability, moisture and other factors) making biodegradation in principle a complex process but to keep it simple all reactions have one aim to follow and that is, mineralization into simple components, whatsoever the compound may be. Thermodynamic feasibility, chemical equilibrium, reaction dynamism and many such other factors play a vital role in biodegradation mechanism. Many intermediates can follow two different pathways for their complete mineralization. The best example is of catechol which can be degraded by ortho cleavage as well as meta cleavage pathway. These differences do reflect some significance at the genetic level. Generally genes that encode enzymes of the ortho-cleavage pathways mostly reside on chromosome/genome, whereas those of meta-cleavage pathways are mostly harboured by plasmids (Houghton and Shanley 1994). This also establishes the importance of other factors playing a role in the mechanism. However, there are certain factors which do tend to favour a particular pathway. Moreover, compounds belonging to same class or group of compounds, having similar kind of core structure or functional groups generally are metabolized to common intermediates and thereafter the pathway is common. Many a times this common intermediates are shared even by many pathways. In many cases these intermediates interconvert depending on reaction dynamism. There are many common

intermediates such as catechol, 4-methylcatechol, protocatechuate, 4-hydroxyl benzoate, maleylacetate, 4-hydroxyl 2-oxopentanoate, homogentisate, glyoxylate, propanoate, cyanuric acid and others for aerobic pathways and benzoyl-CoA, acetyl-CoA and others for anaerobic pathways. Moreover, there are many other intermediates which may or may not be mentioned in the description of this chapter. Certain compounds undergo aerobic biodegradation, in such cases oxygen is generally the electron acceptor. However, many contaminated environmental sites are anoxic and in such cases microorganisms can anaerobically oxidize contaminants with alternative electron acceptors such as nitrate, sulphate, Fe (III) oxides and others. In certain bioremediation reactions contaminants are electron acceptors rather than electron donors, the most prominent example is reductive dechlorination (Lovley 2003). The other important factor that plays a role in degradation of xenobotic compounds is that it may be that, initial reactions require anaerobic environment and final reactions may require aerobic environment. These kinds of need make the biodegradation tricky for certain compounds. The ultimate necessity is that the intermediates formed must be able to enter any of the central metabolic pathways such as glycolysis, Kreb's cycle or others so that they are completely mineralized and does not lead to formation of any dead end metabolites.

Martínková et al. (2009) in their review have discussed the biodegradation potential of the genus *Rhodococcus*, their redundant and versatile catabolic pathways, their ability to uptake and metabolize hydrophobic compounds, to form biofilms, to persist in adverse conditions. The availability of recently developed tools for genetic engineering in *Rhodococci* make them suitable industrial microorganisms for biotransformations and the biodegradation of many organic compounds. The peripheral and central catabolic pathways in *Rhodococci* are characterized for each type of aromatics (hydrocarbons, phenols, halogenated, nitroaromatic, and heterocyclic compounds) and pathways involved in the hydrolysis of nitrile pollutants (aliphatic nitriles, benzonitrile analogues) and the corresponding enzymes (nitrilase, nitrile hydratase) are described in detail. Shen et al. (2010) cloned and sequenced a 9.2-kb DNA fragment encoding the enzymes of a p-nitrophenol catabolic pathway from *Pseudomonas putida* DLL-E4. They found ten open reading frames (ORFs) and functionally verified five ORFs. PnpA is a flavin adenine dinucleotide-dependent single-component PNP 4-monooxygenase which converts p-nitrophenol to para-benzoquinone in the presence of NADH and FAD. PnpC is a 1,2,4-trihydroxybenzene 1,2-dioxygenase which converts 1,2,4-trihydroxybenzene to maleylacetate. The hydroquinone dioxygenase (PnpC1C2) multi-component protein complex was expressed in *Escherichia coli* via plasmid pET-pnpC1C2 containing pnpC1 and pnpC2. This complex converts hydroquinone to gamma-hydroxymuconic semialdehyde. PnpR is a positive regulator involved in hydroquinone degradation in pnp gene cluster. They demonstrated that a pathway encoded by the pnp gene cluster is involved in degradation of hydroquinone and 1,2,4-trihydroxybenzene in *P. putida* DLL-E4.

Manickam et al. (2007) isolated *Xanthomonas sp.* ICH12 capable of biodegrading gamma-hexachlorocyclohexane. They employed DNA-colony hybridization method to detect bacterial populations containing specific gene sequences of the

gamma-HCH degradation pathway and for that linA (dehydrodehalogenase), linB (hydrolytic dehalogenase) and linC (dehydrogenase) from *Sphingomonas paucimobilis* UT26 were used as gene probes against isolated colonies. They carried out gas chromatography-mass spectrometric (GC-MS) analysis for detection of intermediate metabolites (Manickam et al. 2007). From the partial genome of extreme halophilic archaea *Haloterrigena* sp. H13, Ding and Lai (2010) identified genes of (S)-2-haloacid dehalogenase (EC 3.8.1.2) and salicylate hydroxylase (EC 1.14.13.1) that may be involved in 1,2-dichloroethane degradation, naphthalene/anthracene degradation, gamma-hexachlorocyclohexane degradation, 1-/2-methylnaphthalene degradation and benzoate degradation via CoA ligation.

Cyanide is synthesized, excreted and metabolized by hundreds of organisms, including bacteria, algae, fungi, plants and insects. Several cyanide compounds are also produced by industrial activities, resulting in serious environmental pollution (Cipollone et al. 2008). Bioremediation has been exploited as a possible alternative to chemical detoxification of cyanide compounds, and various microbial systems allowing cyanide degradation have been described. Cipollone et al. (2008) have discussed the implicated enzymatic pathways involving hydrolytic, oxidative, reductive, and substitution/transfer reactions involved in detoxification of cyanide by bacteria and fungi. Moreover, critical issues limiting the application of a rhodanese-based cellular system to cyanide bioremediation are also discussed in the review. Dash et al. (2009) have also described the mechanism and advances in the use of biological treatment for the removal of cyanide compounds and their advantages over other treatment processes. They have also discussed various microbial pathways for cyanide removal.

Howsawkeng et al. (2010) investigated possibility of simultaneous activity of superoxide-mediated transformations and heterotrophic aerobic bacterial metabolism in a microbial-MnO_2-catalyzed Fenton-like system. The results demonstrate that bacterial metabolism can occur in the presence of superoxide-mediated transformations. Such coexisting reactions may occur when H_2O_2 is injected into MnO_2-rich regions of the subsurface as a microbial oxygen source or for *in situ* oxidation. However, process control of such coexisting transformations may be difficult to achieve in the subsurface due to heterogeneity. Alternatively, hybrid abiotic reduction-biotic oxidation systems could be used for the treatment of industrial effluents or dilute solvent wastes that contain traces of highly halogenated compounds (Howsawkeng et al. 2010).

Catechols are central intermediates in the metabolism of aromatic compounds. However, degradation of 4-methylcatechol via intradiol cleavage usually leads to the formation of 4-methylmuconolactone (4-ML) as a dead-end metabolite. Mineralization of 4-ML is initiated by the 4-methylmuconolactone methylisomerase. This reaction produces 3-ML and is followed by a rearrangement of the double bond catalyzed by the methylmuconolactone isomerase. 4-methyl-3-oxoadipate enol-lactone hydrolase belonging to metallo-beta-lactamase superfamily, opens the lactone ring. Further metabolism can be assumed to occur by analogy with reactions known from the 3-oxoadipate pathway. 4-methyl-3-oxoadipyl-coenzyme A (CoA) transferase and thiolase transforming 4-methyl-3-oxoadipyl-CoA into methylsuccinyl-CoA and acetyl-CoA. Acetyl-CoA hydrolase/transferase is crucial for channelling

methylsuccinate into the central metabolism (Marín et al. 2010). This modified 3-Oxoadipate Pathway for the biodegradation of methylaromatics in *Pseudomonas reinekei* MT1 is a wonderful example of involvement of enzymes belonging to mostly all the classes of enzymes and thus highlighting the complexities involved in biodegradation.

The work of Salinero et al. (2009) again highlights that there is much to be learned regarding the metabolic capabilities and life-style of microbial species. Metabolic analysis of the soil microbe *Dechloromonas aromatica* strain RCB revealed that it did not harbour the anticipated (previously characterized) enzymes for anaerobic aromatic degradation. On annotations of genes, it was evident that several metabolic pathways have yet to be observed experimentally.

An increasing amount and variety of xenobiotic compounds are released in environment. It is vital to know the fate of these chemical compounds. However, to find that experimentally without any hint will be a very vague approach. Hence, it will be useful to predict whether a particular compound is biodegradable and it can be mineralized by which pathways. UM-BBD besides providing information about enzymes, compounds, reactions, biotransformation rules and microorganisms; it includes a Biochemical Periodic Table (UM-BPT) and a rule-based Pathway Prediction System (UM-PPS) (http://umbbd.msi.umn.edu/predict/) that predicts plausible pathways for microbial degradation of organic compounds (Gao et al. 2010). Finley et al. (2009) have described a computational framework (called BNICE) that can be used for the prediction of novel biodegradation pathways of xenobiotics. The framework was applied to 4-chlorobiphenyl, phenanthrene, gamma-hexachlorocyclohexane, and 1,2,4-trichlorobenzene, compounds representing various classes of xenobiotics with known biodegradation routes. BNICE reproduced the proposed biodegradation routes found experimentally, and in addition, it expanded the biodegradation reaction networks by generating novel pathways to degrade xenobiotic compounds that are thermodynamically feasible alternatives to known biodegradation routes and attractive targets for metabolic engineering.

11.6 Postscript

A multilevel (individual and community) molecular (genome, transcript, proteome and metabolome) analysis has led to an increase in amount of information available about the microorganisms capable of metabolising recalcitrant compounds. Molecular and biotechnological approaches have provided deep insights into microbial metabolic reactions. All this information is useful in several developments such as strain improvements, recombinant strains, protein engineering and cell free systems for enhanced bioremediation strategies. Microbial enzymes are capable of biodegrading recalcitrant compounds, however, as environmental factors play a vital role in regulation of microbial activities it is essential that these criteria are taken in considerations while designing bioremediation strategies. These enzymes are coded by catabolic genes, present in microbial genomes as well as in extracellular

genetic materials such as plasmids. Conversely, it becomes immensely essential as well as useful to characterize the complete genome. The metabolism of xenobiotic compounds is not simple and involves many complex and integrated pathways. Many a times the mainly preferred pathways are not feasible due to thermodynamic or any other chemical disequilibrium, still microorganisms can mineralize the compounds by channelling them into the pathways by some other intermediates. Consequently, elucidating the complete metabolic pathway of biodegradation is the ultimate essentiality in understanding bioremediation processes.

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Chapter 12
Microbial Degradation of Pyridine and Its Derivatives

D. Madhusudan Reddy and Gopal Reddy

Abstract Two thirds of the known organic chemicals have heterocyclic structures. These chemicals are introduced into the environment through their use, storage and disposal of effluents. These heterocyclic compounds seep through soil layers, because of their higher water solubility than homo-cyclic analogs; they are more easily transported to ground water resulting in serious consequences. Their occurrence in higher concentration than the permissible range will have severe impact on ecology and biodiversity. Most of the times, concentrations of these compounds in the environment are kept under check by abiotic or biotic factors. Abiotic factors include incineration or any other chemical means or photo oxidation. Biotic factors includes involvement of microorganisms called microbial bioremediation that employs bacteria, fungi and algae or phytoremediation where higher plant species are used to absorb and accumulate such toxic compounds. Microbial bioremediation has the benefit of being economic, ecologically more benign and safer than the other processes, neither demand extreme reaction conditions or sophisticated equipments.

Alkylpyridines are toxic environmental pollutants commonly found in many surface and ground waters near synthetic chemical industries where they are extensively used to produce many chemical intermediates, solvents, paints etc. They are reported to be found in more concentrations than pyridine and its other derivatives. Despite their occurrence and toxicity, degradation studies of these chemicals are poorly studied when compared to microbial degradation of homocyclic aromatic compounds. Hence, we have carried out an investigation on the microbial degradation of 2-picoline, its parent nucleus pyridine and some of its derivatives under aerobic conditions in our laboratory. Results of the investigation revealed that these

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compounds were degraded predominantly under aerobic conditions with hydroxyl derivatives as intermediate metabolites. In-silico studysupported with wet lab results for detecting the role of dioxygenase in the biodegradation of 2-picoline and other pyridine derivatives. Therefore, we conclude that, under appropriate conditions, bioremediation of 2-picoline and other pyridine derivatives is a potentially feasible method, aided by dioxygenase enzyme system, for the clean-up of environments contaminated with heterocyclic chemicals and 2-picoline in particular. This article therefore aims at giving an overview of the present status of research in biodegradation of pyridine and its derivatives, in particular alkylpyridines.

Keywords Pyridine • Alkylpyrides • 2-Picoline • Biodegradation • *B.cereus GMHS*

12.1 Introduction

Pyridine and its derivatives are important representatives of heterocyclic compounds. Pyridine ring is a major constituent of natural alkaloids, coenzymes such as nicotinamide, nicotine, cavadin etc. (Bernstein et al. 1966; Houghton and Cain 1972). These compounds are produced by plants and animals and are used as insecticides and pesticides. Pyridine compounds such as 2-hydroxypyridine-1-oxide are also reported to have fungicidal activity against *Microsporum* infections and 2,6-dichloro-4-phenylpyridine-3,5-di-carbonitrile is active against *Alternaria*, *Venturia inaequalis*, *Cladosporium fulvum* and *Plasmospora*. Pyridine is widely employed as a solvent in industry and laboratory. It is used as a denaturant in alcohol and antifreeze mixtures, as a solvent for paint, rubber and polycarbonate resins and as an intermediate in the manufacture of herbicides like paraquat, diquat and picloram which are widely used in agriculture (Calderbank 1968; Maya 1981). It is also used as a solvent and intermediate in the preparation of vitamins and drugs, dyes, textile water repellents and flavouring agents in food. Pyridine is produced from coal and recovery from coke-oven gases and coal tar middle oil. It can also be produced synthetically from the vapour phase reaction of acetaldehyde and ammonia, with formaldehyde and methanol (Jori et al. 1983).

12.1.1 Risk Associated with Exposure to Pyridine

The greatest potential exposure to pyridine and its derivatives is in the workplace, where these chemicals are manufactured or used to synthesize other chemicals. Occupational exposure, usually by inhalation or dermal absorption, may occur during their production or use as chemical intermediates. The U.S. Environment Protection Agency (EPA) estimated that 2,49,000 persons were occupationally exposed to pyridine. According to the National Institute for Occupational Safety and Health (NIOSH) 8 h, time weighted average permissible exposure level for

pyridine is 5 ppm (16 mg/m^3) and a concentration of 3,600 ppm is immediately dangerous to life. A concentration of 10 ppm becomes objectionable to unaccustomed individual and a concentration above 5 ppm leads to olfactory fatigue. EPA regulates pyridine as a toxic waste under the Resource Conservation and Recovery Act (RCRA); a maximum pyridine concentration of 5.0 mg per litre of leachate is allowed using analysis determined by the Toxicity Characteristic Leaching Procedure. Pyridine is listed as a chemical known to the State of California to cause cancer under the safe Drinking Water and Toxic Enforcement Act of 1986 (Proposition 65).

12.1.2 Fate of Pyridine in Various Environments

Pyridine exists in atmosphere as vapour. The estimated atmospheric lifetime of pyridine is 23–46 days. In atmosphere, it gets photo degraded by hydroxyl radicals in the troposphere. A large fraction of atmospheric pyridine vapour phase would trend to dissolve in water vapour due to its high water solubility. Much of the atmospheric pyridine is removed by precipitation and if it is dissolved in water, it does not volatilize readily into the atmosphere. The volatility and sorption of pyridine and its derivatives in water varies considerably and is pH dependent. At concentrations less than 20 mg/L, pyridine degradation was virtually completed in 8 days or less. Many of the pyridine compounds, both natural and synthetic, are ultimately degraded therefore their concentrations do not increase substantially in the soil environment. The carbon and nitrogen skeleton of pyridine is thus mineralized and are recycled. Such recycling is essential because continued addition of compounds, which are completely resistant to degradation, can soon lead to the accumulation of concentrations that are potentially toxic or otherwise unacceptable.

12.1.3 Pyridine Derivatives – Alkyl Pyridines

Alkylpyridines are toxic environmental pollutants commonly found in many surface and ground waters near synthetic liquid fuel industries where they are extensively used to produce many chemical intermediates, solvents, paints etc., (Riley et al. 1981; Stuermer et al. 1982; Turney and Goerlitz 1990). They are reported to be found in more concentrations than pyridine and its other derivatives. Despite their occurrence and toxicity, degradation of these chemicals is poorly studied (Sims and O'Loughlin 1989; Kaiser et al. 1996). There have been numerous laboratory studies on alkylpyridines (Shukla 1974, 1975; Korosteleva et al. 1981; Feng et al. 1994) their degradation by many microorganisms (Kost and Modyanova 1978; Shukla 1974, 1984; Sims and O'Loughlin 1989 and Ronen and Bollag 1995) under both aerobic and anaerobic conditions (Rogers et al. 1985; Kaiser and Bollag 1991).

12.2 Critical Review

12.2.1 Pyridine

Though pyridine is considered as toxic, its concentrations in environment, particularly in soils are kept under check by many microorganisms. *Corynebacterium* sp. and *Brevibacterium* sp. isolated by Shukla (1973) and *Nocardia* strain Z1 isolated by Watson et al. (1974) reported degradation of pyridine. The organisms were unable to transform monohydroxylated pyridines. Hydroxylated pyridines were never detected as metabolites ruling out any role of hydroxylases in pyridine metabolism. Formic acid and ammonia were detected as products of degradation in the absence of any metabolic inhibitor. The degradation product of pyridine in the presence of semicarbazide as metabolic inhibitor was succinate semialdehyde. *Brevibacterium* sp. isolated by Shukla (1973) degraded pyridine and produced succinic acid semialdehyde and pyruvic acid when arsenate was added as metabolic inhibitor. The *Micrococcus luteus* reported by Sims et al. (1986) oxidized pyridine to give aliphatic intermediates. Watson and Cain (1975) studied pathway of pyridine degradation with *Bacillus* strain *4*. This organism produced succinic acid semialdehyde as a product when semicarbazide was used as an inhibitor and in presence of cyanide it produced formic acid from the second carbon atom of this heterocyclic ring. Transformation of pyridine by *Bacillus* strain 4 could follow the pathway suggested by Watson and Cain 1975 (Fig. 12.1). Watson and Cain reported a *Nocardia* strain Z1 that could degrade pyridine resulting in accumulation of glutaric acid semialdehyde. The pathway for pyridine degradation proposed by the same authors is given in Fig. 12.2.

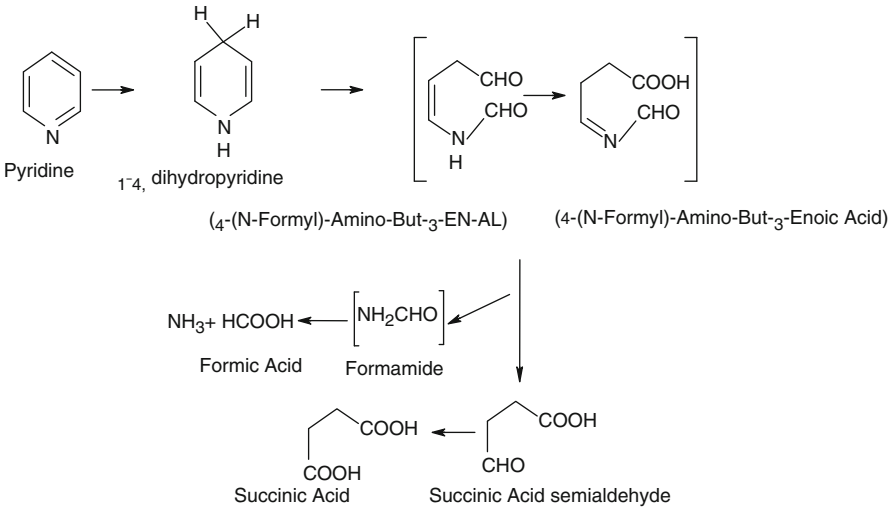

Fig. 12.1 Biotransformation of pyridine by *Bacillus* strain 4 (Watson and Cain 1975)

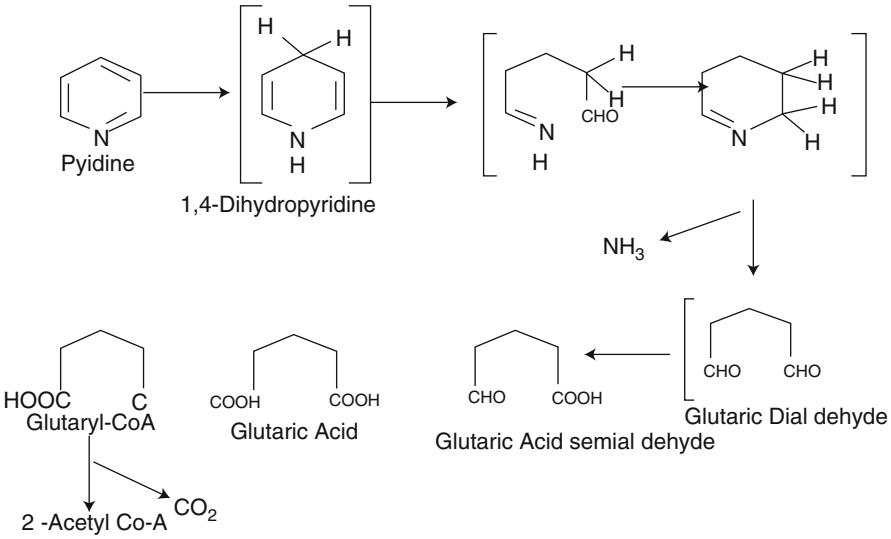

Fig. 12.2 Pyridine biodegradation by *Nocardia* strain Z1 (Watson and Cain 1975)

12.2.2 Hydroxypyridines

Microbial metabolism of hydroxypyridines and other pyridine derivatives are reported by isolating several pure cultures. An *Arthrobacter* sp. degraded 2-hydroxypyridine (Gupta and Shukla 1975), resulting in accumulation of a blue pigment, corresponding to 2, 3, 6-trihydroxypyridine, which eventually converted to maleamate, maleate and pyruvate. Production of similar pigment during 2-hydroxypiridine degradation with other *Arthrobacter* sp. was reported by Ensign and Rittenberg (1963). Kolenbrander and Weinberger (1977) reported 2-hydroxypyridine metabolism by *Arthrobacter crystallopoietes,* A. *pyridinolis* and *A.viridescens* all of which produced extra cellular crystalline pigment. Cell free extracts required reduced NAD, molecular oxygen as well as FMN, suggesting the role of monooxygenase in the degradation activity. The pigment produced was a monopotassium salt of 4, 5, 4′, 5′ tetrahydroxy-3, 3′ diazadiphenoquinone-(2, 2′), structurally related to indigoidine. The role of monooxygenase indicated in the metabolism of 2-hydroxypyridine by the three organisms. In this process plasmid, involvement was detected only in A. *crystallopoietes* and curing experiments supported the same. An *Arthrobacter* sp. was isolated from soil samples of Tarai jungles by aerobic enrichment culture technique in phosphate salt growth medium. This organism utilized pyrrolidine as sole source of carbon, nitrogen and energy for its growth (Ensign and Rittenberg 1963) and metabolized pyrrolidine through Δ-pyrroline, γ-aminobutyraldehyde (GABA), succinic semialdehyde and succinate. The enzymes responsible for pyrrolidine metabolism have been demonstrated in the cell free extracts of this organism.

12.2.3 2-Picolinate

Shukla and Kaul (1973) reported 2-picolinate (pyridine-2-carboxylate) biotransformation by *Bacillus* sp. resulting in the accumulation of 6-hydroxy picolinic acid, 3, 6-dihydroxy picolinic acid and 2, 5-dihydroxy pyridine. Resting cell suspensions of this organism metabolized 2-picolinate to pyruvate and 6-hydroxy picolinate with sodium arsenate as a metabolic inhibitor. A Gram-negative coccus reported by Shukla et al. (1977) transformed 2-picolinate to 6-hydroxy picolinate.

12.2.4 Alkylpyridines (Picolines)

Alkylpyridines or picolines are methylated pyridines, which are considered more toxic than their parent compound pyridine. Their toxicity even lies in position of alkyl group. They are more commonly present in environment than pyridine. However, because of their toxicity they are reported to be more resistant to microbial attack than pyridine. Presence of alkyl group in the ring (in particular 2-picoline) makes the compound selective for only few microorganisms (Shukla 1974, 1975; Korosteleva et al. 1981; Feng et al. 1994). Studies on biodegradation of alkylpyridines were carried out under aerobic and anaerobic conditions.

12.2.5 Biodegradation of Alkylpyridines Under Aerobic Condition

Biodegradations of alkylpyridines were reported by many bacterial and fungal species. Shukla (1974) reported an *Arthrobacter* sp. that degrades 2-picoline and also utilizes 2-ethylpyridine or piperidine as alternate growth substrates. Chromatographic and U.V examination of the fermented broth and methanol extraction of freeze-dried broth failed to show the presence of any metabolite. Even identification of intermediate metabolites using metabolic inhibitors like sodium azide, sodium fluoride, sodium arsenate etc., failed to show any intermediate metabolites. However, a pale yellow pigment having a broad absorption band around 435 nm was reported to be released into the broth during later stages of fermentation that eventually identified as riboflavin. The pathway reported for 2-picoline degradation has some undetected intermediate metabolites (compounds mentioned in parenthesis) as given in Fig. 12.3. O'Loughlin et al. (1995) with an *Arthrobacter* sp. reported similar findings. This organism was able to utilize 2-picoline and 2-ethylpyridine as primary carbon and energy sources. It also utilized 2-, 3-, 4-hydroxyl benzoate, gentisic acid, proto catechuic acid and catechol. Degradation of 2-picoline was accompanied by overproduction of riboflavin. Lee et al. (2001) reported *Gordonia nitida LE31* that could degrade 3-methylpyridine and 3-ethylpyridine. No cyclic intermediates were found, but formic acid was identified as a metabolite. In this degradation pathway, 3-methylpyridine and 3-ethylpyridine were degraded through the enzyme

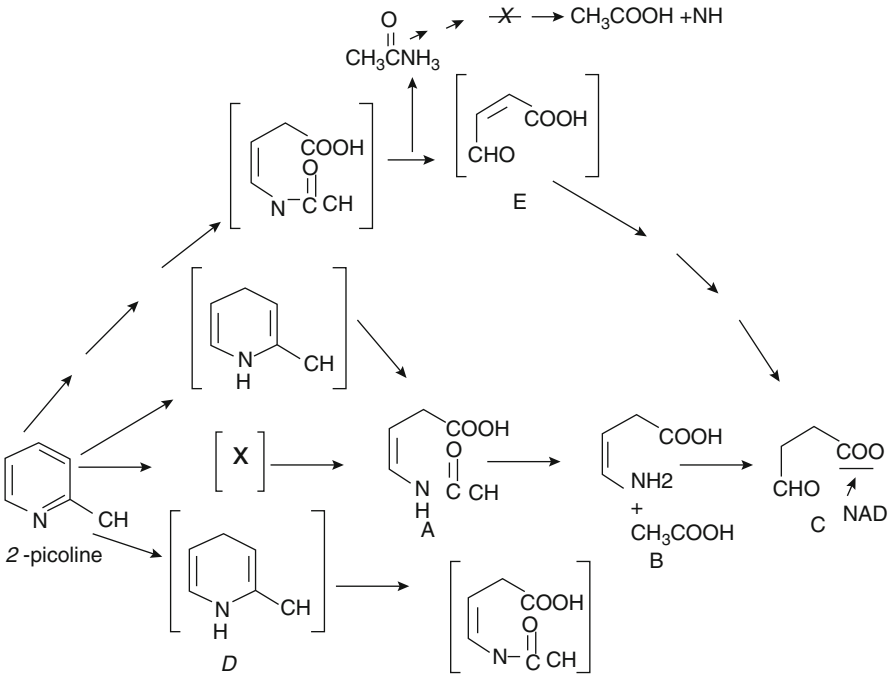

Fig. 12.3 Biodegradation of 2-picoline by *Arthrobacter* species (Shukla 1974) – ***A***. N-acetyl γ-aminobutyric acid; ***B.*** γ-aminobutyric acid; ***C.*** Succinic semialdehyde; ***D.*** 1,4-dihydro-α-picoline; ***E.*** Maleic semialdehyde

system that catalyzed the cleavages of C2–C3 of heterocyclic rings. Coryneform bacteria group reported by Shukla (1975) degraded 2-ethylpyridine, 2, 4-lutidine and 2, 4, 6-collidine. The bacterium also used 2-picoline as a growth substrate after a lag period of about 48 h. However, no metabolites of these pathways were reported. Lee et al. (2001) carried out biodegradation of 3-methylpyridine and 3-ethylpyridine in their laboratory using *Gordonia nitida* LE31. Cells of *Gordonia nitida* LE31 grown on 3-methylpyridine degraded 3-ethylpyridine without a lag time and vice versa. Cyclic intermediates were not detected, but formic acid was identified as a metabolite. The pathway proposed by them was novel involving C2–C3 ring cleavage during biodegradation of 3-methylpyridine and 3-ethylpyridine.

Biodegradation of pyridine, 2-picoline and other pyridine derivatives was studied in our laboratory. Screening of industrial soil samples for isolating efficient degrading microbial consortia by enrichment culturing technique obtained 9 bacterial isolates where bacilli dominated over cocci. Among the isolates, an efficient bacterial isolate was identified by morphological observations as Gram positive, motile and endospore forming bacillus (Madhusudan Reddy et al. 2006). Biochemical and genetic methods identified the bacterium as *Bacillus cereus*. 16S rRNA gene sequence of this bacterium was unique from reported *Bacillus cereus* and its ability to metabolize 2-picoline efficiently designated this bacterium as a novel strain. Hence a strain name *Bacillus cereus* GMHS was given to this isolate and its 16S

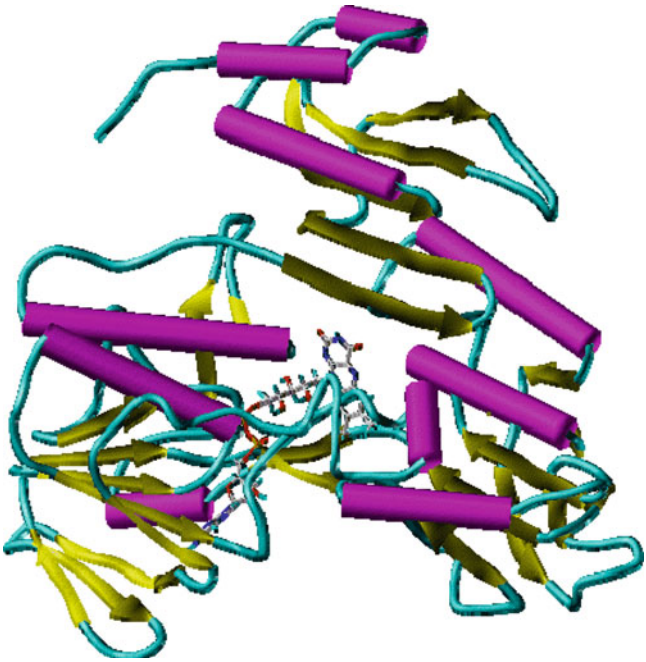

Fig. 12.4 Three dimensional (3D) model for toluene dioxygenase TODA (Energy minimizing and low energy confirmation obtain the structure over the last 1,000 fs of MD simulation. The α-helix is represented in *red* and β-sheet in *yellow*) (Madhusudan Reddy et al. 2008)

rRNA gene sequence is deposited at GENBANK under accession number DQ 351239 (gi no. 84620825) (Madhusudan et al. 2009). Ammonia was accumulated during biodegradation of 2-picoline indicating ring cleavage. No other intermediate metabolites were detected under normal conditions. However, when sodium azide and sodium arsenate were used as metabolic inhibitors, 2-hydroxypicoline and 6-hydroxypicolinic acid were accumulated as intermediate metabolites (Madhusudan Reddy et al. 2009a). A 11 kb plasmid was isolated from *Bacillus cereus* GMHS and role of this plasmid in 2-picoline degradation is evidenced by plasmid curing with acridine orange and hexammine ruthenium (III) chloride. Transformation of this plasmid into *E.coli* DH5α resulted in transfer of degradation character, where untransformed cells were sensitive to 2-pcoline (Madhusudan Reddy et al. 2009b). Biodegradations of most of the aromatic heterocyclics are carried out by a group of efficient oxygenase enzymes (Williams and Sayers 1994). These enzymes produce oxygenated metabolites from these heterocyclics. 2-Picoline degradation was studied in our laboratory and the role of these enzymes was detected using Gibb's reagent. This reagent detects oxygenated metabolites that are formed during biodegradation. Toluene dioxygenase (TODA) was reported for its extended activity in utilizing 4-picoline as substrate for growth (Takeshi et al. 2001). Therefore, this enzyme was used during our study to know its affinity towards 2-picoline and some other selected pyridine derivatives. For this a three dimensional (3D) model for TODA was generated and refined (Fig. 12.4). Active sites on this enzyme were identified and from

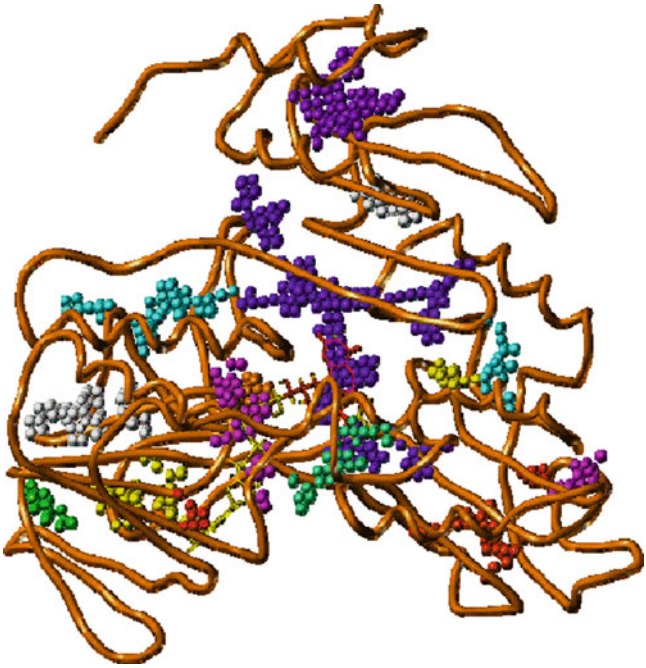

Fig. 12.5 Possible binding sites (active sites) of toluene dioxygenase enzyme (Madhusudan Reddy et al. 2008) (Site 1 represented by *purple*. Site 2 represented by *violet* color. Ste 3 represented by *white* color. Site 4 represented by *cyan* color. Site 5 represented by *red orange* color. Site 6 represented by *yellow* color. Site 7 represented by *magenta* color, Site 8 represented by *green blue* color. Site 9 represented by *green* color. Site 10 represented by *cyan* color. Site 11 represented by *magenta* color. Site 12 represented by *orange* color. Site 13 represented by *white* color. Site 14 represented by *yellow* color)

those sites, the one that is more stable was used to dock with the substrates such as 2-picoline, toluene, 2-hydroxypicoline, picolinic acid and 6-hydroxypicolinic acid (Figs. 12.5 and 12.6). For this study bioinformatics tools such as SYBYL, PROCHECK and GOLD etc., were used. From the results of this study, it was observed that 2-picoline is most preferred substrate next to toluene than other pyridine derivatives (Tables 12.1 and 12.2). This also provided information regarding possibility of evolution of toluene dioxygenase to accept 2-picoline as substrate and possibility of TODA like dioxygenase enzyme system in *Bacillus cereus* GMHS that has a key role in 2-picoline degradation (Madhusudan Reddy et al. 2008). The proposed pathway for biodegradation of 2-picoline by *Bacillus cereus GMHS* is shown in Fig. 12.7.

12.2.6 Biodegradation of Alkyl Pyridines Under Anaerobic Condition

Kaiser et al. (1993), reported a mixed culture that could transform 3-picoline and 4-picoline under anaerobic conditions with sulfate as an electron acceptor. The

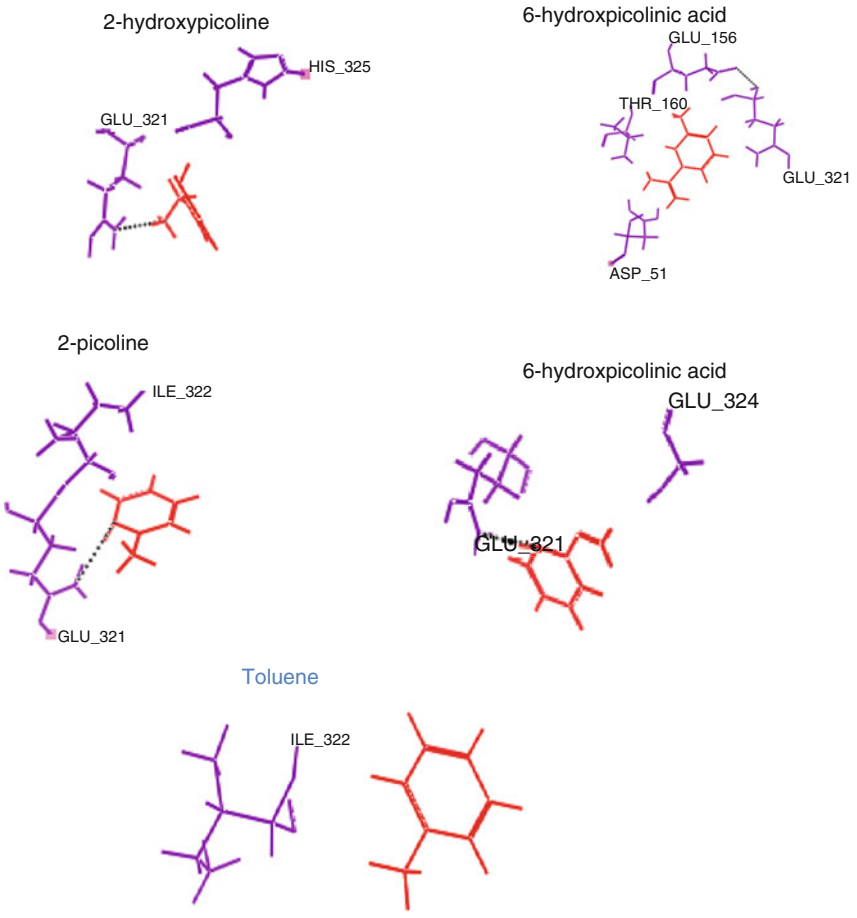

Fig. 12.6 Docking study of toluene dioxygenase (TODA) with selected substrates (substrates are represented in *red* color) (Madhusudan Reddy et al. 2008)

3-picoline degrading culture contained at least three types of bacterial forms where two of them were short rods and long rods. They were reported to be non-motile and spore forming bacteria. The third culture had a streptococcal shape and was non-motile. 4-Picoline degrading culture also contained three types of organisms with one organism as elliptical rods, pointed ends and central spore. Second culture had small coccobacilli and third bacterium was of vibroid shape. 3-Picoline under sulfate reducing conditions was completely degraded to CO_2 and NH_3. 4-Picoline was first transformed to 2-Hydroxy-4-picoline, which after 60 days accumulated sulphide which was completely mineralized after 90 days.

Comparative transformation rates of pyridine derivatives: Existence of a heterocyclic in the environment depends on their structure, concentration, physical and chemical properties. (Grbic-Galic 1990). Carboxylic substituents at any

Table 12.1 Hydrogen bonds and hydrophobic interactions between the substrate and active site residues of toluene dioxygenase (TODA) using molecular operating environment (MOE) (Madhusudan Reddy et al. 2008)

Substrate	Hydrogen bonding		Hydrophobic interactions
Toluene	–		322ILE0.C…PHEN0.C
2-Picoline	321 GLU0.O	1 PHEN0.N	322ILE0.C….1PHEN0.C1
2-Hydroxypicoline	321 GLU0.OE1	2 OH0.O1	
	325 HIS0.O	1 PHEN0.N	
6-Hydroxypicolinicacid	51 ASP0.OD2	3 CO0.O	–
	51 ASP0.OD1	4 OH0.O1	
	156 GLU0.O	2 OH0.O1	
	160 THR0.OG1	1 PHEN0.N	
	160 THR0.OG1	4 OH0.O1	
	321 GLU0.O	2 OH0.O1	
Picolinic acid	321 GLU0.O	1 PHEN0.N	–
	324 GLY0.N	2 COO.O	

Table 12.2 The total energy (E_{total}), electrostatic energy (E_{ele}), steric energy (E_{ste}), of the best-docked conformations of TODA with substrates (Madhusudan Reddy et al. 2008)

Substartes	E_{ele}	E_{ste}	E_{total}
Toulene	−0.289	−2.849	−3.138
2.picoline	−0.917	18.093	17.176
2hydroxy picoline	−1.385	2.968	1.583
6hydroxypicolinic acid	−3.543	13.246	9.703
Picolinic acid	−1.610	26.851	25.241

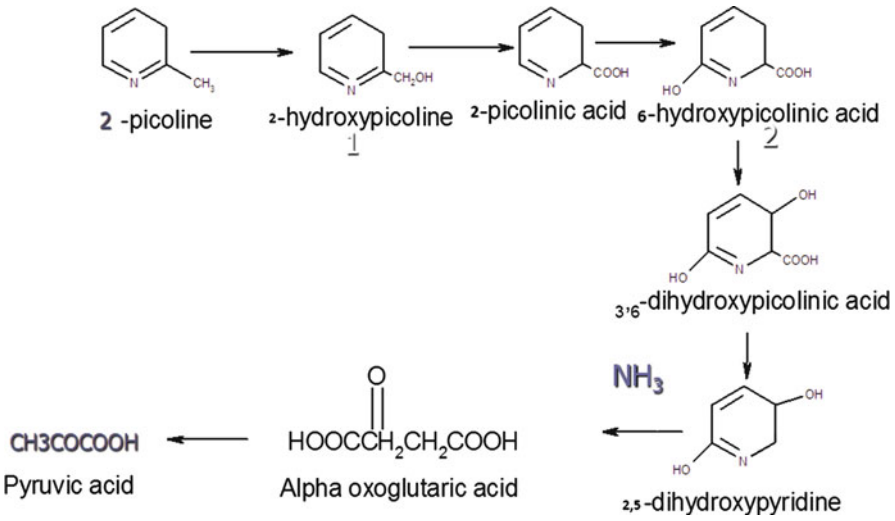

Fig. 12.7 Proposed pathway for biodegradation of 2-picoline by *Bacillus cereus GMHS*. *1&2* are identified hydroxyl intermediates of the pathway (Madhusudan et al. 2009)

position in the pyridine ring greatly stimulated degradation than any other substituents (Naik et al. 1972). Chloropyridines are more resistant to transformation. The transformation rates of pyridine derivatives reported by Sims and Sommers (1985, 1986) is as follows:

Pyridinecarboxylic acids > Monohydroxypyridines > Methylpyridines > aminopyridines > chloropyridines.

12.3 Conclusion

This chapter concludes that pyridine and its derivatives are very important chemicals that are used in making many important chemical intermediates and their ever increasing usage finds them in increasing concentrations in various effluents released by the industries. Though these chemicals are toxic to humans, their concentrations can be kept under check by various microorganisms that are very efficient in mineralizing or transforming them to non toxic compounds. Hence employing microorganisms like *Arthrobacter*, *Nocardia*, *Micrococcus*, *Bacillus* sp. etc. at the contaminated sites can render the sites free from accumulation of toxic chemicals. In-silico study mentioned in this chapter using bioinformatics tools can help in understanding the interactions at enzyme level thereby managing these toxic environments in a better way is possible.

12.3.1 Future Perspectives

Every year around 1,000–1,500 new chemicals are manufactured with perhaps 60,000 chemicals in daily use. Most of these are organic chemicals and pesticides. These compounds due to their extensive use as chemical intermediates are resulting in amassing higher concentration in the environment as recalcitrant. If their concentration is not checked in the environment, every pollutant that was released to the environment would still be here to haunt us. Fortunately, there are many reactions that check their concentrations such as chemical hydrolysis, photo degradation, volatilization, sorption, and most important and economic process bioremediation. Over the last few years, a number of companies have been established already to develop and commercialize biodegradation technologies. Existence of such companies now has become economically justifiable, because of burgeoning costs of traditional treatment technologies, increasing public resistance to such traditional technologies, accompanied by increasingly stringent regulatory requirements. The interest of commercial businesses in utilizing micro-organisms to detoxify effluents, soils, etc. is reflected in "bioremediation" having become a common buzzword in waste management. Companies specializing in bioremediation will need to develop a viable integration of microbiology and systems engineering.

Globally with the ever increasing population, the demand for requirement of various man made products also increases proportionately that poses threat in

release and accumulation of toxic effluents in the environment. Hence, carrying a serious research to isolate potential microbial candidates is a vital step that can check the concentrations in the environment. Critical study of microbial environments especially environments where most of the uncultured micro-organisms are prevailing and using theses microbes, employing techniques like recombinant DNA technology, novel techniques like bioinformatics and nanotechnology tools, managing the contaminated sties would be easy and safe. Construction of gene cassettes responsible for metabolism of toxic chemicals and expression of these cassettes in easily cultivable and routine bacteria like *E.coli* that do not demand expensive nutrients or process to metabolize the toxic chemicals will definitely have advantage. These results can assure us promising improvements in managing the contaminated environments thereby helping us to live in a better environment devoid of toxic pollutants.

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Chapter 13
Bioremediation, Bioconversion and Detoxification of Organic Compounds in Pulp and Paper Mill Effluent for Environmental Waste Management

Monika Mishra and Indu Shekhar Thakur

Abstract Pulp and paper industry is one among the 11 most polluting industry in India are utilizing natural resources as lignocellulose, inorganic and organic materials, and large volume of water in pulping and bleaching stages of the paper manufacturing. In manufacturing processes, 1 tonne of paper generates about 150 m^3 of waste water contains lignin and its degradation products, resin acids, fatty acids and lignosulphonics. In bleaching stages, waste water contains nascent chlorine, chlorinated organic compounds, dioxin, furan, peroxides etc. The coloured compounds and adsorbable organic halogens formed due to environmental factors in waste water exhibit strong mutagenic effects, physiological impairment and they are ecoestrogens. In addition they are recognized as an excellent source of food, fuel, feed, chemicals, vitamins and bioactive compounds after biodegradation and bioconversion of biowaste. This potential is being realized as data from research in the areas of the physiology and chemistry of microorganisms which have been used for treatment of effluent by aerobic and anaerobic methods in different types of bioreactors. The major enzymes, xylanases and ligninases, have been used and commercialized successfully for biopulping and biobleaching purposes for production of ecofriendly pulp and paper, recovery of products, and environmental waste management. Their commercial role as value added products has been established after evaluation of detoxification of toxicants. In this review, the pulping and bleaching processes of pulp and paper, formation of organic compounds, use of microorganisms in degradation and bioconversion of biowaste in different types of bioreactors are discussed. The methods for detection of toxic compounds in the effluent and evaluation of detoxification are also described so that value added products recovered from effluent can be used commercially.

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13.1 Introduction

The rapid increase in population and the increased demand for industrial establishments to meet human requirements have created problems such as overexploitation of available resources, leading to pollution of the land, air and water environments. The pulp and paper industry is the sixth largest polluter (after oil, cement, leather, textile, and steel industries) discharging a variety of gaseous, liquid, and solid wastes into the environment (Ali and Sreekrishnan 2001).

Wood is the major raw material for the forest based industries. One of nature's most important biological processes is the degradation of lignocellulosic materials such as wood and agricultural wastes to carbon dioxide, water, and humic substances through the natural detoxification processes. The virtue of biotechnology lies in its potential to supply more specific reactions, to provide less environmentally deleterious processes, to save energy, and to be used where non-biological chemistry is unfeasible.

There are, at present, about 515 units engaged in the manufacture of paper, paper boards and newsprint in India. The heavy demand for paper has led to the rapid expansion of the paper industries. At present about 60.8% of the total production is based on non-wood raw material and 39.2% on woody material. Annual paper production in India in the year 2009 has been estimated at 5.39 million metric ton. During the production of 1 ton of paper about 150 m^3 of effluent is generated (www.indiastat.com).

Manufacturing of paper is an elaborate process involving mainly two steps: pulping and bleaching. The wood is chopped into small pieces mixed with sodium hydroxide and sodium sulphite (kraft pulping) or acids (sulphite pulping) and heated at very high temperatures (~200°C) and pressure for 1–3 h. The lignin and hemicellulose present in it degrades and cellulose is left as pulp. This pulp is washed with water. The effluent generated at this stage is called black liquor as its dark brown in color due to the presence of lignin, hemicellulose, their degradation products, resin acids, lignosulphonics and phenols. Further the pulp is bleached as it has traces of lignin and hemicellulose which impart it yellow tinge. For bleaching nascent chlorine, hydrogen peroxide and ozone are used. The effluent generated at this stage has adsorable organic halides, chlorophenols, dioxin and furans. The bleached pulp is white in color and is used to make sheets of paper.

The whole process of making paper generates extremely toxic effluent. Use of biological approaches like effluent treatment, biobleaching and biopulping can be used to reduce the toxicity of the effluent. It has been observed that fungi are the

main degraders of lignocellulosic materials, particularly wood. Apart from fungi there are some bacteria, *Bacillus* and *Pseudomonas*, are also capable of wood degradation (Bourbonnais and Paice 1987). A mixed culture of algae was able to decolorize pulp and paper mill effluent (Lee et al. 1978).

13.2 Critical Review

13.2.1 Process of Making Paper

The pulp and paper production is an elaborate process having high demand for water and electricity. The raw material can be wood, bagasse, wheat straw, rice straw and similar agricultural wastes having high fiber content. The steps involved in pulp and papermaking are (Fig. 13.1):

1. Raw material is cleaned and cut in small pieces.
2. Separation of cellulose fibers from lignin and hemicellulose is known as pulping. Pulping can be mechanical or chemical. In chemical pulping two basic methods, Kraft pulping (using sodium hydroxide and sodium sulphite) and Sulphite pulping (using sulphuric acid and bisulphite ions) are used. Pulping contributes maximum to the pollution load.
3. For making low quality paper used for wrapping pulp is directly fed into paper making machines where it is turned into sheets, dried with steam, cut into the desired sizes and packed.
4. For making good quality writing paper, the pulp is bleached to remove residual lignin to make it white. For bleaching chlorine, hydrogen peroxide, oxygen, ozone and similar bleaching agents are used.

The effluent generated in digester house, i.e. pulping stage is dark brown in color due to lignin, its degradation products, lignosulphonics, hemicelluloses, resin acid and phenols (Chuphal et al. 2005). The main problem at this stage is degradation of lignin. It is an amorphous, polyphenolic complex polymer formed from dehydrogenative polymerization of three phenylpropaniod monomers, coniferyl alcohol, sinapyl alcohol and p-coumaryl alcohol (Lin and Dence 1992).

Though lignin is resistant to microbial attack, still it is degraded to humus, water, and carbon dioxide following the death of the plant tissues which indicates that in nature a number of microorganisms exist which are capable of lignin degradation. This property of microorganism is exploited by biotechnology to make the manufacturing process eco-friendly. The pulping process can be made environmental friendly in two ways: treatment of effluent generated at this stage and treatment of raw material, i.e. biopulping.

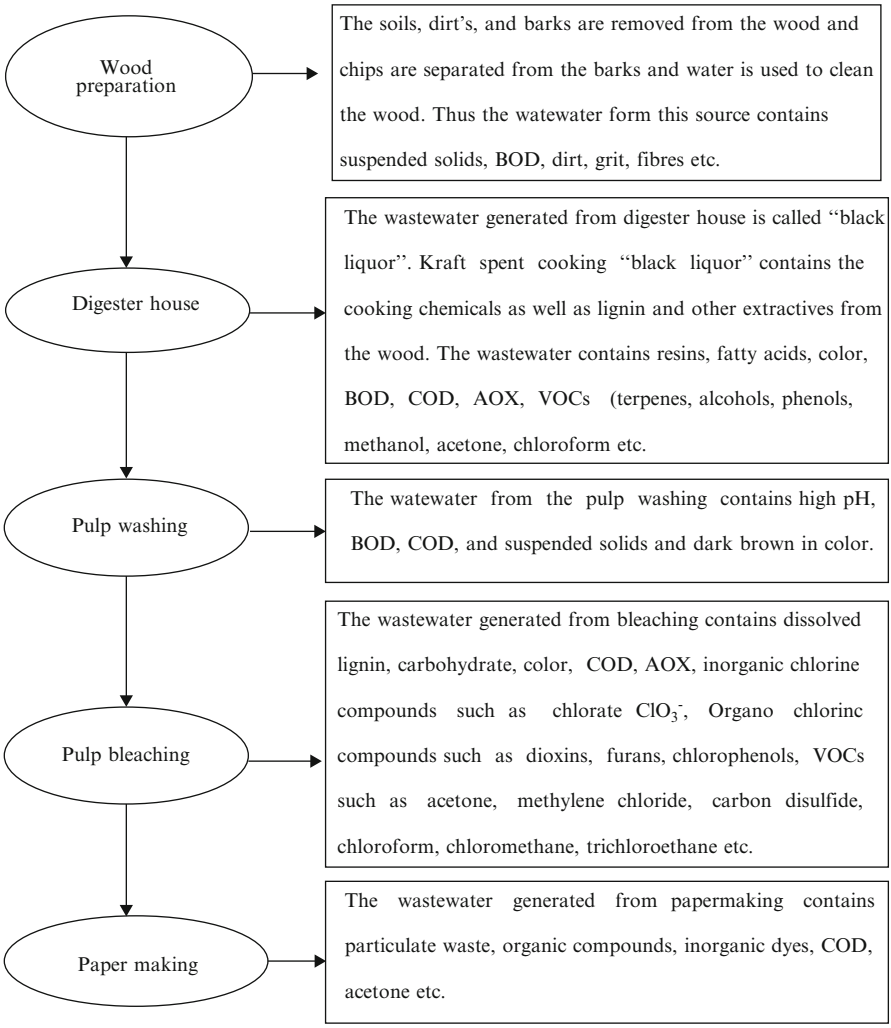

Fig. 13.1 Pollutants from various sources of pulping and paper making (US EPA 1995)

The effluent generated at the pulping stage in pulp and paper mill is highly colored, alkaline and toxic. It has high COD, BOD and TSS. It contains lignosulphonics, resin acid, phenols, lignin and its breakdown products and hemicellulose (Pokhrel and Viraraghavan 2004). These are complex organic compounds, when released in environment without treatment; reacts with a wide variety of other chemicals in presence of light and heat to form highly toxic and recalcitrant compounds (Kinae et al. 1981; Zacharewski et al. 1995). Thus it is obligatory to treat the effluent before disposal into the environment.

13.2.2 Characterization of the Toxic Organic Compounds

Four different families of organic compounds that can be found in pulp and paper mill waters and sludge sediments are:

13.2.2.1 Biocides

Biocides are often used for wood preservation and during paper-making to avoid problems associated with microbial, fungal and algal growth. Biocides used in paper-mills can be of different types: 2,2-dibromo-3-nitrilpropionamide (DBNPA), 2-(thiocyanomethylthio)-benzotiazole (TCMTB) etc. The fate of biocides is as follows: a fraction will degrade (chemically or biologically); a fraction will remain in circulating waters; and, finally, a fraction will be present in the effluent or remain in the solid matter. An additional problem is that, because of their physico-chemical properties, some biocides may retain fibres that can accumulate in the final paper product (Abrantes et al. 1998).

13.2.2.2 Resin and Fatty Acids

Wood extractives include lipophilic (fatty and resin acids (RAs), sterols, steryl esters and triglycerides) and hydrophilic (lignans, low-molecular-mass lignins, lignin-like substances and hemicelluloses) compounds that dissolve in waters during paper production. Among the wood extractives, resin and fatty acids have a great tendency to form pitch deposits and stickies that hamper the machine functioning and decrease the physical properties of the paper i.e. tensile strength, opacity, brightness, etc. (Gutierrez et al. 2001). Resin and fatty acids are not removed by primary flocculation, whereas a decrease of 50% or more is observed after biological treatment (Rigol et al. 2003).

Resin and fatty acids are of different types: linoleic acid, stearic acid, palmitic acid, margaric acid, isopimaric acid, dehydroabietic acid, dichlorodehydroabietic acid etc. which are toxic to aquatic life, causing jaundice in rainbow trout.

13.2.2.3 Surfactants and Plasticizers

Surfactants, such as linear alkylbenzene sulfonates and alkylphenol ethoxylates, are present in effluent because of their use as cleaning agents or as additives in antifoamers, deinkers, dispersants, etc.

The anionic surfactants, linear alkylbenzene sulfonates (LASs), represent 25% of total consumption. The non-ionic surfactants, alkylphenol ethoxylates (APEOs), degrade to nonylphenol (NP) or, to a lesser extent, to octylphenol (OP), which are considered persistent environmental pollutants (PEPs).

LASs have been detected in waste waters of paper-mills at concentrations up to 5,000 μg/L (Rigol et al. 2002) and concentrations of 0.3–10 μg/L have been detected for APEOs such as NP and OP (Rigol et al. 2003). NP and OP are known to cause aquatic toxicity and endocrine disruption in animals. Laboratory experiments, using rat models have shown NP and OP to have negative impact on the hormonal development of mammals, e.g., underdeveloped testis.

13.2.2.4 Chlorinated Compounds

The various chlorinated compounds that are utilized or produced during the bleaching process and are detectable in the effluents and sludge are chlorolignin compounds, chlorophenols such as pentachlorophenol (PCP), chlorobenzenes, chlorinated acetic acids, chlorinated thiophenes, chloroguaiacols, chlorosyringol, chlorovanillin, chlorocatechol, polychlorinated dibenzo-para-dioxin (PCDD), polychlorinated dibenzofuran (PCDF), polychlorinated biphenyl (PCB).

Raw wood material is often treated with pentachlorophenol (PCP) and other chlorophenols that act as wood preservatives. Chlorophenols are highly lipophilic and its benefits are low degradability in outdoor conditions. They have been encountered in water (Virkki et al. 1994) and sediments (Judd et al. 1996) of several paper-mills.

In paper-making, chlorine and chlorinated compounds are also sources of dioxins and furans, which have been detected in sediments in the vicinity of a pulp and paper-mill (Munawar et al. 2000) and in effluents, along with polychlorinated dibenzothiophenes (Sinkkonen et al. 1992).

Chlorinated thiophenes act as weak mutagens. Chlorinated acetones are Ames test positive and mutagenic. 1,3-dichloroacetone has been identified as one of most potent mutagens in effluent. Chloroguaiacols are thought to be precursors of tetrachloro-dibenzo-para-dioxin (TCDD). Chloroveratoles and anisoles are thought to be extremely toxic constituents of effluent with bioaccumulation potential and possible presence in sludge. Chlorophenols and trichloroacetic acid have tested toxic (50% growth inhibition) to a range of plants. Chlorolignins when degrade probably become chlorocatechols, chloroguaiacols and chloroveratoles, which are more toxic. Bacteria in soil are able to “o-methylate” chlorophenols and chlorolignins creating chloroveratoles.

Exposure to chlorinated dioxins and dibenzofurans causes chloracne which is mostly seen on the cheeks, behind the cheeks, in the armpits and groin region. Chloracne persists for more than 10 years. Abnormal reproductive effects such as decreased testosterone, reduced sperm count, male feminization are seen among males while females experience decreased fertility, miscarriage and endometriosis. Other effects that result from exposure to chlorinated dioxins include immune suppression, liver enzyme changes, nervous system damage and thymus, spleen and bone marrow damage (Mandal 2005). Both PCBs and dioxins are known carcinogens (Fig. 13.2).

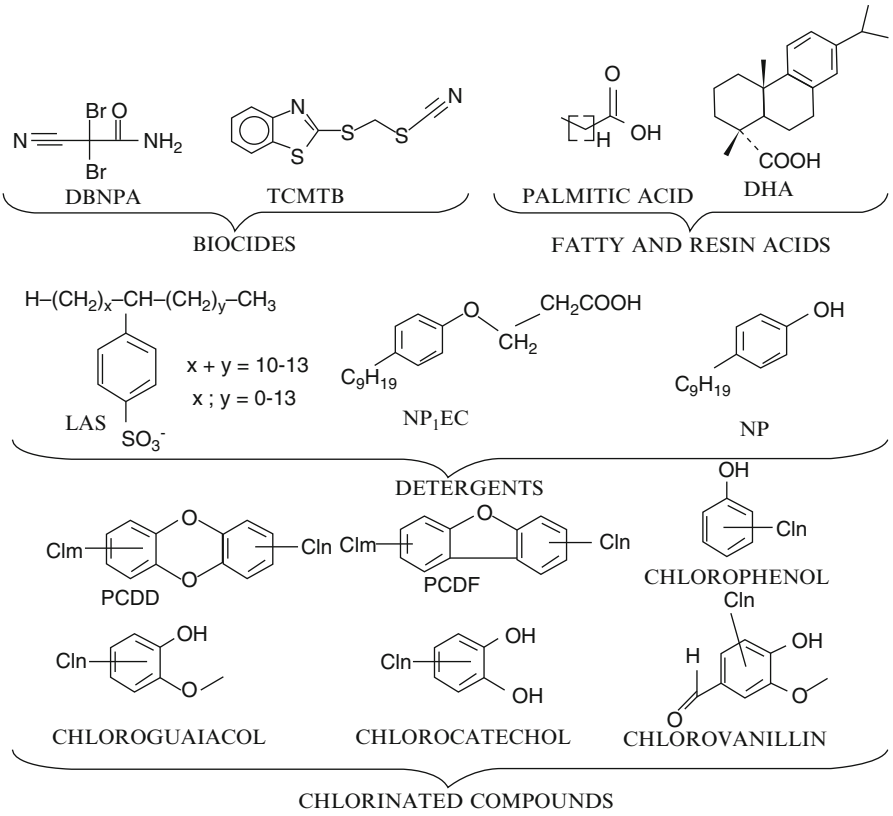

Fig. 13.2 Chemical structure of organic compounds identified in paper-mill effluents (Lacorte et al. 2003)

13.2.3 Biodegradation of Persistant Xenobiotic Contaminants Present in Pulp and Paper Mill Effluents

13.2.3.1 Biodegradation of Chlorinated Dioxins

Lower chlorinated dioxins can be degraded by aerobic bacteria from the genera of *Sphingomonas*, *Pseudomonas* and *Burkholderia* (Field and Sierra-Alvarez 2008). Most studies have evaluated the co-metabolism of monochlorinated dioxins with unsubstituted dioxin as the primary substrate. The degradation is usually initiated by unique angular dioxygenases that attack the ring adjacent to the ether oxygen. Chlorinated dioxins can also be attacked co-metabolically under aerobic conditions by white-rot fungi that utilize extra cellular lignin degrading peroxidases. Recently, bacteria that can grow on monochlorinated dibenzop-dioxins as a sole source of carbon and energy have also been characterized (*Pseudomonas veronii*). Higher chlorinated dioxins are known to be reductively dechlorinated in anaerobic sedi-

ments. Similar to PCB and chlorinated benzenes, halo respiring bacteria from the genus *Dehalococcoides* are implicated in the dechlorination reactions.

13.2.3.2 Biodegradation of Chlorophenols

There are three main pathways involved in the biodegradation of chlorophenols. The first pathway is the monooxygenase catalysed hydroxylation of chlorophenols to form hydroquinones. The second pathway involves the hydroxylation of higher chlorinated phenols to chlorinated hydroquinones. The third pathway is the initial reductive dechlorination of chlorophenols under anaerobic conditions.

Biodegradation of phenol by *Pseudomonas putida* (NICM 2174) and *Pseudomonas pictorum* (NICM 2074) mixed culture exhibited as a versatile inexpensive and potential result to turn a toxic material into harmless products (Annadurai et al. 1999). *Pseudomonas aeruginosa* strain is capable of degrading pentachlorophenol (PCP) (Susella et al. 1991).

Anaerobic dechlorination of 2,4-dichlorophenol has been observed in fresh water sediments in presence of sulphate (Madsen and Aamand 1991). Anaerobic biodegradation of chlorophenols such as 2,3,4,6-tetrachlorophenol and 2,4,6-trichlorophenolin fresh and acclimated sludge has been reported (Boyd et al. 1983). Phenolic compounds such as ortho, meta and para isomers of chlorophenols are converted into phenols by dechlorination under anaerobic condition in digested sludge (Boyd and Shelton 1984).

It has been found that Basidiomycetes such as *Grammathels fuligo* and *Phanerochaete crassa* has capability to degrade chlorinated phenols and pentachlorophenol. At appropriate temperature and pH, *G. fuligo* and *P. crassa* showed superior mycelial growth. Reduction in chlorophenols was due to adsorption.

13.2.4 Removal of Color

Numerous bacterial, fungal and algal cultures are known to decolorize pulp and paper mill effluents (Table 13.1).

13.2.4.1 Decolorization of Effluent by Algae

It has been reported that some algae can decolorize diluted bleach kraft mill effluents (Lee et al. 1978; Tarlan et al. 2002). It was found that pure and mixed algal cultures removed up to 70% of color within 2 months of incubation. All cultures exhibited a similar color reduction pattern consisting of a phase with rapid and accelerating removal rate and a phase with declining rate. Color removal was most effective during the first 15–20 day of incubation, and then gradually dropped off. Complete removal of color did not occur. Color removal by algae is caused by metabolic transformation of colored molecules to noncolored molecules with limited assimilation or degradation of molecular entities. Adsorption is not a major color removal mechanism.

Table 13.1 Cultures used for decolorization of pulp and paper mill effluents

Cultures	Reference
Bacteria	
Pseudomonas ovalis	Kawakami (1975)
Pseudomonas aeruginosa	Blair and Davis (1980)
Bacillus cereus	Bourbonnais and Paice (1987)
Bacillus sp.	Mishra and Thakur (2010)
Algae	
Microcystis sp.	Lee et al. (1978)
Chlorella, Chlamydomonas	Dilek et al. (1999)
Fungi	
Trametes versicolor	Kirk et al. (1976)
Phanerochaete chrysossporium	Eaton et al. (1980)
Tinctoporia borbonica	Fukuzumi (1980)
Schizophyllum commune	Belsare and Prasad (1988)
Aspergillus niger	Kannan (1990)
Gloephyllum trabeum	Galeno and Agosin (1990)
Trichoderma sp.	Prasad and Joyce (1993)
Paecilomyces variotti	Calvo et al. (1991)
Phlebia radiatta	Moreira et al. (1999)
Bjerkandera sp.	Palma et al. (2000)
Cryptococcus sp.	Singhal and Thakur (2009)

13.2.4.2 Decolorization of Effluent by Fungi

Published papers report the use of wide variety of fungi like *Merulius aureus* syn. *Phlebia* sp. and *Fusarium sambucinum* Fuckel MTCC 3788 (Malaviya and Rathore 2007), *Trametes versicolor* (Pedroza et al. 2007), *Paecilomyces* sp (Singh and Thakur 2006; Chuphal et al. 2005), *Coriolus versicolor* and *Rhizomucor pusillus* strain RM7 (Driessel and Christov 2001) for decolorization of pulp and paper mill effluent. The decolorization of the pulp and paper mill effluent by fungi involves two main mechanisms; first use of various enzymes like lignin peroxidase, manganese peroxidaes, laccase, xylanase and second adsorption.

13.2.4.3 Decolorization of Effluent by Bacteria

Along with these fungi, numerous bacteria such as *Pseudomonas* sp., *Flavobacteria*, *Xanthomonas* sp., *Bacillus* sp., *Aeromonas* sp., *Cellulomonas* sp., etc. have been reported to decompose lignin and its derivatives (Kirk et al. 1977; El-Bestawy et al. 2008; Mishra and Thakur 2010). The contributions of bacteria have been reported for utilization of low-molecular weight lignin oligomers as the sole source of carbon and energy that produce enzymes and cleave intermonomeric linkages of lignin (Vicuna et al. 1993). Bacteria play a pivotal role in depolymerizing lignin in aquatic ecosystem because wood degrading bacteria have a wider tolerance of temperature, pH and oxygen limitations than fungi (Vicuna 1988).

Few bacteria have been reported for treatment of pulp and paper mill effluent (Thakur et al. 2001; Chuphal et al. 2005; Thakur 2004). Although many groups of bacteria are able to metabolize the monomeric constituents of the aromatic lignin polymer, their activity on polymeric lignin substrates is limited and low rates of conversion of radiolabelled lignin substrates to CO_2 were observed with most of the bacteria tested (Zimmermann 1990). In pulp and paper industry, bacteria are generally used for degrading chlorinated phenols (Chuphal et al. 2005). Bourbonnais and Paice (1987) tested *Bacillus cereus* and two strains of *Pseudomonas aeruginosa* for decolorization of bleach kraft effluent. Color was primarily removed by adsorption with little depolymerization.

13.2.5 Treatment of Effluent Generated at Pulping Stage

Various physiochemical methods like sedimentation, flotation, screening, adsorption, coagulation, oxidation, ozonation, electrolysis, reverse osmosis, ultra-filtration, and nano-filtration technologies have been used for treatment of suspended solids, colloidal particles, floating matters, colors, and toxic compounds (Pokhrel and Viraraghavan 2004). However, they have disadvantages like high cost and sludge generation. Sludge has to be landfill. This further increases the cost of treatment. Sometimes the sludge is burned to save cost of disposal. On burning, huge quantities of volatile organic toxic compounds are formed. These include dioxins, furfurals and other volatile organic compounds. Thus in general, these processes only change the state of pollutants from liquid to solid then to gas rather than treating them. On the other hand biological methods involve degradation of pollutants, solving the problem permanently. Biological treatment can be divided into aerobic and anaerobic depending on the availability of oxygen. Aerobic treatment involves activated sludge treatment, aerated lagoons and aerobic biological reactors. Anaerobic filter, upflow sludge blanket (UASB), fluidized bed, anaerobic lagoon, and anaerobic contact reactors are anaerobic processes, that are commonly used to treat pulp and paper mill effluents. Among these treatments one thing is common, use of microbes (Pokhrel and Viraraghavan 2004). A number of fungi, bacteria and algae have been reported to have effluent treatment capabilities.

13.2.6 Use of Bioreactor for Decolorization

Many studies have reported high removals of organic pollutants of kraft mill wastewater by sequential batch reactor (SBR) treatment (Franta and Wilderer 1997; Milet and Duff 1998). Substantial removal of COD, TOC, BOD, lignin and resin acids of TMP wastewater using high rate compact reactors (HCRs) at a retention time of 1.5 h had been reported (Magnus et al. 2000a, b). Removal of COD in a moving bed biofilm reactor (MBBR) had been demonstrated (Jahren et al. 2002; Borch-Due

et al. 1997). Berube and Hall (2000) showed that approximately 93% removal of TOC could be achieved by a membrane bioreactor. These studies show the importance of bioreactors in treatment studies. Recently some studies have reported the use of integrative approach. An integrated or hybrid system is designed to take advantage of unique features of two or more processes. A combination of coagulation and wet oxidation removed 51% of COD, 83% of color and 75% of lignin (Verenich et al. 2001; Verenich and Kallas 2001). A combination of ozone and biofilm reactor removed 80% COD (Helble et al. 1999). A combination of activated sludge and with ozonation (as tertiary treatment) removed 87–97% COD, and 97% BOD (Schmidt and Lange 2000). However, all these techniques have high cost of treatment.

13.2.7 Biopulping

Apart from effluent treatment modification in process i.e. use of biopulping as a pretreatment before chemical and mechanical pulping is one economically viable alternative to make the manufacturing process cleaner and greener. Biopulping involves the biotreatment of lignocellulosic material by fungus having lignolytic enzyme system and the subsequent processing by mechanical or chemical pulping (Saad et al. 2008). In wood, cellulose fibers are embedded in hemicellulose and lignin. Paper is made from cellulose thus hemicellulose and lignin is waste. These cellulose fibers need to be separated from hemicellulose and lignin to make paper. Pulping process involves separation of cellulose from lignin and hemicellulose. Also pulping is most polluting step (Pokhrel and Viraraghavan 2004). Pulping technologies have undergone constant improvements due to market demands and new developments in research. The need for sustainable technologies has also brought biotechnology into the realm of pulp and papermaking. Enzymatic processes are being developed to increase pulp brightness, to reduce troublesome pith, to improve paper quality and to purify the effluent (Messner and Srebotnik 1994). Efforts have been made to improve pulp-producing process by using isolated enzymes. These efforts have limited success as lignin, which is the major problem, lacks the regular and ordered repeating units found in other natural polymers.

13.2.8 Biobleaching

Biobleaching of pulps is performed with either hemicellulolytic enzymes, in particular xylanases (Jeffries 1992) or lignin-degrading fungi and their enzymes (Reid and Paice 1994) while xylanases hydrolyze hemicellulose (xylan) in pulp thereby enabling the bleaching chemicals an easier access to lignin (Paice et al. 1992), white-rot fungi and their ligninolytic enzymes directly attack and depolymerize lignin in pulp (Kondo et al. 1994). However, in both cases the aim is to enhance

delignification and therefore facilitate the subsequent bleaching of pulp by applying reduced amounts of bleaching chemicals, especially chlorine and chlorine-containing compounds (Senior et al. 1992).

The interest in xylan degrading enzyme and their application in the pulp and paper industries have advanced significantly over the past few years (Bajpai et al. 1994; Garg et al. 1998; Srinivasan and Rele 1999). In kraft pulping, hemicelluloses and lignin are dissolved and partially degraded during the heating process. In a subsequent phase of the process the pH drops sharply because of the discharge of xylan side groups and xylan precipitates with readsorption of lignin on top of the cellulosic microfibrils. Lignin is colored during kraft pulping and as a consequence, cellulosic fibers become darkly stained. Usually one or more bleaching sequences are needed to remove the dark color caused by the deposition of lignin. Organic chlorine compounds are formed during the chemical bleaching of pulp. These compounds arise mainly from the reaction between residual lignin present in wood fibers, and the chlorine used for bleaching.

Chlorinated organic compounds produced during chemical bleaching technologies are harmful to the environment and need to be substituted by environment compatible procedures. One environmentally safe technique is the use of xylanases. Xylanases cleave and solubilize reprecipitated xylan and lignin located on the surface of the microfibrils. This facilitates pulp bleaching and lowers chlorine consumption thereby reducing discharge of toxic organo-chlorine compounds in the environment (Senior et al. 1992; Tolan and Canovas 1992). The effectiveness of xylanase treatments has been evaluated in at least two aspects: first by determining the amount of sugars after enzyme incubations, where 0.5–1.0% of the pulp carbohydrate content is liberated and second, by observing increased bleachability with conventional methods after xylanase treatments (Viikari et al. 1993). Furthermore, xylanase treatment of kraft pulp releases the lignin-carbohydrate complexes (Yang and Eriksson 1992). Moreover, xylanase treatment helps in increasing brightness of pulp which is very important to develop chlorine free bleaching process.

13.2.9 Mechanism Involved in Decolorization of Pulp and Paper Mill Effluent

There are mainly two major mechanisms involved in decolorization of pulp and paper mill effluent. (1) Enzymatic process and (2) Biosorption.

13.2.9.1 Enzymatic Processes for Lignin and Hemicelluloses Degradation

In pulp and paper industry, cellulose is used for paper production while lignin and hemicellulose end up in effluent. The bacteria capable of degrading lignin and hemicellulose can be used for treatment of effluent. The degradation process

involves use of number of enzymes collectively called ligninase. Ligninase is a generic name for a group of isozymes that catalyze the oxidative depolymerization of lignin. They include lignin peroxidase, manganese peroxidase and laccase, aryl alcohol oxidase, glucose oxidase and NAD(P)H:quinone oxidoreductase etc. Another enzyme xylanase plays a crucial role in hemicellulose degradation. These enzymes are extracellular, are non-substrate specific and aerobic in nature. This is an essential requirement for lignin degradation as it is a randomly synthesized biopolymer that cannot enter inside the cell and degradation involves the cleavage of carbon-carbon or ether bond, that link various sub-units, in oxidative environment (Breen and Singleton 1999). The mechanism of action of these enzymes is as follows:

Xylanase

Xylan, a major constituent of hemicellulose, is composed of β-1, 4-linked xylopyranosyl residues which can be substituted with arabinosyl and methylglucuronyl sidechains. Xylanases (endo-1, 4- β -D-xylan xylanohydrolase; E.C. 3.2.1.8) are a group of enzymes that hydrolyse xylan backbone into small oligomers (Kiddinamoorthy et al. 2008). The xylanolytic enzyme system carrying out the xylan hydrolysis is usually composed of a repertoire of hydrolytic enzymes: β-1,4-endoxylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase, and phenolic acid (ferulic and *p*-coumaric acid) esterase (Fig. 13.3). The presence of such a multifunctional xylanolytic enzyme system is quite widespread among fungi, actinomycetes, and bacteria (Beg et al. 2001).

Due to xylan heterogeneity, the enzymatic hydrolysis of xylan requires different enzymatic activities. Two enzymes, β-1,4-endo-xylanase (EC 3.2.1.8) and β-xylosidase (EC 3.2.1.37), are responsible for hydrolysis of the main chain, the first attacking the internal main-chain xylosidic linkages and the second releasing xylosyl residues by endwise attack of xylooligosaccharides (Subramaniyan and Prema 2002). These two enzymes are the major components of xylanolytic systems produced by biodegradative microorganisms such as *Trichoderma*, *Aspergillus*, *Schizophyllum*, *Bacillus*, *Clostridium* and *Streptomyces* sp. (Bedard et al. 1987; Valenzuela et al. 1997; Yang et al. 1992). However, for complete hydrolysis of the molecule, side-chain cleaving enzyme activities are also necessary.

Xylanases have several different industrial applications including Kraft pulp bleaching in the paper industry, biodegradation of lignocellulose in animal feed, foods, and textiles, as well as biopulping in the paper and pulp industry (Madlala et al. 2001; Schwien and Schmidt 1982).

Lignin Peroxidase (LiP)

Lignin peroxidase is a heme-containing glycoprotein which requires hydrogen peroxide as an oxidant. Fungi secrete several isoenzymes into their cultivation

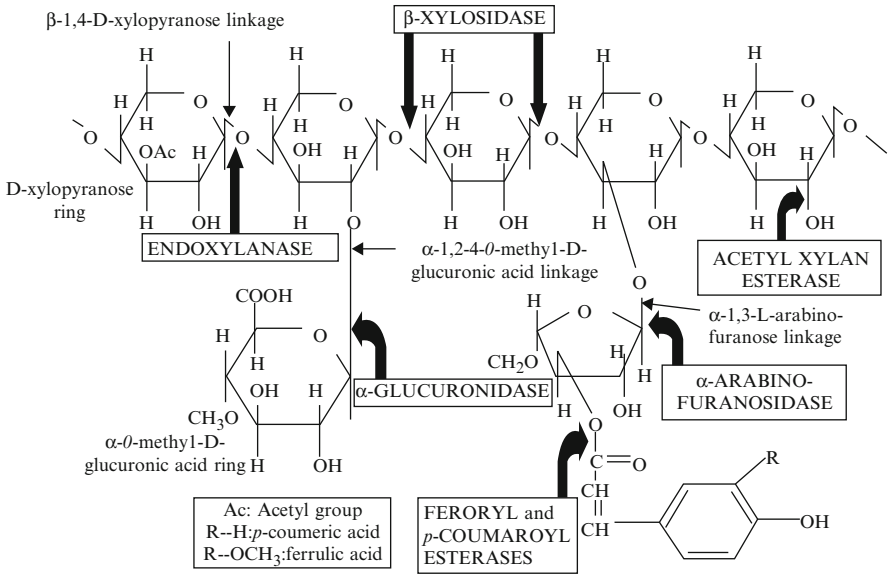

Fig. 13.3 A hypothetical plant xylan structure showing different substituent groups with sites of attack by microbial xylanases (Beg et al. 2001)

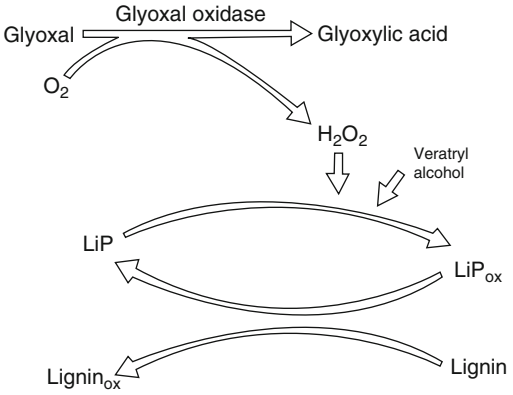

Fig. 13.4 Mechanism of action for Lignin peroxidase *LiP*. *ox* stand for oxidized state of enzyme (Breen and Singleton 1999)

medium, although the enzymes may also be cell-wall bound (Lackner et al. 1991). LiP oxidizes non-phenolic lignin substructures by abstracting one electron and generating cation radicals which are then decomposed chemically (Fig. 13.4). Reactions of LiP using a variety of lignin model compounds and synthetic lignin have thoroughly been studied, catalytic mechanisms elucidated and its capability for C~–C~bond cleavage, ring opening and other reactions has been demonstrated (Eriksson et al. 1990; Higuchi 1989). LiP is secreted during secondary metabolism as a response to nitrogen limitation. They are strong oxidizers capable of catalyzing the oxidation of phenols, aromatic amines, aromatic ethers and polycyclic aromatic hydrocarbons (Breen and Singleton 1999).

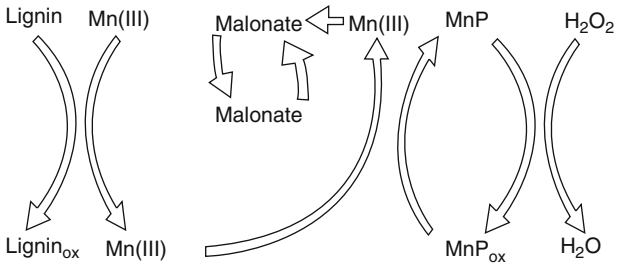

Fig. 13.5 Mechanism of action for Manganese peroxidase *MnP*. *ox* stands for oxidized state of enzyme (Breen and Singleton 1999)

Manganese Peroxidase (MnP)

Manganese peroxidase is also a heme-containing glycoprotein which requires hydrogen peroxide as an oxidant. MnP oxidizes Mn(II) to Mn(III) which then oxidizes phenol rings to phenoxy radicals which lead to decomposition of compounds (Fig. 13.5). Evidence for the crucial role of MnP in lignin biodegradation are accumulating, e.g. in depolymerization of lignin (Wariishi et al. 1991) and chlorolignin (Lackner et al. 1991), in demethylation of lignin and delignification and bleaching of pulp (Paice et al. 1993), and in mediating initial steps in the degradation of high-molecular mass lignin (Perez and Jeffries 1992).

Laccase (Lac)

Laccase (EC No. 1.10.3.2. benzenediol: oxygen oxidoreductase) is a true phenoloxidase with broad substrate specificity. It is a copper containing glycoproteins widely reported in fungi and plants. Most famous are rot fungi like *Phanerochaete chrysosporium, Ceriporiopsis subvermispora, Coriolus versicolor* var. antarcticus*, Pycnoporus sanguineus, Trametes elegans, Bjerkandera adusta, Pleurotus eryngii, Phlebia radiata,* etc. (Baldrian 2006). It has also been reported in some plants like *Acer pseudoplantanus, Aesculus parviflora, Populus euramericana* etc. In plants laccase participates in the radical-based mechanisms of lignin polymer formation (Sterjiades et al. 1992), whereas in fungi laccases probably have more roles including morphogenesis, fungal plant-pathogen/host interaction, stress defense and lignin degradation (Thurston 1994). The presence of laccase has been reported in bacteria; however, such reports remain controversial (Diamantidis et al. 2000).

The reactions catalysed by laccases proceed by the monoelectronic oxidation of a suitable substrate molecule (phenols and aromatic or aliphatic amines) to the corresponding reactive radical. The redox process takes place with the assistance of a cluster of four copper atoms that form the catalytic core of the enzyme (Fig. 13.6); they also confer the typical blue color to these enzymes because of the intense electronic absorption of the Cu–Cu linkages (Piontek 2002).

Lignin is formed via the oxidative polymerization of monolignols within the plant cell wall matrix. Peroxidases, which are abundant in virtually all cell walls,

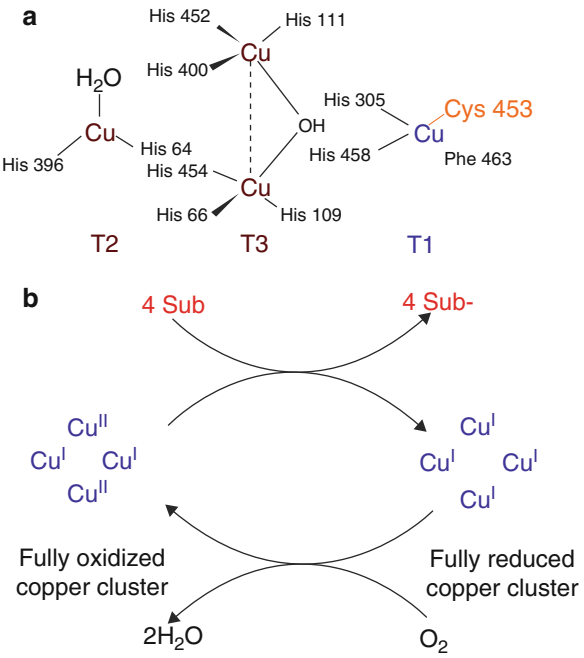

Fig. 13.6 Catalytic action of laccases (Riva 2006)

have long been held to be the principal catalysts for this reaction. Recent evidence shows, however, that laccases secreted into the secondary walls of vascular tissues are equally capable of polymerizing monolignols in the presence of O_2. The role of laccases in lignification has often been debated. Laccase from *Acer pseudoplantanus* was able to polymerize monolignols, in the complete absence of peroxidase (Sterjiades et al. 1992). This shows that laccase was involved in the early stages of lignification, while peroxidases were involved later.

Laccases are able to catalyze electron transfer reactions without additional cofactors, hence their use has been studied in biosensors to detect various phenolic compounds, oxygen or azides. Moreover, biosensors for detection of morphine and codeine (Bauer et al. 1999), catecholamines (Ferry and Leech 2005), plant flavonoids (Jarosz-Wilkołazka et al. 2005) and also for electroimmunoassay Kuznetsov et al. (2001) have been developed. An enzyme electrode based on the co-immobilisation of an osmium redox polymer and a laccase from T. versicolor on glassy carbon electrodes has been applied to ultrasensitive amperometric detection of the catecholamine neurotransmitters dopamine, epinephrine and norepinephrine, attaining nanomolar detection limits (Ferry and Leech 2005). Laccase can also be immobilized on the cathode of biofuel cells that could provide power, for example, for small transmitter systems (Chen et al. 2001; Calabrese et al. 2002).

Aryl Alcohol Oxidase (AAO)

Aryl-alcohol oxidase (EC 1.1.3.7) is a FAD-containing enzyme in the GMC (glucose-methanol-choline oxidase) family of oxidoreductases. AAO partici-

pates in degradation of lignin, a process of high ecological and biotechnological relevance, by providing the hydrogen peroxide required by ligninolytic peroxidases (Ferreira et al. 2009). Thus, the two substrates of this enzyme are aromatic primary alcohol and O_2, whereas its two products are aromatic aldehyde and H_2O_2. This enzyme belongs to the family of oxidoreductases, specifically those acting on the CH-OH group of donor with oxygen as acceptor. The systematic name of this enzyme class is aryl-alcohol: oxygen oxidoreductase. Other names in common use include aryl alcohol oxidase, veratryl alcohol oxidase, and aromatic alcohol oxidase.

$$\text{Aromatic primary alcohol} + O_2 \rightleftharpoons \text{an aromatic aldehyde} + H_2O_2$$

13.2.9.2 Role of Biosorption in Effluent Decolorization

There are two main processes acting during biological decolorization. One is enzymatic action and second adsorption. Biosorption is mainly a physio – chemical process.

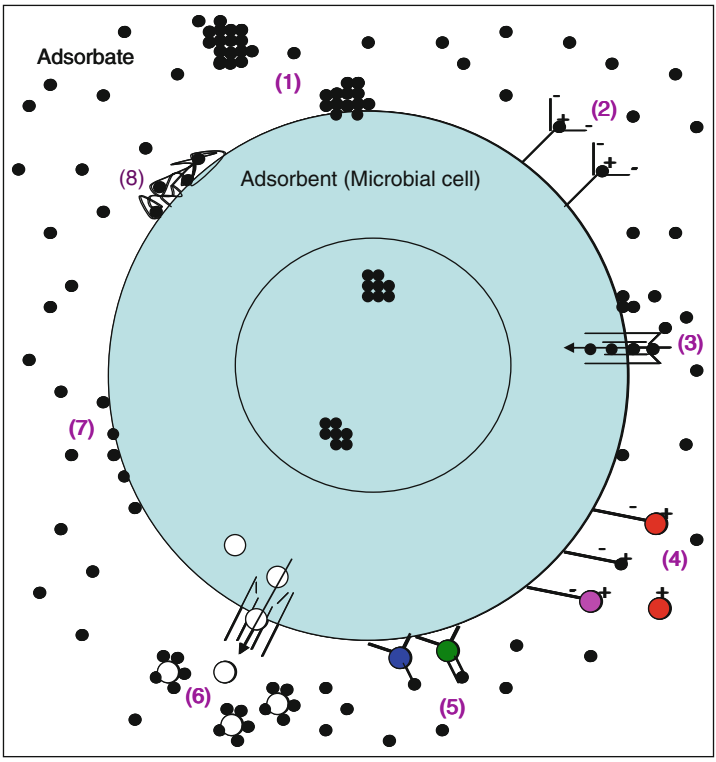

Fig. 13.7 Diagrammatic representation of different mechanisms of biosorption

Microbial cell (adsorbent) is shown in light blue color and effluent, metal or/and dye (adsorbate) are shown in black color circles. (1) Precipitation, on and outside surface, (2) physical adsorption e.g. electrostatic force, (3) active diffusion in cell, (4) ion-exchange, (5) chemical adsorption by bond formation (6) complexation, production of organic acids to form complex with adsorbate, (7) deposition on surface and (8) entrapment in the surface structures of cell involving a biological entity like live or dead biomass of fungi or bacteria (biosorbent) and some chemicals, metals or dyes (sorbate). The biosorption process involves a solid phase (sorbent or biosorbent; biological material) and a liquid phase (solvent, normally water) containing a dissolved species to be sorbed (sorbate, chemicals present in effluent, metal ions). Due to higher affinity of the sorbate for the sorbent species, the latter is attracted and bound by different mechanisms. The process continues till equilibrium is established between the amount of solid-bound sorbate species and its portion remaining in the solution. The degree of sorbate affinity for the sorbate determines its distribution between the solid and liquid phases.

A wide variety of biological materials are used as biosorbents. For example the waste mycelia available from fermentation processes, olive mill solid residues (Pagnanelli et al. 2002), activated sludge from sewage treatment plants (Hammaini et al. 2003), biosolids (Norton et al. 2003), live fungi and bacteria (Srivastava and Thakur 2006 a, b, 2007).

The mechanism of biosorption is complex, involving ion exchange, chelation, adsorption by physical forces, entrapment in inters and intrafibrilliar capillaries and spaces of the structural polysaccharide network as a result of the concentration gradient and diffusion through cell walls and membranes. There are several chemical groups that would attract and sequester the sorbate in biomass: acetamido groups of chitin, structural polysaccharides of fungi, amino and phosphate groups in nucleic acids, amido, amino, sulphhydryl and carboxyl groups in proteins, hydroxyls in polysaccharide and mainly carboxyls and sulphates in polysaccharides of marine algae that belong to the divisions Phaeophyta, Rhodophyta and Chlorophyta.

13.3 Analysis

13.3.1 Contribution of Instrumentation Techniques in Lignocellulose Degradation

To study the degradation of lignin by microbes, a number of instruments are used. They include electron microscopy (EM) and gas chromatography and mass spectrophotometer (GC-MS).

13.3.1.1 Electron Microscopy (EM)

Electron microscopy (EM) (scanning (SEM), scanning-transmission (STEM) and transmission (TEM)) and ancillary techniques (e.g. X-ray microanalysis, electron

diffraction) are now routine procedures which have been successfully applied to an array of problems in lignocellulose biotechnological research. These problems range from conventional studies on morphological aspects of wood cell wall ultrastructure (Fengel and Wegener 1984), biodegradation (Blanchette et al. 1990) and biopulping (Sachs et al. 1989), to enzyme interactions with pulp fibers (Mora et al. 1986) and more recently pitch problems in paper mills (Blanchette et al. 1992). Conventional SEM and TEM have been used essentially to confirm the ability of various microbes to modify and degrade wood cell walls and to visualize these events in time and in space. SEM gives the picture of surface view while TEM gives the idea of the changes taking place inside the wood.

13.3.1.2 Gas-Chromatography Mass Spectrophotometery

Analytical techniques such as pyrolysis-gas chromatography mass spectrometry (Py-GC-MS) are useful for the chemical characterization of lignin-containing materials, providing data on the relative amounts of different types of lignin units (Calvo et al. 1995a, b). Curie-point Py-GC-MS is a rapid microanalytical method for the structural analysis of lignin polymers on a molecular level. The technique requires minimum sample preparation and preserves side-chain information of the phenylpropane structural units. Py-GC-MS has been applied to pulp mill effluents, chlorolignins in xylan (Erik et al. 1993). The main disadvantage of GC relies in the fact that derivatization is necessary and the life of some derivatives is reduced to 12–24 h (Latorre et al. 2003).

GC-MS studies helps in understanding the process of degradation. If samples of different durations are analyzed then it is possible to study the process of degradation as the intermediates formed in between can be detected. Detection of intermediates or end products of degradation gives the conclusive evidence of lignin degradation. Recently, instead of using pyrolysis GC-MS, in which sample is suddenly heated at very high temperature to make it volatile, silylation is done. Samples are treated with silylating agents [BSTFA (N, O-bis (trimethylsilyl) trifluoroacetamide) and TMCS (trimethylchlorosilane)] and trimethyl silyl derivatives are analyzed (Raj et al. 2007).

13.3.2 Denaturing Gradient Gel Electrophoresis (DGGE)

Bioremediation, the use of microbes to degrade environmental contaminants is receiving increased attention as an effective biotechnology to clean up polluted environments as it offers several advantages over the traditional chemical and physical treatments for diluted and widely dispersed contaminants. The establishment of methods to monitor microbes and their genes in the natural environment is desirable because it is necessary to understand the dynamics of microbes that degrade pollutants in order to carry out bioaugmentation efficiently and safely (Tani et al. 2002). To achieve this goal denaturing gradient gel electrophoresis can be used.

In DGGE, DNA fragments of the same length but with different base-pair sequences can be separated. Separation in DGGE is based on the electrophoretic mobility of a partially melted DNA molecule in polyacrylamide gels, which is decreased, compared with that of the completely helical form of the molecule. The melting of fragments proceeds in discrete so-called melting domains: stretches of base pairs with an identical melting temperature. Once the melting domain with the lowest melting temperature reaches its melting temperature at a particular position in the DGGE gel, a transition of helical to partially melted molecules occurs, and migration of the molecule will practically halt. Sequence variation within such domains causes their melting temperatures to differ. Sequence variants of particular fragments will therefore stop migrating at different positions in the denaturing gradient and hence can be separated effectively by DGGE (Muyzer et al. 1993). By comparing the pattern of bands or amplifying the separated bands and then sequencing them, it is possible to track the presence or distribution of microbes of interest.

13.3.3 Detoxification Studies

Bioremediation using microorganisms is very attractive option but it is not always the case, it might increase toxicity (van de Wiele et al. 2005). The measurement for toxicity after microbial treatment suggests whether we should take the microbe for bioremediation further. There are many techniques reported so far to measure the toxicity. Effect of toxicity can be many ways such as genotoxity, carcinogenesis, teratogenesis, mutagenesis and stress caused by toxicant on physological activity. Genotoxic effect can be measured by many ways such as comet assay, end labeling of DNA to visualize the nicking in DNA. For carcinogenesis studies, a comprehensive study is required to established relationship. For mutational effect one can choose S*almonella* mutagenesis test. The physiological stress can be measured by many ways depending on type of stress such as metabolic, neuronal, reproductive stress etc. Some specific biochemical and cell based assays are very much popular due to rapid and reproducible results.

After biodegradation of any compound, it forms an array of secondary metabolites which may cause toxicity. In case, pulp and paper mill effluent has not been classified as potent carcinogen, teratogenic or genotoxic compounds. However, compounds present in it may bind AhR and hence caused the toxicity in terms of CYP activity and apoptosis. Mitochondria play a major role in apoptotic pathway as it is responsible for a variety of key events in apoptosis such as changes in electron transport, loss of mitochondrial trans-membrane potential ($\Delta\psi$), failure of Ca^{2+} control, generation of reactive oxygen species (ROS), and involvement in pro (Bax) and anti apoptotic Bcl-2 family proteins. These events have been proven to very useful indicator in toxicity evaluation of any compound including heterocycles (Ding et al. 2006). The immortal cell lines of human are able to mimic the toxic response in the *in vivo* system with fair reproducibility.

The cancerous cell lines are being used for toxicity evaluation, widely, because it retains the inherency of the organ, tissue, or cell specific responses by cancerous cell lines for long time unlike to normal cell lines. Parameters of toxicity may be morphological deformation, membrane integrity, cell viability, mitochondrial membrane potential ($\Delta\Psi m$) etc. (Rikans and Yamano 2000). These toxic parameters can be assessed by both biochemical assay and microscopic observations. Compounds present in this effluent disrupt the endocrine system and produced stress responses like ROS, O_2^{-2}. The toxicity pathways of dioxin are now well established and a dioxin responsive element (DRE) has been identified but unfortunately, the binding of dibenzofuran to the DRE has not been established. However, it shows binding to aromatic hydrocarbon receptors (AhR) and induces cytochrome P-450 monooxygenases especially CYP1A1 and CYP1A2 (Chaloupka et al. 1994). It is well established that many of these contaminants are acute or even chronic toxins. Chlorinated organic compounds, which include dioxins and furans, have the ability to induce genetic changes in exposed organisms (Nestmann and Lee 1985).

13.4 Future Perspectives

The high polluting potential of pulp and paper industry wastewaters can no longer be ignored. Microbial decolorization and degradation of colored effluents is a cost-effective and promising green technology for treatment of such effluents. Reports of white-rot fungi that show lignin-degrading ability in saline conditions are very few. Industrial effluents are mostly alkaline and rich in carbonates, chlorides, and sulfates. In light of this, the marine fungi and bacteria hold good promise for the application of bioremediation of colored effluents under saline conditions.

In the early 1990s, it was believed that the substitution of elemental chlorine with chlorine dioxide would eliminate the formation of furans and dioxins and reduce adsorbable organically bound halogens levels by almost 90%. However, it has been realized lately that, despite the use of Elemental Chlorine Free (ECF) processes, organochlorines have not been eliminated from discharges, just reduced. The debate between ECF and Total Chlorine Free (TCF) may not be resolved soon, but it is clear that TCF technology has many advantages over ECF and is more eco-friendly in the long run.

13.5 Conclusions

For any reasonable measure of success in treating pulp and paper mill effluents, future abatement programs should include a bilateral strategy for the use of alternate, cleaner technologies (e.g. the replacement of chlorine for bleaching, oxygen delignification and prolonged cooking) on one hand, and the development of economically viable and efficient technologies to treat these effluents on the other.

Pollution from pulp and paper mill effluents is a complex environmental problem; its permanent solution will require comprehensive system considerations as well as multidisciplinary and holistic approaches.

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Chapter 14
Bacteriophage Based Technology for Disinfection of Different Water Systems

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Abstract Water resources are becoming limited due to the contamination problems caused by life threatening human pathogens. Traditional water purification methods, viz, chlorination, radiation and filtration are used for the reduction of pathogenic bacteria in water systems, have many disadvantages. Phage mediated biocontrol of human pathogens in the water bodies has the potential to reduce the risk of spread of pathogens and problem of emergence of antimicrobial resistant bacterial strains through transduction. Successful application of phages requires complete understanding of the microbial community dynamics, and physical and chemical parameters of the system. In addition, constant monitoring for the emergence of resistant bacterial strain is essential. Phage based pathogen removal is effective, provided, phage usage is optimized to deal with the factors affecting phage treatment in different environmental conditions. Phages can be used as potential disinfectant in the natural water bodies alone or in combination with physical and chemical process. Here, the potential application of bacteriophage technology in the water systems, viz., river, swimming tanks, ponds, and lakes to eliminate human bacterial pathogens is discussed.

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14.1 Introduction

Water is an important and vital part that plays an important role in the surrounding environment and in the life of living things. Water covers nearly 75% of the earth's surface. Drinking ample amount of water is necessary for good health. Now-a-days water resources are becoming limited due to contamination problems. Unfortunately, life threatening bacterial pathogens, toxic chemicals, contaminate water that we drink and use for various purposes, thus can be responsible for significant health risks. The drinking water of most communities and municipalities is obtained from two sources; surface water sources particularly rivers, lakes, wells, streams ponds, and ground water sources. These sources are likely to get polluted with the domestic wastes which are suspected as the major sources of water borne diseases (Huttly 1990). Surface waters like rivers, lakes, ponds which are used for drinking and for other purposes are likely to get polluted with domestic waste discharges that contain a wide range of pathogenic micro-organisms which may pose a health hazard to human population (Borrego and Figueras 1997), and health hazards could be severe in heavily populated countries (Table 14.1).

In addition to this, recreational water systems; viz swimming tank and pond systems, also get contaminated with several potent bacterial pathogens, particularly *Salmonella* Spp. and *Vibrio* Spp. When people swim in the swimming pool, they introduce organic contaminants like urea, creatine etc. from urine and sweat.

Table 14.1 List of waterborne bacterial pathogens and possible disease or symptoms

Sr.No	Bacteria	Disease(s) or symptoms
1.	*E. coli* (enteropathogenic strains)	Haemorrhagic colitis
2.	*Clostridium botulinum*	Botulism
3.	*Shigella spp.*	Bacillary dysentery
4.	*Pseudomonas spp.*	Otitis externa, skin infections
5.	*Staphylococcus aureus*	Wound, ear and skin infections
6.	*Mycobacterium spp.*	Swimming pool granuloma, hypersensitivity, pneumonitis, leprosy, tuberculosis
7.	*Salmonella spp.*	Typhoid, salmonellosis
8.	*Leptospira spp.*	Haemorrhagic jaundice, aseptic meningitis, leptospirosis (Weil's disease)
9.	*Vibrio cholerae*	Cholera
10.	*Legionella spp.*	Legionellosis (Pontiac fever and Legionnaire's disease)
11.	*Clostridium perfringens*	Gastroenteritis(food poisioning)
12.	*Listeris monocytogenes*	Listeriosis
13.	*Bacillus anthrasis*	Anthrax

Swimming pools get contaminated with a variety of bacterial pathogens coming from fecal and non-fecal sources. Fecal source may be the swimmer who accidentally discharges enteric pathogens along with feces. Birds and rodents may also contaminate water via fecal contamination route. Non-fecal source includes mucus, saliva, skin and vomiting by the swimmer. Swimming pool water has direct effect on people, who use it. In Washington D.C., in 1954 there had been an endemic of pharyngeal-conjunctival fever, spreading of this infection was mainly via swimming pools. Swallowing contaminated water accidentally is a common cause of diarrhea among swimmers, especially if the pool is crowded and not chlorinated regularly. Insufficient chlorine and clogged filters cannot adequately disinfect water, making it a home to bacteria like *E. coli,* which causes diarrhea and abdominal cramps. Other bacteria like *Staphylococcus* and *Streptococcus sp.* (cause of ear, nose, throat and skin infections), *Pseudomonas aeruginosa* (cause of eye, ear and skin infections) and *Mycobacterium marinum* (cause of skin rashes) also flourish in non-chlorinated water.

A pond ecosystem refers to fresh water ecosystem where there are communities of organism dependent on each other with the prevailing water environment for their nutrients and survival. Usually ponds are shallow water bodies with a depth of 12–15 feet in which the sun rays can penetrate to the bottom permitting the growth of plants there. In contrast to a riverine system (running water), a pond represents a lotic system (standing water). The organic load along with human pathogens brought in by the feeder canal settle down in the pond sediment. In general, pond is more productive with respect to aquatic plants and animals such as molluscs, prawns, fish etc. Pond systems may be natural or artificial. Pond systems are of different types,

1. **Aquaculture Pond System**: Aquaculture is one type of water system where different varieties of fishes are being produced which is nothing but farming of fishes. These systems are suffering from heavy financial losses due to the development of infections caused by pathogenic microorganisms, viz., *Salmonella* spp., *Vibrio* spp.
2. **Wetland and Detention Pond System**: Constructed wetlands and detention pond is a system applied to reduce the pollution load carried by urban storm water. Recent epidemiological studies have shown risk of bacterial infections by swimming in recreational waters polluted with storm water.
3. **Oxidation Pond**: When sewage is treated in oxidation ponds, an attempt is made to create conditions which are similar in their effect to those existing in a river where self purification is known to take place.

14.1.1 Waterborne Bacterial Diseases

Drinking water safety is a worldwide concern. Contaminated drinking water has impact on human health worldwide, especially in developing countries. It is estimated

that 1.1 billion of the world's population does not have access to safe clean water. Lack of clean water for drinking, cooking, washing and for recreational purposes, and lack of sanitary waste disposal systems are responsible for high disease burden. Besides, inadequate water, sanitation and hygiene account for large part of burden of illness deaths in developing countries. Lack of improved domestic water supply leads to diseases through two principal transmission routes. Transmission of waterborne disease occurs by drinking contaminated water. This has resulted in outbreaks of cholera and typhoid. Water washed diseases occur due to lack of sufficient quantity of water for washing and personal hygiene. Skin and eye infections are easily spread in such situations (WHO 2000).

14.1.2 Statistics of Water Borne Diseases

The World Heath Organization (WHO) reported that 80% of diseases and one third of deaths in developing countries are due to consumption of contaminated water. According to UNICEF, 3.8 million children under the age of 5 years died in 1993 from diarrhoeal diseases worldwide. On a global basis, around two million deaths per year are attributed to waterborne diseases and especially diarrhea in children (Gordon et al. 2004). It has been estimated that around 37.7 million Indians are affected annually by waterborne diseases such as viral Hepatitis, Cholera, Jaundice and Typhoid. 1.5 million Children are estimated to die of diarrhea alone and 73 million working days are lost due to waterborne diseases each year. Figure 14.1 and Table 14.2 reflects the cases of waterborne diseases like diarrhea and cholera occurring in various states of India.

14.1.3 Water Treatment Scenario

The drinking water should be free of microbial pathogens, taste, odor, turbidity. To achieve these goals raw water is subjected to various physicochemical processes. Table 14.3 summarizes criteria for different types of waters.

The water is treated in two major steps by using conventional filter plants and softening planting. Figure 14.2 shows conventional filter plant for drinking water treatment (Metcalf and Eddy 2003). Filter plants use coagulation and flocculation as the major processes. Coagulants used are aluminum sulfate, ferric chloride and ferric sulfate. After coagulation, the produced flocs are allowed to settle in a clarifier. Clarified effluents are then passed though sand diatomaceous earth filters. Water is finally disinfected before distribution. Softening plants softens the treated water by precipitating Ca^{2+} and Mg^{2+} ions, which cause water hardening. The softening water is then filtered and again disinfected to kill pathogenic bacteria.

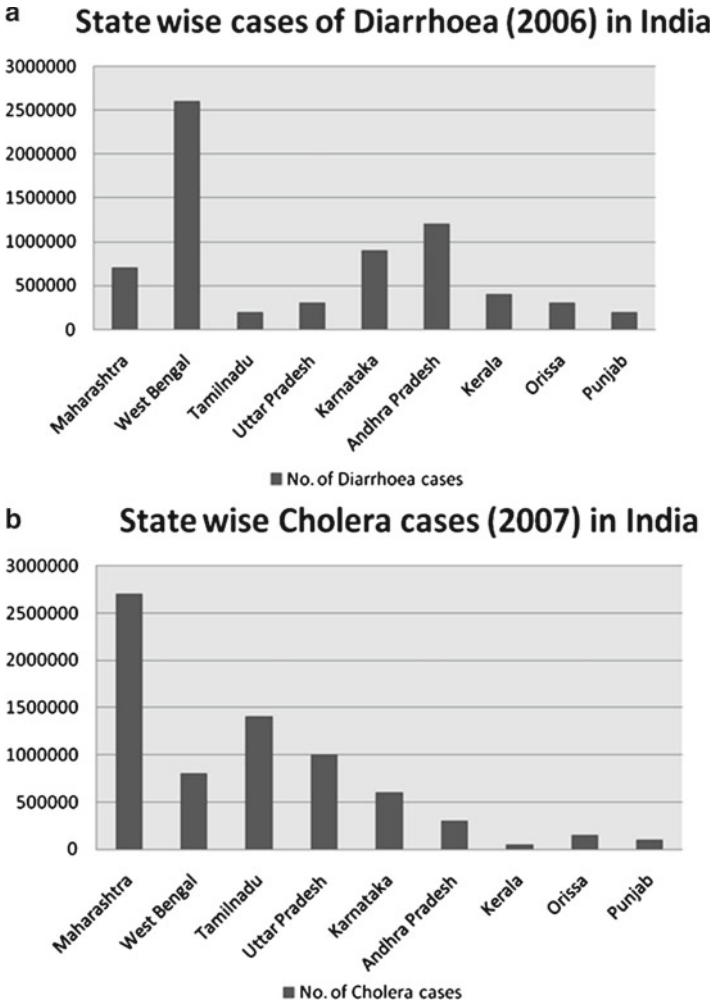

Fig. 14.1 (**a** and **b**) State wise cases of Diarrhea (2006) (**a**) and Cholera (2007) (**b**). West Bengal and Andhra Pradesh showed maximum numbers of Diarrhea cases in 2006, whereas Maharashtra and Tamilnadu showed maximum numbers of Cholera cases in 2007. (Source: chapter on "Drinking water, sanitation and clean living conditions", 11th five year plan document)

14.1.4 Status of Water Treatment Scenario in India

14.1.4.1 Water and Its Quality

Water pollution is a serious problem in India as almost 70% of its surface water resources and a growing number of its ground water reserves are already contaminated by biological, toxic, organic and inorganic pollutants. In many cases, these resources have been rendered unsafe for human consumption as well as other activities

Table 14.2 Cases and deaths due to waterborne diseases in various states of India

	Diarrhoeal disease		Viral hepatitis		Typhoid	
States	Cases	Deaths	Cases	Deaths	Cases	Deaths
Andhra Pradesh	1,215,659	124	17,846	28	135,550	12
Arunachal Pradesh	32,032	30	553	6	9,098	23
Chattisgarh	95,202	13	1,491	2	21,474	6
Gujarat	382,056	4	9,396	16	7,290	0
Haryana	285,342	42	3,983	11	5,688	4
Himachal Pradesh	347,055	28	835	11	26,327	5
Jammu & Kashmir	519,317	32	5,882	0	42,369	0
Karnataka	939,221	1,279	14,980	24	96,147	5
Madhya Pradesh	318,935	88	2,499	9	28,654	29
Maharashtra	695,723	93	43,215	131	39,663	8
Manipur	13,614	17	346	0	2,421	2
Meghalaya	178,260	33	294	2	6,709	1
Mizoram	18,063	20	546	11	1,392	2
Orissa	373,748	40	2,687	38	15,387	9
Punjab	182,451	64	3,829	17	17,008	3
Rajasthan	318,169	21	3,869	78	14,084	131
Sikkim	51,433	8	290	2	428	2
Tamil Nadu	116,062	12	4,523	0	36,973	0
Tripura	150,750	47	2,520	14	18,547	19
Uttarakhand	94,746	6	3,381	0	15,020	2
Uttar Pradesh	284,709	55	3,716	6	42,648	13
West Bengal	2,622,968	964	7,433	205	110,835	70
A&N Islands	22,752	2	213	4	3,055	0
Delhi	94,398	85	4,080	42	13,774	18
Pondicherry	137,443	8	615	7	1,936	1
Total	10,079,263	3,124	146,433	673	726,484	651

Source: chapter on "Drinking water, sanitation and clean living conditions", 11th five year plan document

Table 14.3 Quality criteria for different types of water

Sr. No.	Water type	Criteria
1.	Drinking water source without treatment but after disinfection	1. Total coliform count. MPN/100 ml shall be 50 or less 2. BOD (5 days 20°C) 2 mg/l
2.	Drinking water source after conventional treatment and disinfection	1. Total coliform count MPN/100 ml shall be 500 or less 2. BOD (5 days 20°C) 3 mg/l
3.	Outdoor bathing (Organized)	1. Total coliform count MPN/100 ml shall be 500 or less 2. BOD (5 days at 20°C) 3 mg/l
4.	Propogation of wild life and fisheries	1. PH – 6.5 to 8.5 2. Force Ammonia (as N) 1.2 mg/l

Source: Central Pollution Control Board, India (www.cpcb.nic.in)

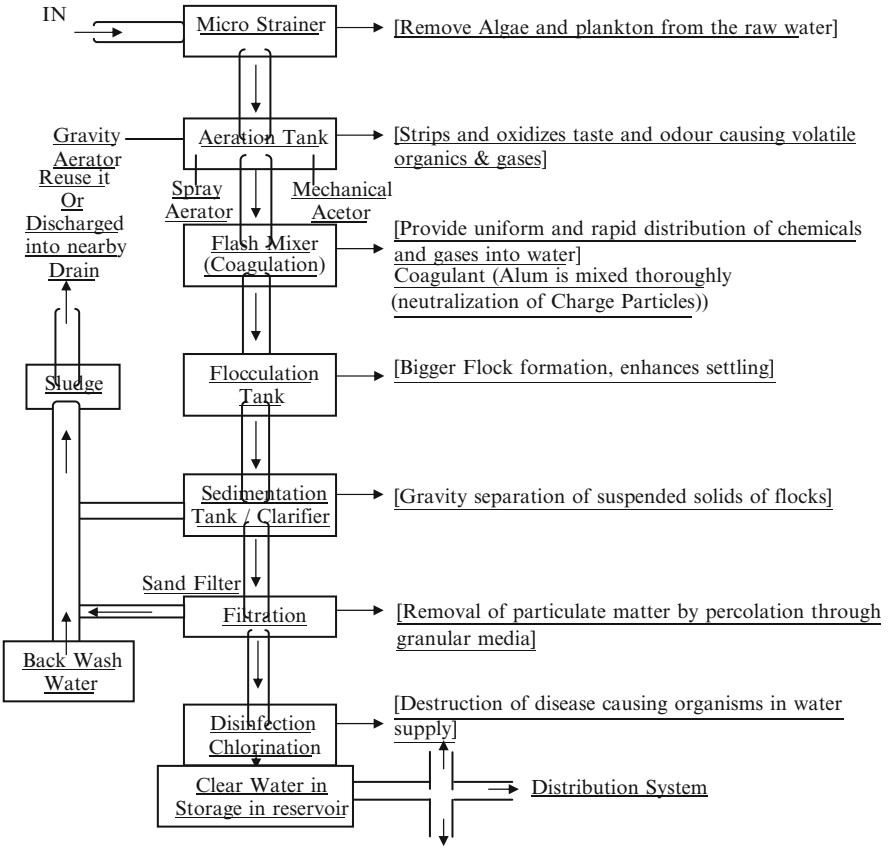

Fig. 14.2 Diagrammatic representation of water treatment process

such as irrigation and industrial needs. India, the land of holy rivers is fast becoming a land of highly polluted and even toxic rivers; in 1995, the Central Pollution Control Board identified severely polluted stretches on 18 major rivers in India (World Bank 1999). The high incidence of severe contamination near urban areas indicates that the contribution of industrial and domestic sectors to water pollution is higher than their relative importance to the Indian economy would imply. All of India's 14 major river systems are heavily polluted, mostly from the 50 million cubic meters of untreated sewage discharged into them each year. The domestic sector is responsible for the majority of the waste water generation in India. Inadequate treatment of human and animal wastes contributes to the high incidence of water related diseases in the country. Combined, the 22 largest cities in the country produce over seven million liters of domestic waste per day and out of that only 80% is collected for treatment (CSE 1999). Water contamination by human waste is often discharged directly into natural water bodies or seeps into the ground water. The level of fecal coli form bacteria in most rivers often exceeds WHO standards and found to be

responsible for intestinal tract diseases among the population. The disease burden is high in India due to the contaminated drinking water supply. Other reasons are poor sanitation, lack of access to freshwater, poor hygiene etc. which are most common in developing countries.

14.1.4.2 Water Treatment Technologies in India

The primary goal of water treatment plant is to produce safe (pathogens free and toxic chemical free) water for human consumption. Unfortunately in many developing countries, the bulk of domestic and industrial waste water is discharged into rivers, lakes, ponds etc. without any treatment or after primary treatment. These surface water resources are used for drinking purposes. A properly designed treatment plant is required to guarantee safe drinking water. In India almost 20% waste water is discharged into water reservoirs which are used as source of drinking water without any treatment. In Latin America about 15% of collected waste water passes through treatment plant. In Venezuela, a below average country in South America with respect to waste water treatment, 97% of countries sewage is discharged raw into the environment (Caribbean Environment Program 1998). In countries, such as Iran and Tehran majority of populations discharge untreated waste into the city's ground water (Massoud and Ahamad 2005).

India is the second most populated nation in the world with the human population exceeding one billion. Growing population pressures and increased development have led to higher populating, over exploitation and degradation of the natural environment. Increased population and Indian economy limits the management of waste water treatment processes.

14.1.5 Disinfection of Water

Disinfection is an important part of the water treatment process. Drinking water as well as waste water is disinfected at different levels. Waste water is treated at tertiary level after biological treatment process. Drinking water is disinfected at primary level only. Disinfection minimizes and reduces human exposure to pathogens and parasites present in water. Traditionally, different disinfectants are employed worldwide. From the early 1900–1970s treatment objectives were concerned primarily with (1) The removal of colloidal, suspended and floatable material, (2) The treatment of biodegradable organics and (3) Elimination of pathogenic organisms, but generally with unspecified goals. Passage of the Federal water pollution control act Amendments of 1972 (Public Law 92–500) as amended in 1977 and 1978 (Clean water act, Public Laws 95–217 and 97–117 respectively), stimulated substantial changes in waste water treatment to achieve the principal objective of these acts, which was to restore and maintain the chemical physical and biological integrity of the Nations "Waters". In effect, the goal of the clean water act was to make the nation's waters "Fishable and Swimmable" (Metcalf and Eddy 2003).

Table 14.4 Characteristics of ideal disinfectant

Sr. No.	Parameter	Desired property
1	Safety	Should be safe to handle and use
2	Cost	Should be cost effective
3	Availability	Should be available in large quantity
4	Penetration	Should penetrate through substrate
5	Stability	Should be stable w.r.t. time
6	Effects on microorganisms	Should be effective at high dilutions
7	Interaction with extraneous material	Should not be absorbed by organic matter other than bacterial cells
8	Corrosion	Should not be corrosive
9	Solubility	It must be soluble in water

Table 14.5 Comparison of ideal and actual characteristics of commonly used disinfectants

Sr.	Characteristic	Chlorine	Chlorine dioxide	Ozone	UV radiation
1	Cost	Low cost	Moderately low cost	Moderately high cost	Moderately high cost
2	Homogenicity	High	High	High	–
3	Interaction with extraneous material	Oxidizes organic matter	High	Oxidizes organic matter	Absorption of organic matter
4	Non-corrosive	Highly corrosive	Highly corrosive	Highly corrosive	–
5	Nan-toxic to higher forms of life	Highly toxic of higher forms of life	Toxic	Toxic	Toxic
6	Penetration	High	High	High	High
7	Safety concern	High	High	Moderate	Low
8	Stability	Stable	Unstable	Unstable	–
9	Solubility	Moderately	High	High	–
10	Toxicity to micro organisms	High	High	High	High

Source: Metcalf and Eddy (2003)

It is important that disinfectant that is used in water treatment processes should be ideal (Table 14.4) and safe to handle and apply. There are different agents that are used as disinfectants: (1) Chemical agents, (2) Physical agents, (3) Radiation. Chemical agents are usually used because of their high efficiency of disinfection. Chemical agents which are commonly used are chlorine and its compounds, bromine, iodine, ozone, phenol and phenolic compounds, alcohols, heavy metals and related compounds, dyes, soaps and detergents, quaternary ammonium compounds. Of these, the most common oxidizing chemicals are chlorine and ozone.

Physical agents include heat and light. Radiation includes use of electromagnetic, acoustic and particle radiations (Table 14.5).

At the beginning of twentieth century chlorination was initiated to provide an additional safe guard against pathogens. The use of chlorination has decreased waterborne diseases to a greater extent. Its usefulness as a disinfectant was first discovered in 1910 when cholera and typhoid rates fell dramatically as a result of chlorination (Christman 1998).

14.1.6 Harmful Effects of Chemical Disinfectants

There is wide spread potential for human exposure to disinfection byproducts (DBPs) in drinking water because everyone drinks, bathes, cooks and cleans with water. Chlorine can cause eye, nose, stomach problems and sinus irritation when ingested via drinking water. Inhalation of chlorine leads to asthma, showering of Chlorinated water can magnify the effects of chlorine inhalation, due to the enclosed nature of shower stalls.

In 1976, the U.S. National Cancer Institute Published results showing that Chloroform, one of the trihalomethanes (THM) that occurs as a by product of drinking water disinfection, was carcinogenic in rodents (U.S. NCI Report). Since that time, there has been a concern that disinfection against microbial risks could also pose chemically induced cancer risks for humans (Melnick et al. 1994). Further more, several epidemiology studies have suggested a weak association between drinking chlorinated water and the occurrence of bladder rectal, and colon cancer (Doyle et al. 1997). In addition, disinfection alternative to chlorination, such as ozonation, produce byproducts for e.g. bromates that are carcinogenic to rodents (Balmier et al. 1995). Recently, it has been shown that consumption of drinking water with high THM levels is associated with adverse reproductive outcomes (Wailer et al. 1998; Xie 2004).

Problems associated with chemical disinfectants on human body are of significance considering the water disinfection scenario. Not only this, pathogenic bacteria found in water bodies are now-a-days becoming resistant to the action of chemical disinfectants. Chlorine resistance was progressively increased in *E. coli.* O157:H7 and thereby adapting to starvation (John et al. 1998). Microbes differ greatly in their sensitivity to disinfectants. Specific protozoa, viruses and bacteria are known to be highly resistant to chemical disinfectant and pose a unique challenge to the water treatment industry.

Micro-organisms produce a gelatinous material known as exopolysaccharide to form biofilms. Biofilm associated bacteria have been reported as being up to 3,000 times more resistant to free chlorine (Lechevallier et al. 1998). One study showed that *Staphylococcus aureus* isolated from healthy and sick persons showed resistance to chloramines (Balakliets et al. 1989). Chlorine resistance in *Salmonella typhi* was recognized 70 years ago (Heathman et al. 1936). Bacterial spores of Genus *Bacillus* and *Clostridium* have been the most resistance (Sagripanti and Bonifacino 1996; Russell 1997). *Pseudomonas aeruginosa* strains show resistance

Table 14.6 Characteristics of bacteriophage as disinfectant

Sr. No.	Characteristics	Lytic bacteriophages
1.	Cost of production	Cost effective production liquid and powder formulations can be possible
2.	Homogenecity	Homogenous
3.	Availability	They are replicate at the site of its host and are thus available where they are most needed
4.	Non corrosive	They are non-corrosive
5.	Safety concern	They are highly safe, no serious side effects have been found
7.	Non toxic to higher forms of life	They are highly specific in their action
8.	Effect on pathogenic bacteria	Lytic phages are found to be highly effective in killing pathogenic bacteria. Phage resistant bacteria remain susceptible to other lytic phages having similar target range

to repeated disinfectant exposure (Lakkis and Fleiszig 2001). In one study, *Pseudomonas aeruginosa* strains showed resistance to quaternary ammonium compounds used to disinfect contact lenses (Sundheim et al. 1998).

Therefore, to overcome all the above mentioned problems associated with the use of chemical disinfectants, an alternative strategy could be to use bacteriophage as biological disinfectant. Bacteriophages fulfill most of the characteristics of ideal disinfectants. Table 14.6 summarizes characteristics fulfilled by bacteriophages as ideal disinfectant.

14.2 Critical Review of Bacteriophage Based Technology for Disinfection of Different Water Systems

14.2.1 Use of Phage as Disinfectant in Water Treatment

Bacteriophages are the viruses of prokaryotes, which can either instantly kill a bacterial cell or integrate its genome into the host genome (Madigan et al. 1997). Bacteriophages are the most numerous organisms on the earth that play a key role in bacterial gene exchange and bacterial pathogenesis and continue to provide important insights into the basic molecular working of life. Through a combination of their antagonistic but metabolically intimate relationship with their bacterial hosts, lytic phages possess ideal properties to serve as agents of both antibacterial biocontrol and bacterial identification. The first report about phages was published by Hankin (1896). He showed that the waters of the Jamuna and Ganges Rivers in India could kill many kinds of bacteria. Bacteriophages get adsorbed on to the host cells and phage entry is mediated by specific receptors such as carbohydrates, proteins and lipopolysaccharides present on the surface of host cell (Marks and Sharp 2000).

Host range for bacteriophages is influenced by the specificity of interaction between phage attachment structures and host cell surface receptors. Host range for aquatic phages is generally assumed to be narrow (Alonso et al. 2002). Surprisingly cyanophages show broad host range (Suttle 2000).

Bacteriophages are ubiquitous in nature; they are highly abundant in the aquatic environment ranging from 10^4 ml^{-1} to excess of 10^8 ml^{-1} (Bergh et al. 1989). Aquatic viruses may have a role in determining the diversity of bacterial communities through control of selected species competing for resources (Weinbauer and Rassoulzadegan, 2003). Bacteriophages are extensively studied with respect to their application in various fields. They are readily exploited in biology fields such as molecular biology, biotechnology, agriculture science, pharmaceutical studies and in variety of industries. Phages were widely used in the early twentieth century to cure human and animal illness. Phages have been used as therapeutic agents against various bacterial infections (Sivera Mirza et al. 2006). There are reports regarding phage mediated "therapy" of bacteria found in association with plants, fungi or their products (Leverntz et al. 2001; Vinod et al. 2006). Phages have been used to treat bacterial infections, especially in Eastern Europe and have been shown to decrease biofilm formation. In the late 1960s, the world Health Organization set up an internal trial of phage therapy for Cholera in Dacca, formerly in Pakistan; now the capital of Bangladesh. It is with the support and review of the National Institution of Health. The high doses of anticholera phage were able to kill bacteria *in vivo* even if they were not able to complete many cycles of replication and amplification (Monsur et al. 1970).

14.2.2 Occurrence and Source of Bacteriophages

The pre-requisite for large scale production of bacteriophages is the source of phage. Bacteriophages occur abundantly in the natural water bodies including rivers, lakes, and ponds as they are ubiquitous in nature. Abundance of bacteriophages, specific to enteric bacterial pathogens in the natural water bodies is reported in many studies. In one study, somatic coliphages, F specific phages and *B. fragilis* phages were measured in the fresh water environments (Araujo et al. 1997). Armon et al. (1997) isolated and studied somatic coli phages and F specific bacteriophages from 1,500 drinking water samples in Israel and Spain. Bacteriophages of Bacteroids were studied for their potential in fecal source tracking (Andrey et al. 2005). Bacteriophages are used as indicators or tracers for the presence of bacteria in waste water treatment systems. Miernik (2004) isolated and studied coliphages from Vistula river, as a potential indicator of fecal pollution of water. Coliphages present in waste water are removed during activated sludge treatment (Bitton 1999). There are few reports suggesting that phages may be active components of activated sludge systems (Hertwig et al. 1999; Thomas et al. 2002). Bacteriophages specific for *Salmonella* group of organisms were isolated from swine effluent lagoons and were characterized (McLaughlin et al. 2006). Once the source of phage is decided, large scale production of phages can be made easily in the laboratory.

14.2.3 Exploitation of Bacteriophages in Various Water Systems

Water Systems (river, lake, pond, and swimming tanks) containing diverse group of pathogenic bacteria, viruses, protozoa and metazoan parasites, even when treated with chemical agents, continue to create serious health problems. Radiation, one of the most efficient water treatment procedures, is very expensive for practical implementation, in case of developing countries. Alternatives such as biocontrol agents may prove effective at such levels. There are reports in which phages have been used to control pathogens in aqueous environment, *in vitro*. EPA (Environmental Protection Agency) worst case water (WCW) microcosm studies were carried out for testing biocontrol of *Salmonella* species with the help of bacteriophages. The WCW provided a consistent and relatively simple, defined, turbid, aqueous matrix, containing high total organic carbon (TOC) and total dissolved solids (TDS) to simulate swine lagoon effluent. Wells containing WCW were loaded with *Salmonella enterica* subsp. *enteric* serrovar *typhimurium* (ATCC14028) and then treated with phages alone and in cocktail combinations. These treatments showed high inactivation rate of *Salmonella* group (McLaughlin and Brooks 2008). In another study river water microcosms were used in plates for testing potential of coliphages and phages specific for staphylococcus aureus against *E. coli* and *Staphylococcus aureus* (Bahadoor 2005). According to Withey et al. (2005), phages have the ability to control environmental waste water process problems such as foaming in activated sludge plant, sludge dewaterability and digestibility.

In the central part of Japan, fate of coliphages in the waste water treatment processes was studied for 10 months. High titer of coliphages (ten times greater than the influent) was detected in the effluent. Bacteriophages have the potential to reduce competition between nuisance bacteria and functionally important microbial populations. Phages are natural predators of bacteria. They are specific and precise in their action of predation. The specificity of interaction between phage attachment structures and host cell surface receptors influences bacterial host range. Host range is generally assumed to be narrow for aquatic phages (Alonso et al. 2002). However broad host range (polyvalent) Cyanophages are widely isolated (Suttle 2000). Such polyvalent phages have been isolated from sewage treatment plants (Jensen et al. 1998). Lytic phages are commercially important in terms of their bacteria killing activity. Lysogenic or temperate phages are not much important commercially; but they do posses research importance in terms of their capacity to integrate their genome into host genome and reside in the host genome in the form of prophage.

Constructed wetland system with bacteriophage application offers attractive alternate for storm water management for reducing load of disease causing viruses to the receiving waters (Yousefi et al. 2004). Bacteriophages were used to decrease the bacterial load from sewage water along with the self purification level in the rivers and lakes (Pretorius 1962). Bacterial contamination of industrial water systems leads to biofouling by biofilms and corrosion from bacterial induced corrosion. Prevention or reduction of process interruptions and general contamination, fouling and corrosion is achieved by the destruction of targeted problematic bacteria with

naturally occurring, non-engineered bacteriophage virulent for targeted bacteria. The use of bacteriophage therapy in aquaculture seems very promising and practical. Few attempts have been made to use bacteriophages to treat diseases in aquaculture. Wu and Chao (1982) examined the effect of a phage, ΦET-1, isolated from pond water in Taiwan, on *Edwardsiella tarda*. In *in-vitro* experiments, phage killed 25 of 27 *E. tarda* strains and reduced the bacterial count to less than 0.1% when a bacterial suspension of 1.2×10^{12} cells/ml was infected with ΦET-1 at multiplicity of infection (MOI) of 0.08 after 8 h.

The studies of Park et al. (1997) and Nakai et al. (1999) have shown that bacteriophage could be used to control *Lactococcus garvieae* infections of yellowtail and other marine fishes.

Use of phages in water treatment processes is very important due to their predation power, and their ability of not being pathogenic or toxic to humans. Specific bacteriophages such as *Salmonella* spp. phages or *Vibrio* spp. phages can be used to remove these pathogens from waste water (Shah et al. 2005; McLaughlin et al. 2006). To understand the scope of phages in waste water treatment processes, it is important to understand the phage and bacterial host relationship. Most of the bacterial strains present in the environment are ever emerging as antibiotic resistant strains during the course of evolution through mutation and conjugation. Such highly pathogenic and resistant bacterial strains can not be easily removed using traditional chemical disinfection process. Also, due to repeated use of chemical disinfectants, chlorine resistant and chlorine degrading bacteria are emerging. Hence for successful application of phages in waste water treatment, it is of prime importance to know the killing power and the ways in which phages can be applied for disinfection.

Phages have been applied almost in all fields to kill nuisance bacteria. Bacterial strains associated with waste water and chlorinated drinking water contains coliforms, enteric pathogens including *Vibrio spp*., *Salmonella spp*., *Shigella spp*. etc. Pathogens are found to have strong ability to persistently adapt to surrounding conditions for survival (Kearney et al. 1994). Hence they can be relatively resistant to traditional methods of pathogen removal. Members of the genus *Salmonella* are known to survive for more than 1 year in the sludge applied to farm land (Mitscherlich and Morth 1984; Edmonds 1976). The bacteria survived upto 16 months on grass treated with sludge in Switzerland (Hess and Breer 1975). It is known that most of the *Salmonella spp.* are resistant to amphicillin, chloramphenicol, and other classes of antibiotics. These strains have been isolated (Kare et al. 1999; Salehi et al. 2005; Murugkar et al. 2002). It has also been found that *Salmonella typhimurium* can grow at pH 4.0 (Lin et al. 1995; Foster and Spector 1995). Similar observations are noted with *Shigella spp.* and *E. coli spp.* (Lin et al. 1995). It shows that, contaminated water associated bacteria have an efficient adoptive behavior against environmental conditions. *Vibrio spp.* is one of the most notorious and highly pathogenic bacterium found in the contaminated water. *Vibrio cholera* responsible for cholera is highly prevalent in estuarine conditions and is related to cholera outbreaks in developing countries, most notably in Bangladesh (Alam et al. 2006).These strains of *Vibrio* are highly pathogenic and frequently mutate to give rise to new antibiotic

resistant and toxic strains. In one study, it has been found that cholera epidemics are self limiting in nature due to phage mediated biocontrol; which can be said to be related to amplification of *Vibrio cholerae* specific bacteriophages due to host (Shah et al. 2005).

Major problem regarding phage mediated biocontrol of bacteria is the efficiency of the phage production. Also, the phages on viable but non culturable bacteria have not been evaluated. It has been found that *Salmonella* spp. exists in stagnant water in dormant form and not in an active form. Most of the phages cannot attack dormant bacteria due to improper adsorption on bacterial surface. One aspect of use of phage is the emergence of bacteriophage insensitive mutants (Connerton and Connerton 2005). However, unlike chemical therapeutic agents such as antibiotics, phages constantly evolve to circumvent their host's defenses and resistant bacteria are often less fit or less virulent than their phage sensitive counter parts (Smith and Huggins 1983).

14.2.4 Phages Effective Against Bacterial Biofilms in Water

Bacterial biofilm consists of bacteria embedded in glycocalyx which is predominantly made up of an exopolysaccharide (EPS) that has the capacity to resist many antimicrobial treatments that results in the public health problems (Xavier et al. 2005; Parsek and Singh 2003; Costerton et al. 1995). Biofilms create major problem in water and waste water treatment process. They predominate in water pumps and water carriage facilities. Microbial EPS is responsible for binding microbial cells and particulate matter together (Bura et al. 1998). Traditional antimicrobials are found unable to penetrate biofilms. It has been observed that antibiotic resistant biofilm forming pathogenic bacteria are ever emerging. Biofilm cells are found to become 10–1,000 times resistant to the effects of antimicrobial agents (Prosser 1987; Cochran et al. 2000). In one study, it has been reported that chlorine, a commonly used disinfectant, did not reach >20% of the bulk media's concentration within mixed *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* biofilm, as measured by a chlorine detecting micro electrode (Beer et al. 1994).This finding confirms that biofilms of *Ps. aeruginosa* and *K. pneumonniae* are impermeable to chlorine, therefore these organisms are found to be resistant to chlorine.

There are many reports on application of phages to disperse biofilms (Timothy and James 2007; Curtin and Donlan 2006; Bedi et al. 2009). In another study, bacteriophages were applied for *In-Vitro* management of biofilm of *Ps. aeruginosa* hospital isolates (Ahiwale et al. 2010).

Narrow host specificity was implicated in the failure of some early phage treatment (Sulakvelidze et al. 2001). It shows the potential impracticality of attempting to develop phage treatments for generalized or broad spectrum control of bacterial populations. Although most phages in aquatic system are specific, it has been reported that in diverse microbial communities, where each species is present at low abundance, polyvalent phages would be expected to be at competitive advantage relative to highly specific phages (Wilkinson 2001). Problems of biofilm forming pathogenic bacteria could be solved with the help of lytic phages in water systems (Fig. 14.3).

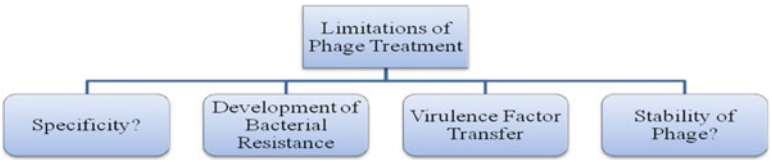

Fig. 14.3 Limitations of phage treatment

14.2.4.1 Stability of Phages

The major problem in phage treatment is inactivation of phages due to various physicochemical factors that include radiations and total solids in the water. In one study, it was reported that phage T6 gets in an aqueous system by high energy photons (Tanooka 1965). In a study carried out by Tanji et al. (2002) to determine fate of coliphages in waste water treatment process, it was suggested that, loss of virus during waste water treatment is due to attachment of viruses to waste water particulate matter which subsequently settles down and becomes a part of settled sludge. More than 97% of coliphages may be associated with suspended particles, which are transferred to sludge during settlement. Hence, poor phage penetration into microbial floc may be one of the reasons of reduced phage treatment as most of the phages get inactivated therefore their infectivity is lost. Schaper et al. (2002) studied comparative resistance of phage isolates of four genotypes of F–specific RNA bacteriophages to various inactivation processes including chlorination, ammonia, extreme pH's, temperature and salt content. It was observed that genotype II, III and IV were inactivated faster than genotype I. Stability of F–specific RNA bacteriophage subgroups were measured in waste water, after inactivation in surface or after waste water treatment and in mixtures of waste water of human and animal origin. Phages showed different degrees of persistence in the environment and to different disinfecting treatment. The greater survival of sub group I and II is treated samples. Chlorination contributes to inactivation of somatic coliphages. Environmental stresses viz. solar radiations and starvation may promote reduction in phage numbers and have deleterious effects on phage replication (Kokjohn et al. 1994). Payne and Jansen (2001) suggested that for successful phage treatment, phage inoculation should coincide with a bacterial population density sufficient to support phage replication. Loss of phage infectivity needs to be fulfilled reapplying phage preparations constantly. It could constraint the practicality of some phage treatments.

14.2.4.2 Host Specificity

As phages are host specific, a particular phage preparation cannot be used to control different types of micro-organisms. In wastewater treatment processes, a variety of types of bacteria can be detected. Also, microbial communities in wastewater differ

from place to place and with respect to time and environmental conditions. Hence, to use phage preparation the user must identify and do a thorough microbial community analysis for getting good results. Narrow host specificity was implicated in the failure of some early phage treatments. Sulakvelidze et al. (2001) showed the potential impracticality of attempting to develop phage treatments for generalized or broad-spectrum control of bacterial populations. Although most phages in aquatic system are specific, it was reported that, in diverse microbial communities, where each species is present at low abundance, polyvalent phages would be expected to be at a competitive advantage relative to highly specific phages (Wilkinson 2001). In another study, sensitivity of 500 bacterial isolates of the villi, a fermented milk product, to bacteriophages was checked, in which all phages showed narrow host range (Saxelin et al. 1986). In one study (Sundar et al. 2009), host specific bacteriophages were isolated from sewage against various human pathogens such as *E. coli*, *Salmonella typhi* and *Pseudomonas aeruginosa*. Each of the phage of *E. coli*, and *Salmonella typhi* was able to infect its original host bacteria but not the other bacteria. In contradiction to the above statements, one experiment suggests that bacteriophage host range is not always genera-restricted and that selection of subpopulations of bacteriophages capable of amplification in alternative genera may provide a tool for selection of broad host-range bacteriophages for the pathogen of interest. One *Klebsiella* and three different *Escherichia* isolates successfully amplified some bacteriophages from the *S. enteritidis* selected bacteriophage pool. Two selected bacteriophages, confirmed to amplify in *Escherichia* or *Klebsiella,* were further evaluated for ability to amplify in ten different *Salmonella* serovars by amplification in broth culture. One had the ability to amplify in six different *Salmonella* serovars, and the other had the ability to amplify in two different *Salmonella* serovars. (Bielke et al. 2007).

14.2.4.3 Development of Host Resistance

Development of phage resistant in host bacteria is a major limiting factor of phage treatment. It has been shown that bacteria rapidly become resistant to phage infection. It has been suggested that the metabolic cost of resistance gives sensitive strains a competitive growth advantage or that low abundance reduces the risk of phage host contact (Chao et al. 1977). According to Lenski and Lewin (1985), in chemostat studies, development of host resistance to phage is inevitable but emergence of new phage variant which can exploit other receptors of the host is relatively rare (Lenski and Lewin 1985). To solve the problem of host resistance, (Jackson 1989) developed a process involving use of host range mutant phages (H–mutants) for disease control. Similarly, in a study done by O'Flynn (2004) a cocktail of three phages against *E. coli* O15:H7 was used and it was observed that the frequency of bacteriophages insensitive mutants (BIM) formation was low, and all mutants reverted to phage sensitive characters. It has been observed that when *E. coli* MG1655 was treated with environmental bacteriophages, phage tolerant subpopulations with increased capacity of biofilm formation were emerged

(Locqua 2006). In another study, impact of phages on two species of (*E. coli* B and *Salmonella enterica* serovar *typhimutium*) bacterial community was reported. The results of this study suggest that, the cost of resistance sometimes allows phages to suppress and maintain low densities of susceptible bacteria when a resistant competitor is present (Harcombe and Bull 2005). Evidence of host resistance in activated sludge process is contradictory. It was reported that out of 48 dominant bacterial isolates only three bacterial isolates showed sensitivity to phages (Ewert and Paynter 1980) from mixed liquor and sewage and approximately 15% sensitivity was observed in activated sludge isolates. In contrast to above studies, Khan et al. (2002) reported that 60% bacterial isolates from activated sludge were phage sensitive. Fuhrman (1990), in a review on marine viruses and their biogeochemical and ecological effects suggested that phage resistance in bacterial hosts may not be prevalent in aquatic ecosystems particularly in oligotrophic conditions The problem of host resistance is likely to complicate use of phage preparations in water disinfection mechanisms, but strategies such as use of phage cocktail and use of mutant phages may solve the problem to a significant level. Use of host specific phages and use competitor bacteria may significantly reduce or may eliminate the target pathogenic bacteria.

14.2.4.4 Transducing Phage Particles

Phages act as vehicles of virulence factors. Temperate or Transuding phages bring about transfer of virulent genes of bacteria from donor cell to recipient cell (Genetic exchange between cells). The process of transduction can be categorized as: (i) Generalized transduction: transuding phage that introduces any region from donor chromosome and, (ii) Specialized transduction: transducing phage that introduces specific gene from donor to recipient cell. Bacterial virulence factors are frequently encoded by bacteriophages. This can confer virulence by transduction mechanism (Saunders et al. 2001). Also, bacteria and phage interactions are found to play an important role in contribution to the transfer of virulence factors; such interactions involve providing helper function to another unrelated phage (Boyd 2001) (Table 14.7). By avoiding the use of lysogenic phages, the possibility of integration of phage DNA into host chromosome can be reduced. Horizontal transfer of virulence factors may result in emergence of highly pathogenic, antibiotic or phage resistant bacterial strains; hence it is very important to assess sensitivity of non-target host to phage.

14.2.5 Isolation, Enrichment and Large Scale Production of Phages

Research of phages for biocontrol of life threatening bacterial diseases is growing. For successful applications of phages as a biocontrol agent, phages need to be isolated and enriched to produce sufficient number for the application. Isolation and

Table 14.7 Bacteriophage encoded virulence factors

Bacterial species	Bacteriophage	Gene	Protein/phenotype
Escherichia coli O157:H7	933, H.19B	stx	Shiga toxin
	ΦFC3208	hly2	Enterohemolysin
	λ	lom	Serum resistance
	λ	bor	Host cell envelope protein
Shigella flexneri	Sf6	oac	O-antigen acetylase
	sfII, sfV, sfX	gtrII	Glucosyl transferase
Salmonella enterica	Sop EΦ	sop E	Type III effector
	Glfsy-2	sodC-1	Superoxide dismutase
	Glfsy-2	nanH	Neuraminidase
	Glfsy-1	glpA	Insertion element
	ε^{34}	rfb	Glucosylation
Vibrio cholera	CTXΦ	ctxAB	Cholera toxin
	K139	glo	G-protein like
	VPIΦ	tcp	TCP pillin
Pseudomonas aeruginosa	ΦCTX	ctx	Cytotoxin

Source: Boyd (2001)

enrichment are two important steps in large scale production of phages. Phage Enrichment involves mixing environmental samples (source of phages) and specific host strain in enrichment media with overnight incubation. After incubation, lytic phages are detected as clear zones or areas (plaques) on lawn of bacteria used as a host (Adams 1959). In nature, phages of culturable as well as nonculturable bacteria exist, but we can isolate phages of only 20% of bacteria in the environment which are culturable *in vitro* (Andreottola et al. 2002). Repeated purification of phages using single host strain increases the specificity for that particular strain, potentially narrowing the host range of phage to be used. Purified phages can be checked against various pathogenic bacterial species to check the host range of phage. Such cross reactivity studies provide important insights about usability of phage in a mixture of bacteria.

Large scale production of phages was carried out in the laboratory between 1950 and 1970 (Marks and sharp 2000). Large scale production of M2 Phage of *E.coli* was carried out in the laboratory and around 75 mg phage per liter of the culture medium was produced (Sargeant and Yeo 1966). High titer liquid and powder phage formulations can be prepared in the laboratory. Phage formulations to be used should be easily miscible in water to get better results.

14.2.6 Commercialization of Phages

Although phages have potential for application in waste water treatment, phages for waste water treatment are not yet commercialized. Most of the phage application studies are at experimental level. It seems difficult to apply phages in wastewater treatment process, due to limitations in large scale production of bacteriophages.

Phages have been commercialized for use in phage therapy, in agriculture and in medical field. The first company to produce phages specifically for control of bacterial plant diseases was Agriphi, Inc., established by Jackson (1989). Also many companies are producing commercial preparation for the treatments of wound sepsis, infections of respiratory tract, diarrhea and dysentery (Sulakvelidze et al. 2001; Moris et al. 2004). Many companies are in process of development of effective phage formulation in disinfection processes.

Phage preparation can be monovalent (contain only one type of bacteriophage) or polyvalent (more than one type of phages or cocktail of phages). Phages can be formulated in various ways viz, powder, liquid and semi solid form. During 1930–1940, Eli Lily and Co. manufactured several therapeutic phage products for treating various bacterial infections. Other major companies involved in therapeutic phage production in the U.S.A. include E.R. Squibb and Sons and Swan–Myers (non Abbot Laboratories).

14.2.7 Commercially Available Phage Formulations

1. Aqueous embodiments of *Salmonella* bacteriophages are available in phosphate buffered saline, Luria Bertani Broth or Chlorine free water. *Salmonella* bacteriophage is used to control biofilm growth in municipal and personal water systems, as well as biofilms present in refrigerated environments (Sulakvelidze 2008).
2. Omni Lytics and Intralytix Inc. are developing phage products to be used in water, food and agriculture.
3. List Shield™ (formerly LMP–102), is a natural product that contains cocktail of six bacteriophages targeted at food borne bacterium called *Listeria monocytogenes*.
4. Eco Shield™ (formerly ECP–100), is a cocktail of three different bacteriophages against food borne bacterium *Escherichia coli* O157:H7.

Phage formulation on an industrial scale was carried out at Bacteriophage Institute, Tbilishi, Georgia. Special phage mixtures were developed against bacterial strains causing nosocomial infections in various hospitals. They were very effective in sanitizing water taps and equipment.

14.3 Analysis

Water resources are really becoming limited due to the contamination problems caused by life threatening bacterial pathogens. Traditional methods that are employed for the decontamination of water resources leave behind many health risks. Besides, many developing countries including India do not employ proper treatment to get safe water. Many times, untreated waste water is directly discharged into natural

water systems. Thus, almost all water resources have contaminated either by pathogenic bacteria or by disinfection process byproducts. Therefore, there should be an efficient alternative strategy to meet with such problems. The interest in phage research has increased over past few years. Phages have been employed in almost every field as a biocontrol agent to prevent and treat bacterial infections.

Phage as disinfectant is now a current field for research. Studies on potential of phages as disinfectant in various water systems have been carried out. Phages have been applied successfully as disinfectants in pond water, swimming pools, industrial water systems, aquaculture system and in the treatment of waste water. Phages could be applied as disinfectant during disinfection step, along with other physicochemical processes (aeration, coagulation, flocculation, sedimentation and filtration). As bacteriophages are host specific, complete understanding of nuisance bacterial community dynamics is essential. Polyvalent or monovalent phage preparation could be employed depending upon nuisance bacterial community dynamics. Application of phages would be rather easy in stagnant water systems, viz., pond, lake, well, groundwater, swimming pool, aquaculture system than in running water like river. In running water systems, applied phage titers may get diluted, therefore may not disinfect the water efficiently.

There are some limitations on the use of phages as disinfectant in water systems. Successful strategies should be developed to encounter the problem of host cell resistance and penetration of phages through flocs or suspended matter. Also, Strategies against risk of pathogen emergence through transduction should be developed to overcome the problem of phage resistance in bacteria. Thus for successful phage therapy in water systems, substantial research regarding waste water microbial ecology, phage-host interactions and low cost phage preparation technology is needed. Our understanding of microbial ecology of water systems and interactions of physicochemical factors with phages would prove to be essential for getting insights into biocontrol of pathogens in various water systems.

14.4 Future Perspectives

It is well known that bacteriophages have been successfully applied as therapeutic agents to treat many notorious bacterial pathogens. There are few reports in which phages have been applied in food and agriculture field to kill infectious bacterial pathogens. Traditional chemical disinfectants that are used in water treatment process can form by-products that are carcinogenic in nature. Besides, antimicrobials resistant bacterial strains are ever emerging that are showing resistance to traditional chemical disinfectants. Therefore, now this is the time to think of an alternative antibacterial strategy. It seems possible that phages can be used in environmental problems especially in case of water borne epidemic diseases where it is difficult to control the pathogenic bacteria sources by chemicals. There are very few reports in which bacteriophages have been used as disinfectant in water bodies to kill bacterial pathogens. Bacteriophages could be applied as disinfectant to overhead tanks or

public places tanks which are built for supplying drinking water in urban and rural areas. Phages can be used as disinfectant, instead of chlorine for stored water in home during rainy season. Use of phages in water treatment requires full understanding of waste water microbiology and phage- host interactions. Although phages are effective antimicrobials, phage therapy can not be the sole option as an anti microbial agent. Combination of phages and antibiotics can be used; provided proper research should be done. Bacteriophage based Technology for disinfection of pathogenic bacteria may provide long term and cost effective control. Successful application of phages in various water systems requires high titer of phage because of the potentially large volume of water systems to be inoculated. Therefore in future, one might think of large scale preparation of high titer phage formulation. Carriers (liquids and powders) that are used in the formulations should be easily miscible in water to get better results.

14.5 Conclusion

Drinking water contamination is a serious problem in developing countries. Contaminants may arise by improper waste water treatment. Traditionally, waste water disinfection is done by chemical and physical methods. Because of improper treatment and emergence of chemical resistant and tolerant bacteria, water disinfection may not work efficiently, leaving behind pathogenic bacteria in the drinking water. Biocontrol of bacteria by their viruses, popularly known as phages, is one the most reliable method to control pathogens. Phages are specific, sometimes show broad host range; they are easy to prepare and use; and they do not infect eukaryotic cells. Phages do also possess some limitations which may result in emergence of phage resistant or highly virulent bacteria. Stability of phages in waste water treatment plant is also a crucial thing which limits phage usage. Evidences presented in this review strongly support that phage could be used as an effective disinfectant for prevention of many kinds of water borne bacterial infectious diseases, especially those caused by bacteria resistant to antimicrobials.

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Chapter 15
Microbial Mining of Value Added Products from Seafood Waste and Their Applications

Divya Prakash, N.N. Nawani, and Balasaheb P. Kapadnis

Abstract Waste management is the current focus of the decade towards a greener and cleaner environment. Waste management involves effective, safer and efficient methods that can degrade waste and reduce the release of pollutants. The seafood industry generates around 312 tons waste annually that poses a serious crisis for degradation and waste management. The marine waste contains valuable products like chitin, chitosan, their oligosaccharides, proteins, pigments, etc. which can be extracted and used for commercial applications. The chemical methods employed for their production have numerous disadvantages like low recovery, high cost and the release of hazardous effluents, thus biological conversion which is cost effective, efficient and environment friendly, is the best alternative to recover these value added products. This review describes the value added products that are biologically extracted from marine waste and their applications in the field of biotechnology.

Keywords Microorganisms • Waste • Chitin • Chitosan • Oligosaccharides • Protein hydrolysate • Biotechnological applications

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15.1 Introduction

Waste management involves several processes like collection, effective degradation of waste, recovery of valuable products and efficient disposal of the residual waste. Effective waste management is the removal of wastes without altering the environment. Improper waste management brings about further increase in wastes leading to contamination of air, soil and water.

The total waste generated by both developing countries and developed countries is alarming (CalRecovery Inc 2004). Along with the biodegradable wastes like food industry, agricultural wastes, hazardous wastes are also generated all around the world. The other factor contributing to this is the economic difference between the developed and developing countries. The important factor responsible for this is the technology advancement in the developed countries which dispose the waste in a more effective manner thereby, recovering value added products and reducing the release of pollutants. For example, in many European countries, Poly Vinyl Chloride (PVC) is mainly recycled to make other reusable products like mixed plastics, etc. and increase its usage period. The remaining waste is used to utilize the energy for useful purposes and the non-degradable part is used for landfill after proper waste treatment (www.pvc.org). Compared to physical, chemical and biological treatment of wastes, the microbial conversion is favored since it is more efficient and does not require any energy. Microbes constitute a ubiquitous group of living entities that are present almost everywhere. Microbes prove to be the most suitable candidates since; they are capable of surviving in the most hostile conditions, are less expensive and are capable of immobilizing, removing, degrading the contaminants. They have always been an integral part of the traditional wastes and sewage management but now, their scope to efficiently and effectively degrade wastes is gaining importance due to the advancement in biotechnology.

Microbes play an important role in food webs and biogeochemical cycling in different ecosystems. They can be also exploited for their ability to produce novel enzymes and metabolites with potential biotechnological applications. Microbes are also known to be involved in the global cycling of bio-elements such as nitrogen, carbon, oxygen, phosphorous, iron, sulphur and trace elements. Microbes are extremely diverse and they are constantly exposed to extremes in pressure, temperature, salinity and nutrient availability. Extreme environments contain highly diverse bacterial communities (Sogin et al. 2006). These microbes have the potential to possess unique biochemical properties. Enzymes isolated from microbes from such environments possess diverse biochemical and physiological characteristics that allow these microbes to adapt and thrive in extreme conditions. Microbial mining of waste for effective waste management and recovery of valuable products is very important to balance the delicate ecology.

15.2 Critical Review

The aim of waste management in an effective way would only reduce the wastes that are generated and recover value added products from waste. The solid waste that is generated is more than that from water. The solid wastes are even more difficult to degrade and handle. A major portion of this solid waste is contributed by the food industries. These include agricultural, food processing and seafood industries. The food industry is not profoundly polluting but it does contribute to the wastes generated and the energy utilized. The seafood industries around the world play a significant role in the social and financial wellbeing of nations. In addition, it also provides food to the growing population. It has emerged as on of the profit making industry along with providing employment to people. The global seafood market is projected at US$ 100 billion per annum (Tidwell and Allen 2001). Also, the world requirement for seafood increases by 3% each year (FAO 2000). The world largest seafood consumption is by Japan, followed by European Union (World Nutrition Forum). The top five consumed species are salmon, shrimp, tilapia, catfish and crab.

There are many types of fishes and other marine species that are used for the production of seafood. Some species like the tuna, sardines are most commonly canned. Other species include salmon, clams, oysters, shrimps, octopus, and crabs. One of the major agro-industry is the shellfish industry and it has acquired great importance due to the growing demand of shrimps and crabs. But this also requires knowledge of handling and processing of all processes involved from transportation up to the storage. The commercial processing of all these species involve including removing the scales, gills, intestines or shells, filleting, chilling, freezing or packaging seafood.

In commercial processing of the seafood, the meat recovery is only about 25% (w/w) whereas the remaining part consists of 40% (w/w) solid waste. An estimated 312 tons of seafood waste is generated annually and its disposal causes a serious threat to the environment due to its adverse effects (Rattanakit et al. 2002). The solid waste from the seafood industries which is mainly the head, shells, etc. contains about 30–35% tissue protein with Calcium carbonate and chitin. The waste water and the solid wastes generated by the seafood processing industries have enormous environmental impacts. In this sector, the seafood processing industry also contributes the major amount of waste as the wastes are generated in every step. The processing of seafood and generation of wastes by various steps is depicted in Fig. 15.1 (Stevens et al. 1998).The organic wastes from the solid wastes and waste water from the seafood processing industries released in the water bodies establish the changes in the community and biodiversity of the flora and fauna (Vezzulli et al. 2002, 2003). The pollutants that are released during the seafood processing are many. These include smoke and steam released during the seafood processing. There is also release of odor which pollutes the air and combines with the atmospheric air to form Methane, ammonia and mercaptans (National Pollutant Inventory 1999).

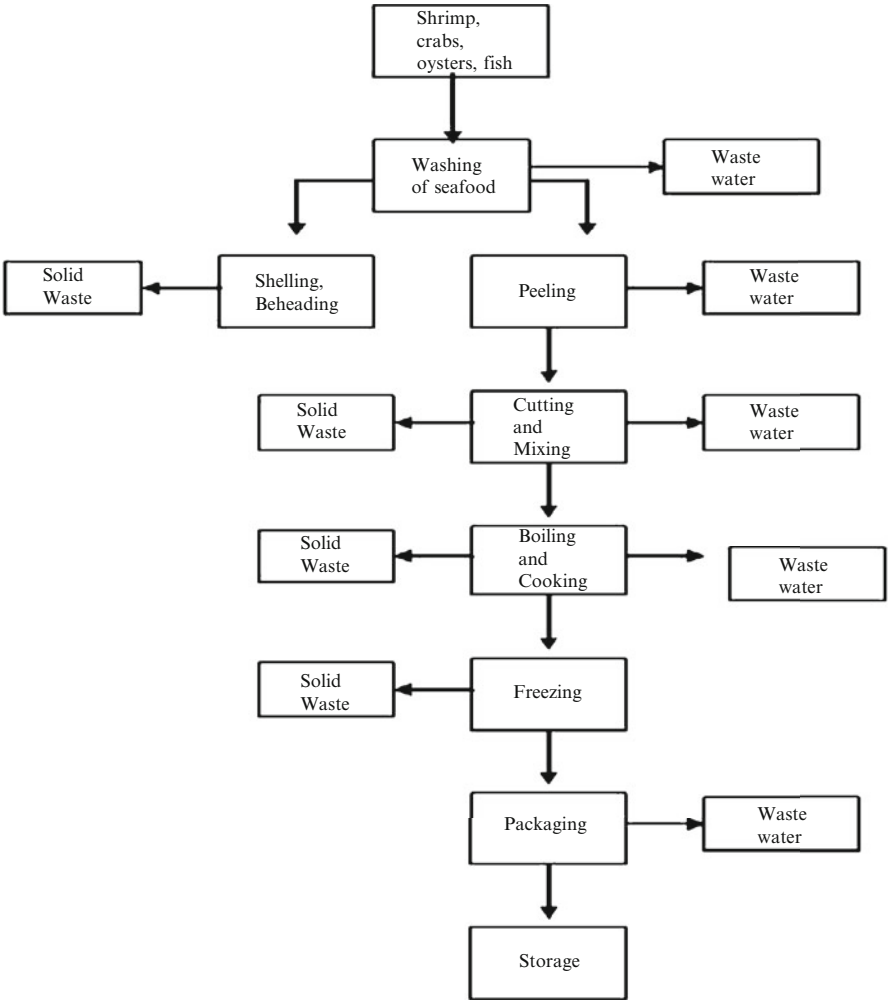

Fig. 15.1 Solid waste and waste water generated during seafood processing

The effluent water generated from these industries is rich in organic matter due to proteins, suspended solids and other matter along with other minerals. If the effluent is discharged without treatment, they can cause eutrophication and oxygen depletion due to high levels of Chemical Oxygen Demand (COD) and Biological Oxygen Demand (BOD) in the solid waste and waste water as shown in Table 15.1.

Similarly, the solid waste from the seafood industries contain many value added products like chitin, chitosan, proteins, pigments etc. as shown in Table 15.2. This can be utilized for various applications. But there is also a serious problem for the disposal of the formidable quantity of the solid waste generated (Nirmala 1991).

Table 15.1 Characteristics of seafood waste

Parameters	Crab waste	Shrimp waste	Waste water
Nitrogen	55 g/kg	77 g/kg	76 g/l
Chemical Oxygen Demand (COD) [ppm]	1,100	790	700
Biological Oxygen Demand (BOD) [ppm]	1,400	490	400
Suspended solids	700 g/kg	780 g/kg	187 g/l
Total organic carbon	190 g/kg	220 g/kg	254 g/l
Calcium	36 g/kg	105 g/kg	NA*
Sodium	360 g/kg	65 g/kg	NA*
pH	6.7	6.8	6.8

Overcash and Pal (1980), Nwanna et al. (2004)
*NA** Not Available

Table 15.2 Chemical composition of seafood waste

Composition	Crab waste (%)	Shrimp waste (%)
Chitin	24.4	15.4
Protein	24.3	9.4
Lipids	1.3	12.7
Ash	43.8	64.7
Minerals	14.9	NA*
Moisture	NA*	NA*

Synowiecki and Al-khateeb (2003), Nawani et al. (2010)
*NA** Not Available

Some part of it is used as animal feed but major part of it is discarded causing serious ecological threat. The waste is highly perishable and the organism colonizing them could be a nuisance and pose public hazard. The disposal of these wastes into the water has been banned.

The seafood industry in India is one of the major food processing industries. India is the fourth country in the world in terms of fish production and second in aquaculture (FAO 2000). The wastes generated by these industries accounts to nearly 48–56% of the total weight (Sachindra et al. 2005). Presently only a small amount is being utilized in the dry form as animal feed and for the manufacture of chitin and chitosan. But a huge quantity of this waste is unutilized and is wasted causing environmental pollution (Sachindra et al. 2006). In developed countries like Europe, laws are governed by a variety of European Commission (EC) and this regulates the waste management of seafood industries in Europe (Read et al. 2001).

The seafood wastes generated from the seafood industries can be utilized as shown in Fig. 15.2. The waste generated from the worldwide production and processing of shellfish is a serious problem of growing magnitude. This abundant waste may pose environmental hazard due to the easy deterioration of the proteins in them.

Some of common ways of disposal currently carried out is listed below. But most of them have disadvantages and are not economically profitable.

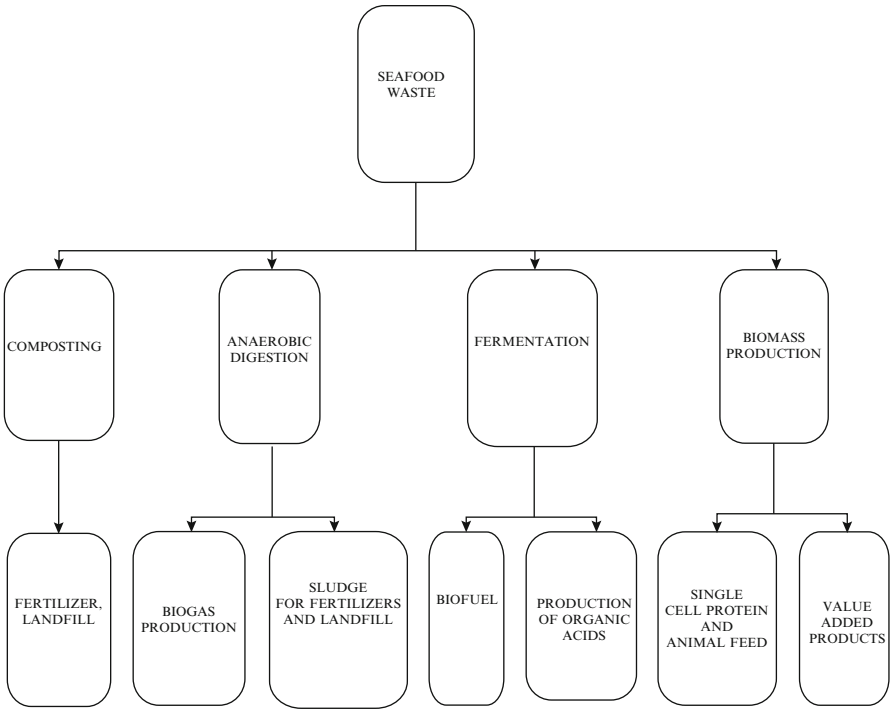

Fig. 15.2 Disposal of seafood processing sobid waste

15.2.1 Land Application of Shellfish Waste

Landfill is the most common method of disposal of shellfish waste since it is rich in organic matter. It has been shown that it is beneficial to the farmers (ADAS UK Ltd 2006). But it attracts secondary pests like flies and rodents. In addition, the availability of land is also decreasing due to increasing population.

15.2.1.1 Incineration

Waste is combusted in special combustion plant with additional fuel to ensure complete combustion of materials but there is requirement of energy and no energy harnessing.

15.2.1.2 Fertilizers

There are a number of fertilizers at present in existence that are formed from seafood waste. A number of methods are employed to produce fertilizers including

composting, rendering, drying at high temperatures and digestion. The seafood is broken down into its liquid and solid phases, which produces a nutrient rich fertilizer (Knuckey et al. 2004).

All the above processes however do not recover the value added products from seafood waste. Due to its high organic content, seafood waste is often classified as a certified waste and this makes it even more costly to dispose. This practice is under increased scrutiny due to environmental issues (Jespersen et al. 2000; Anon 2002). Due to all the above reasons, there is an increasing concern and cost burden for the whole seafood industry. Continued production of the shrimp head waste without corresponding development of technology utilizing the wastes has resulted in waste collection, disposal and pollution problems (Nwanna et al. 2004). Seafood waste is mostly alkaline in nature having a pH of 7.5–8.0. This is mainly responsible for the growth of putrefying microbes leading to odor and spoilage. The conversion of this waste into an ensilage provides an eco-friendly and alternate means of disposal. In order to do so, chemical and biological methods are employed. But the chemical conversion of the VAP from seafood waste has several disadvantages:

- The extraction process is relatively expensive due to the chemicals and solvents used.
- The chemical process itself produces significant waste which again requires appropriate disposal.
- There are health and safety concerns about the products recovered from it.
- It is difficult to control the quality of the products recovered.
- The yield of the VAP is less.
- Excess period of time results in degradation of chitin and chitosan.

Due to the above cited reasons, the biological conversion of the seafood waste is favored. Biotechnology provides this opportunity for the recovery of VAP by the usage of microbes and their enzymes. The various valuable products (VAP) that can be recovered from seafood waste especially shrimp and crab waste are chitin, chitosan, their oligosaccharides, proteins, pigments, etc. as shown in Fig. 15.3.

Each of these by-products has extensive applications in various fields of Biotechnology. So their recovery from seafood waste is the only way to dispose the enormous amount of waste produced around the world every year. The various VAP that can be recovered from seafood waste and their applications are listed below.

15.2.2 Chitin

Chitin is the second most abundant biopolymer on earth after cellulose. The production of chitin by aquatic life is estimated to be 10^6–10^8 tons (Cauchi 2002). It has a very high molecular weight and is composed of *N*-acetylglucosamine units linked to proteins by strong covalent glycosidic bonds (Chang and Tsai 1997). Chitin is insoluble in water and many other solvents. Therefore, in its pure form, it has limited applications. Chitin in the shells of seafood animals is tightly bound to the proteins,

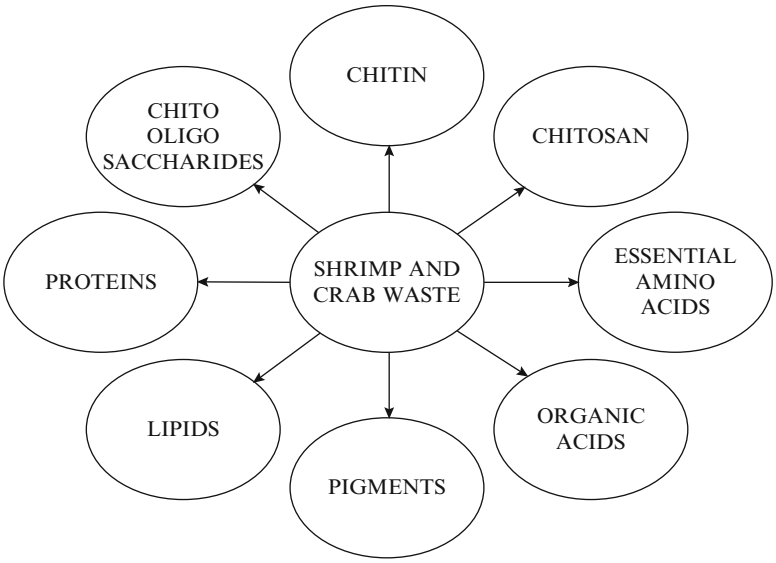

Fig. 15.3 Value added products that can be recovered from seafood waste

minerals, pigments and lipids. In order to recover the various by-products from the waste, the chitin has to be removed efficiently. The chemical conversion of seafood waste involves the usage of acids and alkali to deproteinase and demineralize the waste in order to recover the valuable by-products that are bound to the hard shells and other parts of the waste. Chemically, the waste is treated with 4% sodium hydroxide (NaOH) to eliminate the proteins and this is carried out at elevated temperatures (70–120°C). After fractionation, the protein slurry is dehydrated to be supplemented in feed. The solid fraction contains the chitin, pigments and calcium carbonate. In the next step, the solid fraction is treated with 4% hydrochloric acid (HCl) which converts the insoluble calcium Carbonate into soluble calcium chloride. Demineralization is generally performed by acids including HCl, HNO_3, H_2SO_3, CH_3COOH, and HCOOH but HCl is the most preferred reagent. It has also been found that deproteinization followed by demineralization is found to be suitable for optimum chitin recovery (Aytekin and Elibol 2009). In addition to the use of acids like Hydrochloric acid and alkali like Sodium Hydroxide, Coward-Kelly et al. (2006) have demonstrated the usage of Calcium hydroxide to recover chitin, amino acids and carotenoids which had numerous advantages like reduced cost for resurgence and Calcium levels in the ultimate product could be helpful as animal feed.

Due to the usage of excess acid and alkali for the recovery of chitin from seafood waste is of low quality and quantity. To overcome this, the microbial resurgence is preferred. Ensilation of seafood waste is brought about by the action of microbes which bring down the pH of the waste from alkaline to acidic. This results in a liquor fraction which is rich in proteins, minerals and carotenoids. The solid fraction

is rich in chitin. The reduction in pH is ideal for the removal of proteins and minerals to yield the VAP along with higher yield. This process is achieved by the following processes:

- Lactic Acid Fermentation
  Lactic acid bacteria is only of the most important and useful bacteria. Lactic acid fermentation combined with chemical treatments has been studied as an alternative to chemical extraction for chitin recovery reducing the amount of alkali and acid required. In this process, protein and calcium removal is achieved by enzymatic action on the tissues and by solubilization of calcium by organic acids, respectively (Cira et al. 2002). Lactic acid is produced by the breakdown of glucose resulting in the lowering of pH. This also prevents the production of amines which is responsible for odors, detaches the protein from the solid component and prevents the growth of spoilage bacteria. This can also be carried out by food grade *Lactobacilli* (GRAS). The protein recovered is suitable for human consumption and can be used as a flavor enhancer. But this is possible not before 16 h of fermentation. Microorganisms studied for bioconversion of seafood waste by the production of lactic acid and proteases include *Lactobacillus plantarum, Pseudomonas aeruginosa, P. maltophilia, Bacillus subtilis, Lactobacillus paracasei, Lecanicillium fungicola and Penicillium chrysogenum* (Oh et al. 2007). Rao et al. (2000) showed that the critical factors that affected lactic acid fermentation leading to the deproteination and demineralization of seafood waste included 6.7% *L. plantarum* inoculum, 5% glucose and initial pH of 6.0 adjusted with acetic acid. This resulted in 75% deproteination and 86% demineralization. However, when acetic acid was replaced with citric acid, 88% deproteination and 90% demineralization was achieved. But in case of the usage of acetic acid, the quality of chitin was of high standard. Co-fermentation was also carried out with *Lactobacillus paracasei subsp. tolerans* KCTC-3074, a lactic-acid-producing bacterium, and *Serratia marcescens* FS-3, a protease-producing bacterium by Jung et al. (2006) useful for chitin extraction from crab shells. The level of demineralization and deproteination was 94.3% and 68.9%, respectively, at the end of fermentation. This opens new avenues for the usage of two different microbes for co-fermentation for the recovery of VAP.
- Protease Production
  Another important method for the bioconversion of seafood waste is the application of proteolytic organism or proteases. Since chitin is relatively inert to chitinases, the deproteinized waste can be used for the recovery of other VAP. The cheap protein hydrolysate can be utilized as a source of carbon for the production of chitinases and for the recovery of chitooligosaccharides. *Pseudomonas aeruginosa* K187 produced chitinases and proteases when grown on squid pen wastes and produced chitooligosaccharides and biofertilizers after 5 days of fermentation as reported by Wang et al. (2010). An anaerobic spore forming bacterium was isolated from sewage sludge and ground meat of *Penaeus monodon* and *Crangon crangon* shells. This was used for deproteination and decalcification along with *Lactobacillus casei* MRS1. The efficiency of this process was

more than 90% and the time requirement for this was 2 days when compared to the deproteination time required by *S. marcescens* FS-3 (Jung et al. 2006). This two-stage fermentation process is not commonly practiced but the quality of chitin obtained is much higher. The demineralization efficiency was 99% using glucose as a carbon source (Xu et al. 2008).

- Demineralization
  A homofermentative bacterium *Pediococcus acidolactici* CFR2182 was found to exhibit a very high rate of efficiency of demineralization (72.5%). This organism also produced only lactic acid which may be responsible for this effect when grown on shrimp biowaste according to Bhaskar et al. (2007). Using Response Surface Methodology (RSM), conditions were optimized for demineralization. Choorit et al. (2008) concluded that 5% addition of pretreated shrimp shells with chlorine solution into the culture broth of *Pediococcus* sp. L1/2 resulted in 83% demineralization in 36 h. Oh et al. (2007) reported an organic acid and protease producing strain *P. aeruginosa* F722 that was useful for bio-extraction of chitin from crab shells. A high value of demineralization (91%) was achieved with 10% glucose and 10% inoculum concentration of *P. aeruginosa* F722 after 7 days of fermentation.

Chitin and chitosan are produced by many companies throughout the world on a large scale. Chitin is mostly non-toxic and easily biodegradable. Due to this, it finds many important applications in the field of medicine and veterinary. Some of the applications are listed below:

- Antiviral
- Anti-infectious
- Bacteriostatic
- Fungistatic
- Antitumor
- Anticholesterolemic agent

In addition, it can be used as a packaging material, stimulant of the immunological system, blood anticoagulant and for wound healing (Synowiecki and Al-khateeb 2003). Due to its biodegradability property, it can be used for the controlled release of drugs, in the preparation of medicines, nutrients, cosmetics, agrochemicals and toiletries. Chitin also finds application in the removal of heavy metals and other contaminants from water bodies and also in the clarification of fruit juices. It is also employed in the separation and recovery of proteins. Since it is a source of glucosamine and *N*-acetylglucosamine, it is used in the rebuilding of extracellular matrix.

Due to the reactivity of the amino groups for substitution, it can be used as for enzyme immobilization and as a matrix for column chromatography. The incorporation of chitin into the coating of water resistant textiles results in the large increase in its water permeability (Synowiecki and Al-khateeb 2003).

Since chitin is largely insoluble in water, substitution of the side chain groups yield products that are more soluble depending upon the length of the chain which is substituted and hence, have wider applications as described by Vincendon (1997). The reaction of the hydroxyl groups of chitin with alkyl-, acyl-halides or isocynates

yield ethers, esters or carbamate derivatives. Chitosan, the *N*-acylated form of chitin is more readily digested than natural chitin.

15.2.3 Chitosan

Various modifications in the chitin structure can render it soluble and improve its properties. Chitosan is the deacetylated form of chitin. The deacetylation is at C-2 atom of the basic unit (Hirano 1999). The degree of deacetylation varies between 60% and 100%. Chitosan exhibits polyelectrolyte properties in acidic conditions. It is also biocompatible and biodegradable (Shahidi and Synowiecki 1991). In addition, it is non-toxic and antithrombogenic. Chitosan unlike chitin is readily soluble in acidic solvents and forms a viscous solution. But its viscosity depends upon its molecular weight and degree of acetylation along with temperature (Synowiecki and Al-khateeb 2003). The viscosity increases with temperature making it a more suitable candidate for biological applications compared to chitin. The chemical deacetylation of chitin into chitosan is carried out by strong chemicals like 50% sodium hydroxide and at high temperatures as high as 70–90°C. Depending upon the degree of deacetylation, several treatments are carried out. Chitosan with a degree of deacetylation of 70–90% is a pre-requisite for superior quality chitosan. The chitin and chitosan should contain low quantities of ash and protein (Stevens et al. 1998).

Chitin can be deacetylated into chitosan using the fungal enzyme, chitin deacetylase (CDA). This enzyme has been isolated from the fungus *Absidia coerulea* and characterized. Although with surplus solid chitin as a substrate the enzyme activity is high, only a small percentage of the substrate is transformed. This problem has not yet been solved. Using crude proteases from *Bacillus cereus* SV1, chitin and chitosan were recovered along with protein hydrolysates as reported by Manni et al. (2010). Until now, researchers have found a wide range of microorganisms that can produce chitinase or chitosanase, including *Aspergillus, Penicillium, Rhizopus, Myxobacter, Sporocytophaga, Bacillus, Enterobacter, Klebsiella, Pseudomonas, Serratia, Chromobacterium, Clostridium, Flavobacterium, Arthrobacter* and *Streptomyces* (Zhang and Kim 2010). This indicates the abundant applications of microbes for the recovery of VAP from seafood waste.

Chitosan has found several applications in gene delivery, tissue building engineering and cell culture (Sashiwa and Aiba 2004). Chitosan and its derivatives are used in various cosmetics, toothpaste, body creams and hair-care products. Their usage is also extended in antiseptic creams owing to their anti-microbial activities. The antimicrobial activity depends on the average molecular weight of chitosan and its derivatives. Biofilms of chitosan are used in packaging industries (Synowiecki and Al-khateeb 2003). Some of its applications (Synowiecki and Al-khateeb 2003) in the human body are listed below:

- Antimicrobial activity
- Osteoinduction
- Immunostimulation

- Anticholesterolemic activity
- Antiulcer activity
- Growth stimulation
- Wound healing.

Chitosan is a cationic polysaccharide in nature, nontoxic and biodegradable in the human body. This makes its application extensive for biomedical applications. However, chitosan does not dissolve in neutral and basic aqueous media and therefore, its biomedical usage is limited. Chemical alteration of chitosan provides derivatives. The various substitutions in the amino group of chitosan extend its applications in the field of biotechnology. These are soluble at neutral and basic pH. Moreover, chemical modification can be employed to attach a range of functional groups and to control hydrophobic, cationic, and anionic properties. This would greatly help in building biomolecules which can be used in various fields of biotechnology. Artificial glycopolymers prepared from chitosan may be used to block influenza viruses or blocking agents for acute rejection during organ transplantation (Sashiwa and Aiba 2004). Similarly dendrimers having chitosan backbone could be potential inhibitors of viral pathogens. Cyclodextrins bound to chitosan have enormous applications in the field of drug delivery, cosmetics and analytical chemistry (Sashiwa and Aiba 2004). Sulfobenzoyl chitosan was used as a natural preservative of oysters which increased its storage life at temperatures of 5 °C and inhibited *Pseudomonas, Salmonella, Aeromonas,* and *Vibrio* strains. Treatment with chitosan solution protected potatoes against contamination by pathogens. Carboxymethylated chitosan was employed as a substrate for synthesis of heparin-like blood anticoagulant (Synowiecki and Al-khateeb 2003).

The other chemically modified forms of chitosan have found several applications as adsorbent matrix (Sreenivasan 1998), removal of heavy metals (Li et al. 2003), hydrogels for drug delivery (Martin et al. 2003) and anti-tumor agents (Sashiwa and Aiba 2004).

15.2.4 Oligosaccharides of Chitin and Chitosan

The oligosaccharides of chitin and chitosan are low molecular weight that is released from chitin and chitosan due to action of acids, alkalis or enzymes. The chitooligosaccharides from chitin and chitosan are oligomers of varying length Squid pens were converted to chitooligosaccharides by the production of proteases and chitinases as reported by Wang et al. (2009). A combination of enzymes from *Trichoderma atroviride* and *Serratia marcescens* was used for the production of GlcNAc, Glucosamine and chitobiose from langostino shell chitin which indicated synergistic action amongst enzymes from prokaryotic and fungal enzymes as reported by Donzelli et al. (2003). Nawani et al. (2010) used a cocktail of protease and chitinase for the production of chitobiose. Using different organisms or enzyme complexes, we can recover suitable chitooligosaccharides of varying length.

Their solubility and mode of action depends mostly on their molecular weight, degree of acetylation, degree of polymerization and nature of chemical modification. Oligomers of *N*-acetylglucosamine (NA-COS) can be used *in vitro* and *in vivo*. NA-COS of different molecular weights were extracted from crab chitin and their action against oxidative stress was determined by Ngo et al. (2009). It was concluded that NA-COS with molecular weight of 1–3 kDa was more effective than those of less than 1 kDa in weight. They showed inhibition against DNA and protein oxidation. This studies pave the way for the usage of these NA-COS as a potential antioxidant in the removal of free radicals causing oxidative stress. In addition, they also exhibit the following properties useful in the field of biotechnology:

- Enzyme inhibitory effect (Ngo et al. 2008), (Je and Kim 2005)
- Immunostimulation (Seferian and Martinez 2000), (Shon 2001).
- Anticoagulant (Vongchan et al. 2003)
- Antimicrobial (Chang et al. 2007; Jeon et al. 2001)
- Anticholesteremic, anticancer (Kim et al. 2006)
- Wound-healing (Freier et al. 2005).
- Antitumor (Wang et al. 2008)
- Antioxidant activity (Wang et al. 2009)

Low molecular weight chitooligosaccharides prepared enzymatically with chitosanases had proliferating effect on pancreatic cells thereby, having antidiabetic activity (Liu et al. 2007).They are also found to play a significant role in cell signaling. They were found to stimulate mitogenic responses to platelet-derived growth factor and induce protein kinase phosphorylation in vascular smooth muscle cells (Inui et al. 1995). The absorbability of several COS from rat GI tract and pharmacokinetics of chitobiose and chitotriose administered intravenously or orally to rats has been studied (Chen et al. 2005).

15.2.5 Pigments

Shell fish waste is one of the important sources of carotenoid pigments (Shahidi et al. 1998). These lipid soluble pigments produce yellow-red color in plants and animal products. This class of pigments include carotene, Asthaxanthin, its esters, zeaxanthin, lutein, etc. (Khanafari et al. 2007). Amongst them, Asthaxanthin is the most important. It is a ketocarotenoid (3, 3'-dihydroxy-β, β-carotene-4, 4'-dione). It has a similar structure to β-carotene but is a more potent antioxidant than β-carotene. It is highly polar but non-toxic. It is the oxidized form of β-carotene responsible for the pink/reddish pigmentation in crustaceans and salmons (Stepnowski et al. 2004).

The carotenoid content in the shrimp waste from India was found to be between 335–153 μg per gram of the waste depending upon the species (Sachindra et al. 2005). So the recovery from seafood waste could be exploited on a commercial value and it would be a cheaper substitute for synthetic carotenoid pigments. Seafood waste forms the cheapest medium for the extraction of these valuable

pigments. The carotenoids have many industrial applications. Methods have been developed for its extraction from crustacean wastes using vegetable oils (Shahidi and Synowiecki 1991; Sachindra and Mahendrakar 2005). Li et al. (2005) extracted carotenoids from fish eggs using acetone. Charest et al. (2001) used alcohol as co-solvent in supercritical CO_2 extraction of astaxanthin from crawfish shells. Using a solvent mixture, carotenoids have been extracted from shrimp waste (Sachindra et al. 2001, 2006). But the levels of these solvents need to fulfill the criteria of the FDA (Food and Drug Regulation 2005). In view of this, Astaxanthin was recovered by using hot pressurized Ethanol which is an eco-friendly way reducing the release of harmful effluents (Quan and Turner 2009). Removal of astaxanthin also yields high quality of chitin and chitosan.

There are reports indicating that the acid fermentation could facilitate availability or extraction of carotenoproteins, carotenoid pigments and valuable fatty acids (Cremades et al. 2003). The oil mining yield of carotenoids can be improved by hydrolysis of waste with protease prior to oil extraction and bacterial protease alcalase was found to be better than other proteases. *Lactobacillus plantarum* and *Lactobacillus acidophillus* produced astaxanthin more competently than the chemical methods indicating that biological resurgence of extraction is environmentally and economically feasible (Khanafari et al. 2007).

Astaxanthin is a xanthophyll which has inherent properties like antioxidant and anti-carcinogenic, as well as Vitamin A precursor (Stepnowski et al. 2004). Due to its outstanding antioxidant activity, astaxanthin has been attributed with extraordinary potential for protecting organisms against a wide range of ailments such as cardiovascular problems, different types of cancer and some diseases of the immunological system (Quan and Turner 2009).This pigment finds wide applications in aquaculture, food colorant and cosmetics (Gimeno et al. 2007; Bhaskar et al. 2007). Recently, Sachindra and Mahendrakar (2010) reported that the carotenoids extracted from shrimp waste could be successfully used as coloring agent in fish sausage. In addition, it also enhanced the flavor of the product.

15.2.6 Lipids

Seafood waste has considerable amounts of lipids which can be used for the extraction of retinol, Cholesterol and α-tocopherol. The removal of lipids was up to 76.9% by using lactic acid fermentation compared to the removal of lipids by chemical treatment (46.1%) as reported by L´opez-Cervantes et al. (2006). Similarly the removal of calcium was higher by using bioconversion (Beaney et al. 2005). Retinol, α-tocopherol and cholesterol are amongst the nutritionally important lipids in foods Retinol is also known as Vitamin A. It is an important micronutrient required for vision, growth, reproduction, and maintenance of the immune system (Ball 2000; De Vries and Silvera 2002). α-Tocopherol (Vitamin E) is an important antioxidant and is required for muscular and reproductive functions (Lopez-Cervantes et al. 2006). Cholesterol is another important lipid which is a precursor of bile acids, steroid hormones, and Vitamin D (Lopez-Cervantes et al. 2006).

15.2.7 Amino Acids

Protein hydrolysates, extracted from seafood waste, have importance in the food industry due to the high protein content. Shrimp by-products have been identified as an animal protein source of great nutritional value (Armenta and Guerrero-Legarreta 2009). Essential amino acids can be extracted from these protein hydrolysates and used as a nutritional supplement. The essential amino acids are Methionine, Lysine, Leucine, Tryptophan, Phenylalanine, etc. to name a few of them. Protein hydrolysates have important applications in the field of pharmaceuticals, human and animal nutrition, or cosmetics. Hydrolysis of the proteins decreases the peptide size, making the most of the amino acids available (Gildberg and Stenberg 2001). The liquid hydrolysate recovered by the lactic acid fermentation after the removal of the solid substrate contains a high content of essential amino acids and ash, suggesting that it has a high nutritional content, as well as a low metal and microorganism content. The products obtained from processing shrimp by-products can be incorporated as high value supplements in human and animal diets (Bueno-Solano et al. 2009). The chitin acid hydrolysate may be used as a carbon and energy source for the growth of *Saccharomyces cerevisiae* K1V-1116, proving that shrimp-shell waste may be used for the production of single cell protein (Ferrer et al. 1996).

Protein hydrolysates from shrimps exhibited antioxidant activity (He et al. 2006). From studies carried out by Katayama and Mine (2007), it was shown that amino acids exhibited protection of hydrogen peroxide induced tissue oxidative stress in human epithelial cells. Fermented shrimp waste contains a large amount of essential amino acids (Lopez-Cervantes et al. 2006). Protein hydrolysates produced by the enzymatic processing inhibited the Angiotensin-I-converting enzyme as studied by Cheung and Li-Chan (2010). They also concluded that every 1,000 g of wet shrimp waste, approximately 30 g of peptide/amino acid materials were recovered in the soluble hydrolysates, representing 12% of the solid matter in the shrimp waste.

15.2.8 Organic Acids

The other valuable by-products recovered from seafood waste are organic acids like Lactic, formic and citric acids.

In addition, there have also been reports on antifungal chitinases (Chang et al. 2007), Protein hydrolysates having anti-oxidant properties (Wang et al. 2009) and anti-microbial compounds produced from seafood waste (Wang et al. 2005). This opens new avenues for the efficient and effective treatment of this seafood waste for the recovery of these valuable products. Hence, the seafood waste can be used as a cheap substrate for the production of these important enzymes which have biotechnology applications.

It has been shown that the use of shrimp shell for arsenic removal appears to be technically feasible. Shrimp shell, as a natural material, is ideally available with low cost and is environmentally friendly. Moreover, being composed entirely of aquacultural and fishing industry waste, it helps in reduction of waste generation (Lin et al. 2009).

15.3 Analysis and Future Perspectives

The seafood processing waste is an unutilized resource which can be harnessed to recover valuable products which have very important biomedical and biotechnological applications. The seafood waste can be used as a cheap medium for the growth of many proteolytic and chitinolytic microbes and at the same time, for the simultaneous recovery of Value Added Products (VAP). There are scanty reports on the utilization of microbes for the production of various VAP from seafood waste which requires a great amount of research in the area of optimization of parameters and production of these VAP. Since biological resurgence is environmentally safer and greener, it should be exploited on a large scale. The VAP can be used in developing countries for nutritional supplementation and other applications. The usage of these VAP as an antioxidant and their role in the inhibition of oxidative stress and tumor requires further research as it would greatly help in the well being of humans. This would be more economically feasible besides providing employment to people. The by-products can be used for other applications which would reduce the economic burden. This would require the scientific community to develop methodologies on a large scale to streamline the overall process using different potent microbes. Since the biological conversion of the seafood waste is more favorable, its implementation should be encouraged. Microbes are the ideal candidates to bring about the effective conversion, since they produce a variety of enzymes which favor the bioconversion of seafood waste and the cost of production is much economically feasible compared to the other methods currently employed. The bioconversion of seafood waste with the help of microbes or their enzymes is still in an infant stage. With the help of molecular biology techniques, the microbes or the recombinant enzymes can be employed for the large scale production of enzymes, VAP, etc. The waste generated is very less compared to the chemical conversion. The microbes also derive their food and the waste is also converted in an efficient and effective manner. This serves as an ideal path for effective waste management of seafood waste. This would reduce the disposal of seafood waste unutilized and increase the production of valuable products in an environmental friendly way. The VAP have enormous applications in the field of biotechnology.

15.4 Conclusions

The biological conversion of the enormous seafood waste is the most adequate way to dispose and recover the value added products from it. But this requires the seafood processing industries and the scientific community to work together as disposal of seafood waste by the conventional methods will lead to further environmental pollution. Microbes and their enzymes provide the perfect solution to this ever increasing problem of waste disposal. This natural resource of microbes must be utilized on a large scale to effectively degrade the waste and recover the valuable products that the seafood waste has to offer to mankind.

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Chapter 16
Management of Heavy Metal Pollution by Using Yeast Biomass

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Abstract In recent years, the management of heavy metal pollution has become a major issue. Toxic heavy metals pose a serious threat to the environment and living forms. A number of conventional methods have been developed for the recovery of toxic heavy metals. On account of some disadvantages associated with these methods, biosorption onto microbial biomass has become an attractive alternative. Among microorganisms, yeasts have received considerable attention on account of yeast biomass being easily obtainable from inexpensive media. This is also abundantly generated as a by-product from the fermentation industry. Based on the published literature, the principles, methodologies and techniques involved in the management of heavy metals by yeast systems are summarized in this chapter. Adsorption capacities of different yeasts for a variety of heavy metals have been compared. Dependence of yeast biomass metal-binding capacities on parameters such as pH, temperature, contact time, competitive metal ions, agitation, initial metal ions and biomass concentrations have been explained. Isotherms, equilibrium models and kinetics have also been extensively discussed. Mechanisms involved in the biosorption by yeasts and the future prospects of this biotechnologically relevant topic have been highlighted.

Keywords Biosorption • Yeast biomass • Heavy metal ions • Adsorption isotherms • Kinetics • Mechanisms • Industrial effluent

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16.1 Introduction

Heavy metals are continuously discharged into wastewaters by several industrial processes. Lead, cadmium and mercury are considered to be the "big three" heavy metals. They are highly toxic and have a great impact on the environment (Volesky and Holan 1995). Other metal ions such as copper, chromium, zinc, nickel and cobalt are metal ions essential for metabolism. However, these metal ions are toxic to organisms beyond specific threshold concentrations (Gadd 1993). Heavy metal ions are released into the environment by several industrial processes involved in energy and fuel production, fertilizer and pesticide industry, metallurgy, iron and steel technologies. Electroplating, electro-osmosis, electrolysis, metal surface treating, leather making, photography, aerospace and atomic energy installations are other reasons contributing towards metal pollution (Volesky 1990; Bishop 2002; Wang 2002). The continuous production of metal-containing wastewaters poses a serious threat to the environment and public health. This is mainly due to their entry and bioaccumulation in food chains (Holan and Volesky 1994). The management of such hazardous metals is thus of prime importance.

The conventional methods used for the removal of heavy metals are chemical precipitation, lime coagulation, ion exchange, reverse osmosis and solvent extraction (Rich and Cherry 1987). These methods have certain limitations and are often not economically viable in the developing countries. Biosorption is a promising alternative to the traditional methods due to its effective performance, eco-friendly nature and low cost (Gadd 1990; Volesky and Holan 1995). Biosorption can be defined as "a non-directed physicochemical interaction between metal species and microbial cells" (Shumate and Stranberg 1985). The biosorption process involves a solid phase (biomass) and a liquid phase (water) containing a dissolved species to be sorbed (a metal ion). Due to higher affinity of the sorbent (biomass) for the sorbate (metal ions) species, these ions are attracted towards and bind to the former by a variety of mechanisms. This process continues until equilibrium is attained between the solid-bound sorbate species and the portion remaining in the solution (Alluri et al. 2007). Heavy metal removal by biosorption has been extensively studied during last several decades (Volesky 1990). Recently, some reviews on heavy metal biosorption have been published to understand the mechanisms involved in the process and to apply this technique in the management of heavy metal pollution (Volesky and May-Philips 1995; Wang and Chen 2006; Alluri et al. 2007; Gadd 2009).

Biosorption is an emerging technology that employs a variety of biomass as biosorbents for the treatment of the wastewater contaminated with different heavy metals. Microorganisms such as algae, fungi, yeast, moulds and bacteria have been used for the removal of these heavy metals (Volesky and Holan 1995). Living as well as dead yeast cells have been used as biosorbents in the removal of toxic metal species (Volesky et al. 1993; Gadd 1990). Yeast biomass is inexpensive and a by-product of large-scale fermentations. These cells also have the potential to accumulate

a broad range of heavy metal ions. They are thus considered to be economically attractive biosorbents for the treatment of a wide variety of metal-containing industrial effluents. Several yeasts such as *Saccharomyces cerevisiae, Candida utilis, Yarrowia lipolytica, Rhodotorula glutinis* and *Loddermyces elongisporus* have been applied for the removal of toxic heavy metal ions (Norris and Kelley 1979; Rehman et al. 2008; Bankar et al. 2009a; Cho and Kim 2003). Moreover, the efficiency of biosorbents can also be increased by pre-treatments, granulation and immobilization. The bead-like matrices thus produced entrap heavy metals. These can be subsequently stripped of metal ions and recycled for further use (Wang and Chen 2006). Biosorption using yeasts has thus emerged as an efficient, simple, eco-friendly and cost-effective promising technology for the management of heavy metal pollution.

16.2 Advantages of Yeast as Biosorbents

Although all biological materials have an affinity for heavy metal ions, the selection of the biosorbent is an important step for a process to be successful. A common rationale for the feasibility of the biosorption process is to identify efficient biosorbents that are inexpensive. Among the different types of microorganisms that have been used as biosorbents, the use of yeast cells has the advantages listed below:

1. Large quantities of yeast biomass can be obtained easily by cultivating them on inexpensive substrates (Kapoor and Viraraghavan 1995). A classical example is the well known yeast *S. cerevisiae*, a by-product of the alcohol fermentation industry. Another example is that of the non-conventional yeast *Y. lipolytica* that extensively degrades hydrophobic substrates as well as wastes derived from enzymatic, pharmaceutical, food and dairy industries. Therefore, in comparison with other microbial biomass, yeast biomass is easily available and of low cost (Wang and Chen 2006; Bankar et al. 2009b).
2. Non-pathogenic and non-toxic yeasts are used in the food, dairy and beverage industries. The biomass therefore obtained is generally regarded as safe (Bankar et al. 2009b). Such waste biomass is acceptable by the public at large for the removal of heavy metal ions from industrial wastewaters.
3. Yeasts are ideal model systems to study metal-microbe interactions at the molecular level. They display resistance towards heavy metal stress, display specific metal sorption capacities and accumulate a broad range of metals (Bankar et al. 2009b; Wang and Chen 2006). On account of the aforementioned features, yeasts have been used as model systems for investigations related to biosorption mechanisms at the molecular level (Eide 1998). Moreover, yeasts such as *S. cerevisiae* and *Y. lipolytica* are easily manipulated at the genetic and morphological level. They can be modified suitably to enhance their biosorbent capacities and thus aid in the removal of heavy metal ions from industrial wastewaters (Bankar et al. 2009b; Wang and Chen 2006).

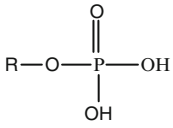
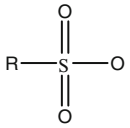
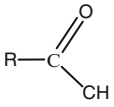
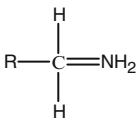
16.3 Surface Characterisation of Biosorbents

Yeast biomass has been used extensively for the biosorption of toxic heavy metal ions from bulk aqueous solutions. Metal uptake by biosorption is facilitated through the interactions of metal ions with the functional groups of cell wall (Goksungur et al. 2005). This is a metabolism-independent process and occurs rapidly within few minutes by one or a combination of the following mechanisms: complexation, coordination ion exchange, physical adsorption or inorganic micro-precipitation. The mechanisms involved in metal biosorption by yeasts are complicated and not fully understood (Wang and Chen 2006). However, identification of functional groups present on the biosorbent is helpful in speculating the possible mechanisms involved in metal biosorption process (Ting and Teo 1994).

The surface chemistry of yeast biomass has been studied by using methods such as the potentiometric titration and chemical treatments. Potentiometric titration is a qualitative or semi-quantitative method used to determine the nature of acidic sites available on the yeast cell wall. The potentiometric titration of *S. cerevisiae* with regard to H^+ and OH^- ions has been studied well (Parvathi et al. 2007; Cui et al. 2010). Parvathi et al. (2007) have indicated the importance of two acidic and one alkaline functional group in the process of metal biosorption. The two acidic groups were postulated to correspond to the carboxylic and phosphate groups (Loukidou et al. 2004) and the alkaline one to saturated amines (Romero-González et al. 2001). Cui et al. (2010) have used the proton-binding model to analyse the potentiometric titration data. The three-site model (two types of negative groups and one type of positive group) fitted well to the experimental data with a high correlation coefficient ($R^2 = 0.99$). Form this data, it was postulated that the functional groups on brewer's yeast surfaces may be sulfonate, carboxyl and amine groups. The functional groups determined by potentiometric titration are listed in Table 16.1.

Figure 16.1 shows the FTIR spectra of *Y. lipolytica* an efficient biosorbent. A number of absorption peaks were observed, suggesting a complex nature of the yeast surface. The FTIR spectrum obtained after the process of metal biosorption was altered. Analysis of these spectra revealed the significance of functional groups

Table 16.1 Functional groups present on yeast cell surface estimated by potentiometric titration (Padmavathi et al. 2007; Cui et al. 2010)

Functional groups Formula	Phosphate	Sulfonate	Carboxylic	Amine
				
Charge	Negative	Negative	Negative	Positive
pK_H values	6.63 (0.02)	2.42 (0.20)	4.78 (0.39) 5.6 (0.04)	10.62 (0.91) 9.51 (0.03)

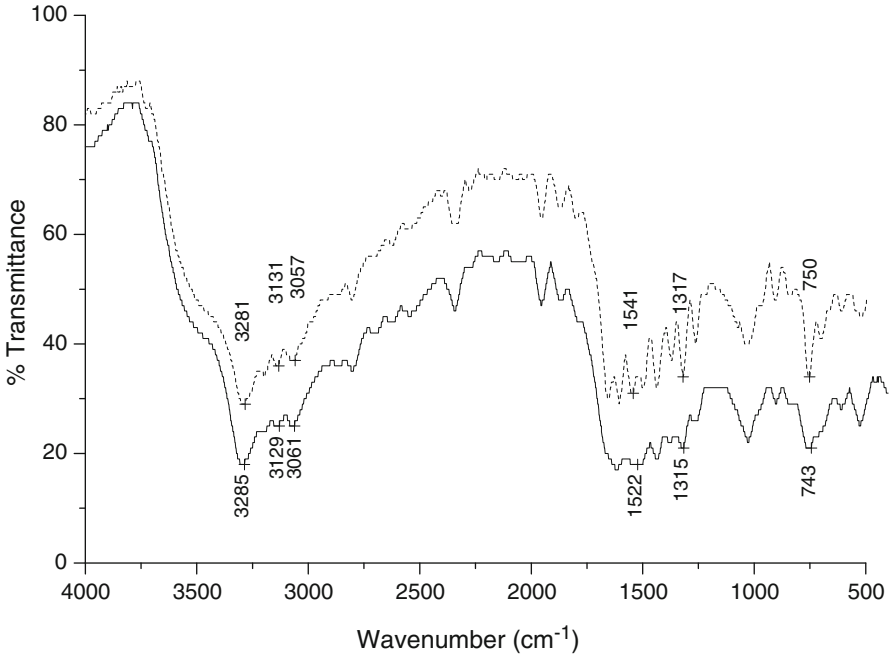


Fig. 16.1 FTIR spectra of *Yarrowia lipolytica* before (*solid line*) and after (*dotted line*) metal biosorption

such as carboxyl, carbonyl, hydroxyl and amino on yeast cell surface in the biosorption process. Several studies on FTIR spectra give information on the yeast cell components, reflected as specific peaks in the fingerprinting regions as follows (i) **Sugar region:** peaks observed between 790 and 1,180 cm^{-1} (ii) **Nucleic acid region:** peaks between 1,200 and 1,290 cm^{-1} (iii) **Protein region:** peaks between 1,400 and 1,700 cm^{-1} (iv) **Chitin region :** peak observed around 2,900 cm^{-1} and (v) **Glucan:** possible peak at 1,074 cm^{-1} (Brugnerotto et al. 2001; Galichet et al. 2001; Padmavathy et al. 2003).

The aforementioned functional groups can be modified by treatment of yeast cells with chemicals such as formaldehyde-formic acid (HCHO-HCOOH), ethanol, dithiopyridine, benzene, triethyl phosphate and nitromethane as depicted in Fig. 16.2.

Methylation of amine groups was carried out by the treatment of biosorbent with HCHO-HCOOH (Kapoor and Viraraghavan 1997). This modification prevented the participation of amine groups in the metal biosorption process thereby decreasing the efficiency of lead and chromium biosorption by 53.38% and 28.28%, respectively, (Parvathi et al. 2007; Parvathi and Nagendran 2008). Ethanol treatment of the biosorbent causes the esterification of carboxylic groups (Drake et al. 1996). This treatment led to a lowering of lead and chromium biosorption by 96.34% and 64.35%, respectively, (Parvathi et al. 2007; Parvathi and Nagendran 2008).

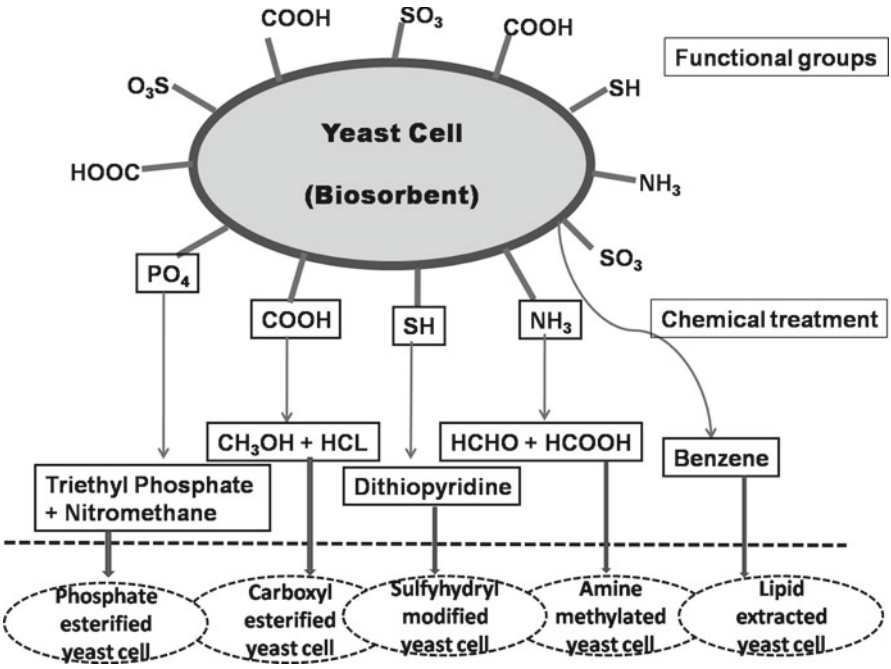


Fig. 16.2 Chemical treatment and modification of functional groups on yeast biomass

Triethyl phosphate and nitromethane treatment of the biosorbent causes an esterification of the phosphate groups (Markowska et al. 1975). This treatment in turn reduced the efficiency of Pb biosorption by 17.61% (Parvathi et al. 2007). Treatment of the biomass with benzene extracted the lipid fraction (Tobin et al. 1990). Due to this treatment, a 35.68% reduction in lead biosorption was observed (Parvathi et al. 2007). On the basis of the results obtained with the different treatment procedures, the significance of the abovementioned functional groups in the metal sorption process were confirmed (Parvathi and Nagendran 2008).

16.4 Critical Review of Metal Biosorption by Yeast Biomass

16.4.1 Biosorption Capacity of Yeast Biomass

Yeasts have received a lot of attention as biosorbents due to their good metal biosorption capacities and specificity for metal uptake. A large variety of toxic heavy metal ions such as Pb^{2+}, Cr^{3+}, Cr^{6+}, Zn^{2+}, Cu^{2+}, Co^{2+}, Cd^{2+} and Ni^{2+} have been effectively removed by yeasts (Table 16.2). Amongst the different species of yeasts, the *S. cerevisiae* biomass has been extensively studied for heavy metal biosorption

Table 16.2 Metal biosorption capacity of different yeasts

Yeast strain	Heavy metal	Biosorption capacity (mg/g) (mmol/g)	References
S. cerevisiae	Pb^{2+}	55.71	Parvathi et al. (2007)
R. glutinis	Pb^{2+}	73.5	Cho and Kim (2003)
C. albicans	Pb^{2+}	833.33	Baysal et al. (2009)
Beer yeast	Pb^{2+}	0.03	Han et al. (2006)
Brewer's yeast	Pb^{2+}	96.4	Kim et al. (2005)
S. cerevisiae	Cu^{2+}	6.4	Al-Saraj et al. (1999)
Brewer's yeast	Cu^{2+}	48.9	Kim et al. (2005)
Beer yeast	Cu^{2+}	0.02	Han et al. (2006)
S. cerevisiae	Cu^{2+}	0.17	Chen and Wang (2007)
S. cerevisiae	Cd^{2+}	1103.2	Breierová et al. (2002)
Sp. roseus	Cd^{2+}	2359.7	Breierová et al. (2002)
Cr. laurentii	Cd^{2+}	5328.5	Breierová et al. (2002)
Brewer's yeast	Cd^{2+}	96.4	Kim et al. (2005)
A. pollulans	Cd^{2+}	1,264	Breierová et al. (2002)
R. rubra	Cd^{2+}	4711.5	Breierová et al. (2002)
H. anomala	Cd^{2+}	6,534	Breierová et al. (2002)
Brewer's yeast	Cd^{2+}	5.34	Cui et al. (2010)
P. fermentans	Cd^{2+}	1632.7	Breierová et al. (2002)
S. cerevisiae	Cd^{2+}	0.137	Chen and Wang (2007)
Cy. capitatum	Cd^{2+}	1600.9	Breierová et al. (2002)
K. fragilis	Cd^{2+}	19.36	Hadi et al. (2003)
Brewer's yeast	Ni^{2+}	10.1	Cui et al. (2010)
S. cerevisiae	Ni^{2+}	0.108	Chen and Wang (2007)

(Brady and Duncan 1994; Volesky and May-Philips 1995). This is on account of the reasons listed as follows (i) it is easily available in large quantities at a very low cost (ii) it is a by-product of the brewing and wine making industries (iii) it has the potential to take up a broad range of heavy metals under a wide variety of external conditions (Blackwell et al. 1995; Volesky and May-Philips 1995; Ferraz and Teixeira 1999; Kim et al. 2005; Wang and Chen 2006).

The metal uptake rates of the biosorbent are usually determined at the equilibrium state of the system. The metal uptake rate q, is usually expressed in milligrams of metal sorbed per gram of the dry biomass of yeast or mmol g^{-1} or meq g^{-1} (Kratochvil and Volesky 1998). Biosorption capacities of yeast biomass for metal ions has been standardised by different ways. Biosorption capacities of different yeasts listed in Table 16.2 cannot be compared owing to the differences in conditions used for studies. For the purpose of evaluating the performance of yeast biomass, comparisons of metal uptake 'q' at similar equilibrium concentrations need to be made (Kratochvil and Volesky 1998). It should be noted that there is no standard method of determining the dry weight of yeast biomass during different studies. For example, *R. glutinis* dry weight estimates have been made after drying biomass at 70°C for 24 h (Cho and Kim 2003). In the case of *S. cerevisiae*, the

temperature time relations of 80°C for 8 h (Parvathi et al. 2007), 80°C for 48 h (Kim et al. 2005), and 105°C to a constant weight (Chen and Wang 2008) have been used. This difference in methodology in turn would affect dry weight estimates of yeast biomass and subsequently other biosorption values. There is thus a need to use standard conditions for such studies to make comparisons possible.

16.4.2 Factors Affecting Metal Biosorption

The biosorption capacities of different biosorbents are in general influenced by three factors (i) metal ionic characteristics (ii) nature of the biosorbents and (iii) biosorption conditions. Metal ionic properties in aqueous solutions are crucial inherent factors that influence the uptake of metal ions (Tobin et al. 1984; Remacle 1990; Brady and Tobin 1995; Tsezos et al. 1996). There are several reports that describe the significance of environmental conditions in the biosorption process.

The biosorption capacity of yeast biomass is strongly influenced by pH. This factor affects the solubility of metals as well as the binding sites for metal ions on the cell wall (Fourest and Roux 1992). It is generally observed that the biosorption capacity of metal cations increases with an increase in pH of the sorption system. At lower pH values, proton concentrations are high and metal binding sites on the biomass acquire a positive charge. Under such conditions, metal cations and protons compete with each other for the limited binding sites. This decreases the sorption of metal ions. On the other hand, at higher pH values, negative charge of functional groups on cell wall increases due to deprotonation of the metal binding sites thereby enhancing metal sorption. The optimal pH value for different sorption systems vary with the type of biomass and metal ions (Brady and Duncan 1994; Lopez et al. 2000). Optimum sorption of Pb^{2+} by *R. glutinis* biomass was observed in the pH range of 4.5–5.0 (Cho and Kim 2003). The optimum pH for Cu^{2+} sorption by *S. cerevisiae* was found to be in the range of 5.0–9.0 (Volesky 1990). Mapolelo and Torto (2004) have reported that the biosorption capacities of Cd^{2+}, Cr^{3+}, Cu^{2+}, Pb^{2+} and Zn^{2+} were enhanced at pH values greater than 5.0. However, optimum pH for Cr^{+6} biosorption by *Y. lipolytica* was observed at pH 1.0 (Bankar et al. 2009a). The reason for the enhanced adsorption of Cr^{6+} at low pH was that negatively charged $[HCrO_4]^-$, $[Cr_2O_7]^{2-}$, $[Cr_4O_{13}]^{2-}$ and $[Cr_3O_{10}]^{2-}$ ions were the dominant species under these conditions. There is another report supporting the fact that negatively charged Cr ion species are effectively adsorbed on the positively charged active sites on the sorbent (Özer and Özer 2003).

Temperature is another important factor affecting on biosorption of metal ions. Due to an increase in the operation cost, biosorption processes are usually not carried out at high temperatures (Wang 2002). Adsorption processes are normally exothermic and biosorption capacity thus increases with decreasing temperatures (Kapoor and Viraraghavan 1997). Optimal temperature for the biosorption of Pb^{2+}, Ni^{2+} and Cr^{+6} by *S. cerevisiae* was found to be 25°C. A decrease in biosorption at higher temperatures might be due to damage of the active sites in the biomass (Özer and

Özer 2003). However, there are a few reports on enhanced metal biosorption with increasing temperatures. For example, the biosorption of Cr^{6+} by *S. cerevisiae* and *Y. lipolytica* was better at higher temperatures (Goyal et al. 2003; Bankar et al. 2009a). These reports suggest that higher temperature may be enhancing the affinity of metal ions and binding sites of the yeast biomass.

For practical applications, contact time is an important factor that needs to be considered. The biosorption of Cu^{2+}, Zn^{2+} and Pb^{2+} by *S. cerevisiae* was rapid and reached equilibrium within hours (Kapoor and Viraraghavan 1997). The biosorption of Cr^{6+} by two strains of *Y. lipolytica* was also rapid (5–15 min) and equilibrium was reached within 2 h (Bankar et al. 2009a). Similarly, the equilibrium time of 2 h was also reported for biosorption of Zn^{2+} and Cu^{2+} by *S. cerevisiae* biomass (Bustard and McHale 1998). In another report, Pb^{2+} biosorption by *R. gutinis* biomass, equilibrium was attained in 30 min (Cho and Kim 2003). Most of the reports suggest that biosorption is a rapid process. Such short solution–biosorbent contact time durations are significant in the development of viable commercial processes (Volesky 1990).

Effect of biomass concentrations on biosorption has been reported by several authors. Cho and Kim (2003) studied the effect of biomass concentration on Pb^{2+} biosorption by using *R. gutinis* biomass. The percentage sorption of Pb^{2+} increased rapidly with increasing biomass concentration up to 2 g/l. On the other hand, the specific Pb^{2+} sorption was decreased with increase in biomass concentration. Similarly, the specific uptake of Cr^{6+} by *Y. lipolytica* decreased with an increase in biomass (Bankar et al. 2009a). This is explained by the fact that with increased biomass, there are increased electrostatic interactions leading to formation of agglomerates. In such aggregates, metal binding at active sites is not achieved (Cho and Kim 2003). At lower biomass concentrations, specific biosorption is increased possibly due to an increased metal to biosorbent ratio (Puranik and Paknikar 1999; Ariff et al. 1999).

Effect of initial concentration of metal ions also greatly influences the biosorption process. Banker et al. (2009a) reported that biosorption of Cr^{6+} increased with an increase in the initial metal concentration. This might be due to two reasons (i) the initial concentration of metal ions may provide a favorable driving force that increases adsorption process (ii) more number of metal ions may compete for the limited binding sites on biomass (Zhou et al. 2007).

The influence of competing ions on biosorption capacities of yeasts is also significant. This is because industrial effluents often contain a variety of metal ions such as calcium, magnesium, sodium and potassium. The presence of competing ions affects the sorption of heavy metal ions onto the biomass and reduces the binding capacity to some extent (Mogollon et al. 1998; Matheickal and Yu 1997). Cho and Kim (2003) have studied the effect of competing ions such as Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+} on the Pb^{2+} sorption capacity of *Rhodotorula glutinis*. These metal ions, at 50 mM concentrations, decreased the biosorption efficiency of the yeast biomass from 8% to 1%. Chen and Wang (2007) have also studied the competition between ten different metal ions by the waste biomass of *S. cerevisiae*. The maximum biosorption capacity (q_{max}) as determined by the Langmuir isotherm model decreased

in the following order (in millimole/g): Pb^{2+} (0.413) > Ag^{+}(0.385) > Cr^{3+}(0.247) > Cu^{2+} (0.161) > Zn^{2+} (0.148) > Cd^{2+}(0.137) > Co^{2+} (0.128) > Sr^{2+} (0.114) > Ni^{2+} (0.108) > Cs^{+}(0.092). Thus when commercial biosorption processes are being designed, these conditions need to be considered and optimized for the bioprocess to be effective.

16.4.3 Immobilisation of Yeast Biomass for Biosorption

In industrial applications or in technical operations, the use of freely dispersed yeast biomass is not recommended on account of the disadvantages listed below. Free biomass often creates problems in the operation of reactors by blocking flow lines and clogging filters. There are also difficulties associated with the separation of biomass and effluent. Moreover, the use of free dispersed biomass is often expensive for such applications (De Rome and Gadd 1991). Immobilized cell systems on the other hand, offer some advantages over the use of freely dispersed biomass. These include ease of regeneration and reuse of the biomass, easier solid-liquid separation, higher biomass loading and minimal clogging in continuous flow systems (Gadd 1988). Among the various immobilization methods available, physical entrapment of biomass within a polymeric matrix in the form of beads or granules with optimum size, mechanical strength, rigidity and porosity are popular.

Matrices such as polyacrylamide and alginate are found useful for biomass entrapment (Brady and Duncan 1994). Polyacrylamide gels have been widely used at the laboratory-scale. Marinkova et al. (2007) have reported that the Cu^{2+} and Cd^{2+} biosorption efficiencies were increased after *Trichosporon cutaneum* R57 was immobilized in polyacrylamide gels. This biosorption efficiency was better as compared to the free cells, as well as in bicomponent solutions. However, it must be noted that that polyacrylamide gels are not suitable for large-scale processes due to their toxicity, high cost and the limited mechanical strength of the resulting biosorbents (Tobin et al. 1994; Macaskie 1990; Hu and Reeves 1997).

The removal and recovery of caesium, strontium and uranium from aqueous solution by *S. cerevisiae* immobilized in calcium alginate has been reported (De rome and Gadd 1991). The use of alginate also has some drawbacks. These include (i) dissolution in buffers containing calcium chelates (ii) instability at low pH (Bauer et al. 1996) and (iii) poor mechanical strength (Hanaki et al. 1994).

Another alternative that has been investigated for the immobilisation process is polyvinyl alcohol (PVA). PVA offers several advantages over other polymer materials. These include low cost, non toxic nature, high stability, durability and mechanical strength (Chen and Lin 1994). Ting and Sun (2000) have reported that the use of PVA based matrices is promising in Cu^{2+} biosorption by *S. cerevisiae*. There is however scope for researchers to investigate other matrices, that would have desirable properties for the immobilisation process and application.

16.4.4 Improvement in Metal Biosorption

Biosorption of heavy metals by live yeast biomass is found to be higher than that by dead biomass (Avery and Tobin 1992; Volesky et al. 1993). However, some reports have highlighted that dead biomass offers several advantages for industrial applications. These include ease of storage, insensitiveness to metal toxicity and lack of nutritional requirement (Gadd 1990; Volesky 1990). Yeast cells that have been modified (killed) by chemical and physical conditions display different properties for heavy metal uptake as compared to the original yeasts (Lu and Wilkins 1996). In order to enhance the biosorption efficiency of yeast biomass, following pre-treatment methods have been used (i) **Physical methods:** These include vacuum and freeze drying, boiling, autoclaving and mechanical disruption. (ii) **Chemical methods**: They include the treatment of biomass with acid, alkali, detergents, caustic, methanol or formaldehyde. These methods have improved metal biosorption to some extent. Cell walls of yeast cells play a crucial role in the biosorption process. Metal biosorption is often enhanced by heat or chemical sterilization or by crushing the biomass. The processed biomass offers a larger surface area, exposes the intracellular components and thus makes more surface sites available for metal binding (Errasquin and Vàzquez 2003). Machado et al. (2009) have studied the metal biosorption efficiency of *S. cerevisiae*. The experiments were performed by using live and heat-killed cells for removal of Cu^{2+}, Ni^{2+} and Zn^{2+} from aqueous solutions. Heat-killed cells showed higher biosorption efficiency than live cells for heavy metal removal. This study suggested that modified heat-killed biomass was more suitable for large scale processes.

Another approach that has been studied is related to the use of magnetically modified yeast cells (Safarik et al. 2002; Azevedo et al. 2003; Safarikova et al. 2005). Magnetic nanoparticles were located on the cell wall surface of modified non-growing cells and accumulated inside the periplasmic space in growing cells (Azevedo et al. 2003).

Figure 16.3a shows a representative SEM image of magnetically modified yeast surfaces of *S. cerevisiae*. Such yeast surfaces were rough and offered a large surface area for the biosorption of mercury. Biosorption capacities of the magnetic yeast cells were 29.9 mg/g for Cu^{2+}, 76.2 mg/g for Hg^{2+}, 14.1 mg/g for Ni^{2+} and 11.8 mg/g for Zn^{2+}. These modified yeast cells exhibited a metal ion affinity sequence as follows: $Hg^{2+} > Cu^{2+} > Ni^{2+} > Zn^{2+}$ (Yavuz et al. 2006). Yeast cells have also been modified by nano-TiO_2. Figure 16.3b shows a representative SEM image of baker's yeast composites with nano-TiO_2. The cell surfaces of the composite baker's yeast were rough (Zhang et al. 2008). Such composites were formed due to attractive forces (coordination, hydrogen bonds, Van der Waals' attractive force, dipole attraction) among the polar groups (C═O, C–O, C–N, O–H or N–H) on the baker's yeast cell, and the unsaturated Ti atoms and hydroxyl groups (–OH) on the surface of the nano-TiO_2 (Chien et al. 2004; Oliva et al. 2003). The composite adsorbent showed higher adsorption efficiency for Cu ions than either the baker's yeast biomass or the nano-TiO_2 alone. The yeast biomass thus modified showed enhanced metal

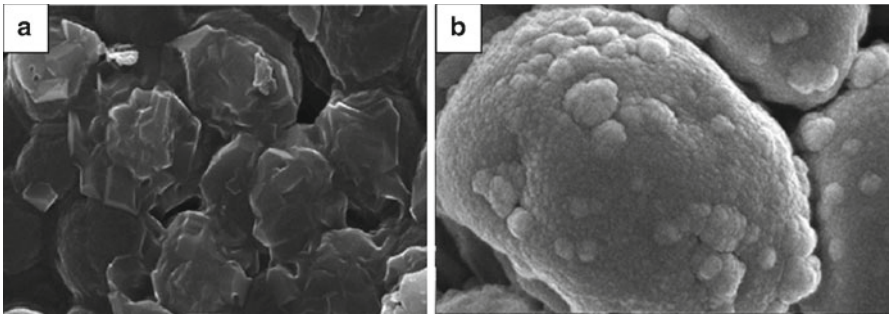


Fig. 16.3 Representative SEM images of (**a**) the magnetic yeast biomass (Yavuz et al. 2006) (**b**) composite adsorbent of yeast and TiO_2 (Zhang et al. 2008)

biosorption. In the future, there is scope for further investigations on the modification of adsorbents with different nanoparticles for enhancing their biosorption capacities for metal ions.

16.4.5 Biosorption Equilibrium Isotherms Models and Kinetics Models

The preliminary assessment of solid-liquid sorption system is based on two types of investigations**:** (i) equilibrium batch sorption tests and (ii) dynamic continuous-flow sorption studies (Volesky and Holan 1995). Recently, some reviews have described the equilibrium isotherms and kinetics models for biosorption studies (Wang and Chen 2006; Das et al. 2008; Wang and Chen 2009; Gadd 2009). The equilibrium of biosorption process is often described by fitting the experimental points with models (Gadd et al. 1998). Depending on the mechanism of metal ion sorption, equilibrium isotherm models are categorised into two groups: (i) the empirical models (ii) mechanistic models. The empirical models for single solute systems used to describe the biosorption equilibrium are listed in Table 16.3.

Langmuir and Freundlich models are the two most widely accepted models for single solute system. Langmuir model is popular due to its simplicity and the good agreement it has with experimental data. The Langmuir isotherm was derived originally from studies on gas adsorption to activated carbon. This model assumes that (i) all binding sites possess an equal affinity for the adsorbate, (ii) adsorption is limited to the formation of a monolayer, and (iii) the number of adsorbed species do not exceed the total number of surface sites i.e. there is a 1:1 stoichiometry between surface adsorption sites and adsorbate (Langmuir 1918). It is likely that none of

Table 16.3 The common adsorption isotherms

Isotherm types	Description/importance	References
1. Langmuir	L type, based on monolayer adsorption of solid. Originally derived for adsorption of gases in monolayer to activated carbon Isotherm is applicable to biological systems. It is widely accepted for biosorption	Langmuir (1918)
2. Freundlich	F type, developed for heterogeneous surfaces. It is a widely used in biosorption	Freundlich (1906)
3. Scatchard plots	Originally describe the attraction of proteins for small molecules and ions. It is also used to describe metal biosorption equilibrium	Kapoor and Viraraghavan (1995)
4. Langmuir-Freundlich	It is developed for homogenous surface, when biosorption process is cooperative due to absorbate-adsorbent interactions	Sips (1948)
5. BET model	BET describes the multi-layer adsorption at the adsorbent surface and assumes that Langmuir equation applies to each layer	Brunauer et al. (1938)
6. Tempkin	It assumes that the heat of adsorption decreases linearly with the coverage due to adsorbent-adsorbate interaction	Tempkin and Pyozhev (1940)
7. Dubinin-Radushkevich (D-R) isotherm	It assumes that the characteristics of the sorption curves are related to the porosity of the adsorbent	Dubinin (1960)
8. Redlich- Peterson isotherm	It describes adsorption on heterogeneous surfaces. It contains three parameters and incorporates the features of the Langmuir and the Freundlich isotherms into a single equation	Redlich and Peterson (1959)

these assumptions apply to biological systems. The Langmuir model saturated monolayer isotherm, can be described as follows:

$$q_e = \frac{q_{max}\, bC_e}{1 + bC_e}. \tag{16.1}$$

q_e is equilibrium metal sorption capacity; C_e is equilibrium solute concentration in solution; q_{max} and b are Langmuir constants related to maximum sorption capacity (monolayer capacity) and bonding energy of adsorption/affinity, respectively.

Another empirical model is the Freundlich model that defines adsorption to heterogeneous surfaces, i.e. surfaces possessing adsorption sites of different affinities (Freundlich 1906). The equation is given as:

$$q_e = K_F C_e^{1/n} \tag{16.2}$$

K_F is biosorption equilibrium constant, representative of the sorption capacity; and n is a constant indicative of biosorption intensity. More detail or examples on these empirical models can be obtained from relevant references (Parvathi et al. 2007; Cho and Kim 2003; Baysal et al. 2009; Al-Saraj et al. 1999; Han et al. 2006; Kim et al. 2005; Breierová et al. 2002; Hadi et al. 2003; Cui et al. 2010; Chen and Wang 2007). Table 16.4 summarizes the parameters of Langmiur and Freundlich models as reported in different references.

The BET model describes the multi-layer adsorption at the adsorbent surface. The model assumes that the Langmuir isotherm applies to each layer (Brunauer et al. 1938).

$$q_e = \frac{BQ^{\circ}C_e}{(C_S - C_e)\left[1 + (B-1)C_e / C_s\right]} \tag{16.3}$$

C_s is the saturation concentration of the adsorbed component; B is a constant indicating the energy of interaction between the solute and the adsorbent surface, and Q^o is a constant indicating the amount of solute adsorbed forming a complete monolayer. These models give information of metal uptake capacity and difference in metal uptake between various species (Kapoor and Viraraghavan 1995; Volesky and Holan 1995). These empirical models however do not explain the mechanisms involved in metal uptake.

Kinetic models on the other hand, are useful for understanding the mechanisms of metal biosorption and in evaluating the potential of biosorbent for removal of metal ions. Two simplified kinetic models were developed to understand the mechanisms of metal biosorption. The first is the pseudo-first-order model proposed by Langergren (1898). The pseudo-first-order expression based on solid capacity can be written as follow:

$$\frac{d_{qt}}{d_t} = k_1(q_e - q_t) \tag{16.4}$$

$$\log(q_e - q_t) = \log q_e - \frac{k_1}{2.303}t \quad V_1 = K_1 q_e V_1 = K_1 q_e$$

Where q_e is the amount of adsorbed metal ions on the biosorbent at time t; k_1 is the rate constant of Lagergren first-order biosorption; V_1 is the initial sorption rate.

The pseudo-second-order model assumes that the rate-limiting step in biosorption may be chemical sorption. It also states that the adsorption capacity is proportional to the number of active sites occupied on the sorbent (Ho and McKay 1999).

$$\frac{d_{qt}}{d_t} = k_2(q_e - q_t)^2 \tag{16.5}$$

$$q_t = \frac{t}{\frac{1}{k_2 q_e^2} + \frac{t}{q_e}} \quad V_2 = k_2 q_e^2$$

where k_2 is the rate constant of second-order biosorption; V_2 is the initial adsorption rate.

Table 16.4 Parameters for the application of Langmuir and Freundlich model to the biosorption of metal ions on yeast biomass

		Langmuir			Freundlich			
Yeast biomass	Metal	q_{max} (mg g^{-1})	b (l mg^{-1})	r^2	K_F	1/n	r^2	References
Y. lipolytica 3589	Cr^{6+}	109.7	0.01	0.99	0.53	1.13	0.96	Bankar et al. (2009a)
Y. lipolytica 3590	Cr^{6+}	150.5	0.01	0.97	0.15	3.96	0.99	Bankar et al. (2009a)
S. cerevisiae	Cr^{2+}	94.34	0.41	0.98	31.1	0.31	0.97	Bingol et al. (2004)
R. glutinis	Pb^{2+}	73.5	0.02	0.99	–			Cho and Kim (2003)
S. cerevisiae	Pb^{2+}	60.2	0.06	0.99	3.8	0.84	0.99	Goksungur et al. (2005)
Brewer's yeast	Hg^{2+}	133.3	0.11	0.99	30.4	0.35	0.92	Yavuz et al. (2006)
Brewer's yeast	Ni^{2+}	5.34	0.10	0.98	1.1	2.9	0.96	Cui et al. (2010)
Brewer's yeast	Cd^{2+}	10.7	0.33	0.98	3.8	3.5	0.94	Cui et al. (2010)
Waste beer yeast	Pb^{2+}	55.71	0.08	0.99	0.51	1.18	0.99	Parvathi et al. (2007)
S. cerevisiae	Cd^{2+}	31.75	0.09	0.99	3.08	0.69	0.96	Goksungur et al. (2005)

As shown in Table 16.5, the pseudo-first-order and pseudo-second-order equations were used to analyse the experimental data of Pb^{2+}, Cu^{2+}, Cd^{2+} and Hg^{2+} biosorption by brewery yeast. The correlation coefficient (r^2) values for the pseudo-second-order rate equation at all Pb^{2+}, Cu^{2+}, Cd^{2+} and Hg^{2+} concentrations were greater than 0.99 and substantially higher than that of the pseudo-first-order equation. The values of q_e increased as the initial metal ions (Pb^{2+}, Cu^{2+}, Cd^{2+} and Hg^{2+}) varied from 10 to 200 mg/l (Kim et al. 2005; Yavuz et al. 2006). Cui et al. (2010) reported the pseudo-second-order model fits better the adsorption kinetics data of Ni^{2+} on brewer's yeast than the pseudo-first-order model. The values of r^2 were greater than 0.99 for adsorption of Ni^{2+}. As shown in Table 16.5, there was a good agreement between the experimental and the calculated q_e values suggesting the applicability of this model to describe the biosorption of Pb^{2+}, Cu^{2+}, Cd^{2+}, Hg^{2+} and Ni^{2+} onto the yeast biomass. This indicated that the pseudo-second order mechanism was predominant and chemisorption might be the rate-limiting step that controlled the biosorption process. During the course of the biosorption process three possible mechanisms may take place: (i) an external surface mass transfer or film diffusion controlling the early stages of the biosorption process (ii) this may be followed by a reaction or constant rate stage and (iii) finally a diffusion stage in which the biosorption process slows down considerably (Öncel et al. 2005; Allen et al. 2005).

16.4.6 Biosorption Mechanism(s) by the Yeast Cell

The mechanism involved in metal sorption is complicated and not fully understood. Several factors affect the mechanism of biosorption. These include the type of biomass, status of biomass (living or dead), metal-solution chemistry and environmental conditions. Recently, several reviews describing the mechanisms involved metal biosorption have been published (Volesky and May-Philips 1995; Wang and Chen 2006; Alluri et al. 2007; Gadd 2009). Metals interact with living as well as dead microbial cells. Metal uptake by yeast cells and other microbes occurs by two methods (i) a metabolism independent process and (ii) a metabolism dependent manner. The first step of initial biosorption is rapid and takes place within a few minutes after contact with heavy metal ions. This step is independent of temperature, metabolic energy and inhibitors (Blackwell et al. 1995; Avery and Tobin 1992; Brady and Duncan 1994). The initial metal binding is thought to involve the microbial cell wall and in some cases, extracellular polymers. Metal binding is attributed to adsorption, complexation, ion-exchange, precipitation and crystallization within the cell wall structure (Mowell and Gadd 1984). The second step (bioaccumulation) is slow, dependent on metabolic energy, temperature and metabolic inhibitors (De Rome and Gadd 1987; Blackwell et al. 1995). Thus living as well as dead cells can be used in metal biosorption for industrial applications (Volesky et al. 1993; Gadd 1990). Depending on the location of the metal, the mechanisms of metal biosorption can be classified as (i) extra cellular accumulation/precipitation, cell surface sorption/

Table 16.5 The Pseudo-first-order and Pseudo-second-order kinetic parameters for different metal concentrations

	Pseudo-first-order model				Pseudo-second-order model				
Metal	(mg/l)	q_e (mg/g)	k_1 (1/min)	r^2	$q_{e,cal}$ (mg/g)	k_2 (g/mg min)	r^2	q_e^{exp} (mg/g)	References
Pb^{2+}	10	6.53	3.96	0.97	8.04	0.93	0.99	7.44	Kim et al. (2005)
	50	17.76	4.77	0.95	34.97	0.57	0.99	33.82	
	100	44.05	4.93	0.98	64.10	0.19	0.99	60.54	
	200	61.04	5.87	0.98	78.74	0.17	0.99	74.86	
Cu^{2+}	10	1.83	3.75	0.85	7.32	5.81	0.99	7.22	Kim et al. (2005)
	50	7.73	3.94	0.96	23.26	1.45	0.99	22.83	
	100	20.12	4.24	0.99	35.71	0.53	0.99	34.52	
	200	23.58	4.33	0.99	42.02	0.42	0.99	40.58	
Cd^{2+}	10	1.55	1.66	0.98	2.37	2.94	0.99	2.37	Kim et al. (2005)
	50	5.11	2.10	0.99	6.48	0.61	0.99	5.90	
	100	7.63	2.28	0.97	9.51	0.41	0.99	8.47	
	200	8.47	2.33	0.98	11.49	0.40	0.99	10.25	
Hg^{2+}	25	2.3	0.05	0.96	29.1	0.0025	0.99	38.5	Yavuz et al. (2006)
	50	76.5	0.06	0.87	54.0	0.0015	0.99	49.6	
	100	146.6	0.05	0.93	91.7	0.0005	0.99	79.6	
	200	120.8	0.06	0.86	90.1	0.0008	0.99	82.4	
Ni^{2+}	15	1.55	0.02	0.93	1.84	0.0219	0.99		Cui et al. (2010)

precipitation and (ii) intracellular accumulation (Veglio and Beolchini 1997). In this chapter, based on the available literature, these mechanisms have been discussed.

16.4.6.1 Extracellular Accumulation of Metal Ions by Yeast Cell

Several microorganisms have the ability to produce extracellular polymeric substances (EPS). These are composed of polysaccharides, glucoprotein, lipopolysaccharide and soluble peptides. There are several reports on metal biosorption by EPS derived from bacteria such as *Bacillus megaterium*, *Acinetobacter*, *Pseudomonas aeuroginosa* and Cyanobacteria (Lui et al. 2001). Wang and Chen (2006) have concluded that the biomolecules constituting EPS have different functional groups for binding heavy metal ions. EPS produced by yeast cells also plays a role in metal biosorption. Breierová et al. (2002) have studied Cd^{2+} tolerance and its accumulation in eight yeast species namely, *Aureobasidium pullulans, Cryptococcus. laurentii, Sporobolomyces roseus, Hansenula anomala, Cystofilobasidium capitatum, Pichia. fermentans, S. cerevisiae* and *Rhodotorula rubra*. The adaptation of these yeasts to toxic concentrations of Cd^{2+} was dependent on the production of extracellular glycoproteins. The highest Cd^{2+} tolerance was observed in *H. anomala*, while the lowest tolerance was in *S. cerevisiae*.

As shown in Table 16.6, the biosorption capacities of yeasts for Cd^{2+} ions were dependent on the structural elements of exopolymers and on the yeast species. Under conditions of Cd^{2+} stress, the saccharide moieties of exopolymers contained higher content of mannose and lower contents of glucose, galactose and arabinose when compared to exopolymers secreted by cells that were without the metal stress. Extracellular glycoproteins of *H. anomala* showed adsorption of about 90% of the total content of Cd^{2+} ions bound by yeast cells, while extracellular glycoproteins of *S. cerevisiae* adsorbed only 6% of the total Cd^{2+} ions. This difference in metal uptake may be due to a variation in composition of the saccharide moieties in the extracellular glycoproteins. The composition of the extracellular glycoproteins changed during the process of adaptation to the Cd^{2+} ions as shown in Table 16.6. Differences in the phosphorus content in the exopolymers isolated from yeast cells with and without metal stress conditions were also observed. Under the stress of metal ions, exopolymers showed a higher amount of phosphorus than the exopolymers obtained under optimum conditions (without metal ions). Under stress of Cd^{2+} ions, the content of glutamic acid was affected, while the contents of other amino acids in protein moiety of exopolymers were unaltered (Breierová et al. 2002). Stress proteins contained strongly hydrated amino acids with non-polar or polar residues (Jaenicke 1991). An increase in the content of glutamic acid under other stress conditions have also been reported (Stratilová et al. 1998). Higher amount of glutamic acid in the protein moiety under stress of metal ions was possibly to eliminate the toxic effect of Cd^{2+} ions (Breierová et al. 2002). Most of cell wall proteins are O- or N-glycosylated. The O-chains are joined to serine or threonine residues are short and linear while the N-chains are joined to aspartic acid are represented by highly branched oligosaccharides (Klis 1994). Under stress conditions, levels of serine and

Table 16.6 Composition of exopolymers produced by yeast strains with or without Cd^{2+} stress and their sorption capacity (%) (Breierová et al. 2002)

Composition of expopolymers (%)						Sorption capacity (%)
Yeast strain	Glc	Gal	Man	Ara	P	
A. pullulans						
With Cd^{2+}	68.1	6.1	25.7	–	4.3	4.1
Without Cd^{2+}	72.3	9.3	18.4	–	3.2	
Cr. laurentii						
With Cd^{2+}	30.5	12.4	28.5	28.5	6.2	51.8
Without Cd^{2+}	20.6	22.4	41.6	15.4	3.6	
Sp. roseus						
With Cd^{2+}	32.2	35.2	21.3	11.3	5.1	15.4
Without Cd^{2+}	32.7	6.2	42.4	15.7	6.1	
H. anomala						
With Cd^{2+}	20.4	4.3	56.8	18.5	6.4	33.3
Without Cd^{2+}	33.7	5.8	48.2	12.3	4.7	
Cy. capitatum						
With Cd^{2+}	34.5	19.0	28.7	17.8	7.7	45.8
Without Cd^{2+}	63.2	8.2	23.2	5.4	4.9	
P. fermentans						
With Cd^{2+}	75.3	5.2	19.5	–	6.6	6.7
Without Cd^{2+}	66.3	6.2	27.5	–	5.8	
S. cerevisiae						
With Cd^{2+}	25.2	10.6	64.2	–	6.2	5.6
Without Cd^{2+}	33.1	10.8	56.1	–	5.0	
R. rubra						
With Cd^{2+}	32.8	17.5	38.3	11.4	9.4	0.8
Without Cd^{2+}	31.2	8.6	39.2	21.0	5.4	

threonine were decreased and the level of aspartic acid was increased (Breierová et al. 2002; Breierová et al. 1996).

Suh et al. (1999) have reported that the accumulation of Pb^{2+} by the yeast *Aureobasidium pullulans* was only on the cell surface was due to presence of EPS. More than 90% of Pb^{2+} ions were adsorbed onto the excreted EPS. It was also observed that biosorption capacities and initial rate of Pb^{2+} accumulation in living cells were higher than that of dead cells due to presence of EPS in cells of *A. pullulans* (Suh et al. 1998). Metal sorption by exopolymers is mostly dependent on the composition of the saccharide moiety of these polymers (Breierová et al. 2002). FTIR studies of these exopolymers have revealed the presence of hydroxyl, carboxyl and amine groups. Metal biosorption by exopolymers possibly occurs via ionic interactions and by physical entrapment (Breierová et al. 2002). Other yeasts such as *Rhodotorula glutinis* KCTC 7989 also secrete extracellular polymeric substances (EPS). The EPS secreted was a novel acidic heteropolysaccharide, which was composed of neutral sugars (85%) and uronic acid (15%). The neutral sugars

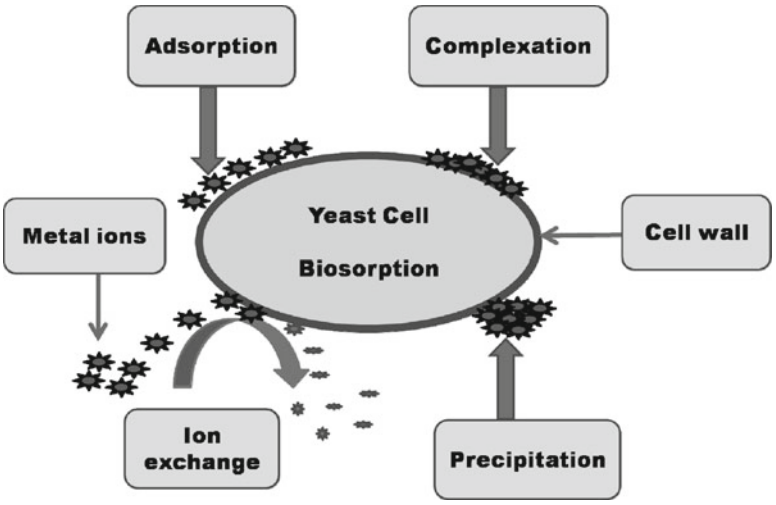


Fig. 16.4 Summary of the possible mechanisms involved in metal biosorption in yeasts

identified were mannose, fucose, glucose, and galactose in a ratio of 6.7:0.2:0.1:0.1. Uronic acids gave a net negative charge to the polymer and contributed to the binding capacities of the polymer for metal ions. This polysaccharide enhanced the adsorption capacity of *R. glutinis* (Cho et al. 2001). Thus, a variety of studies have confirmed the role of EPS in the process of metal binding.

16.4.6.2 Cell Surface Sorption

Wang and Chen (2006) have emphasized on the fact that the cell wall is the outermost part of cell that interacts with metal ions. There are two different ways of metal uptake by cell wall: stoichiometric interaction between functional groups such as carboxylic, phosphate, amine as well as phosphodiester and physicochemical inorganic deposition via adsorption or inorganic precipitation. Other mechanisms such as complexation, ions exchange, inorganic microprecipitation, oxidation and/reduction, adsorption are proposed by several authors (Fig. 16.4).

In order to implicate the role of the cell wall in metal uptake, its structure needs to be understood. The yeast cell wall is a complex layered structure consisting of an inner and outer layer (Fig. 16.5). The inner layer is mostly composed of β-glucan and chitin and the outer layer is made up of α- highly glycosylated mannans associated with mannoproteins (Cabib et al. 2001; Lesage and Bussey 2006). The chemical analysis of the yeast *Y. lipolytica* revealed that the cell wall is composed of 70% neutral carbohydrate, 7% amino sugars, 15% protein, 5% lipids and 0.8% phosphorus (Vega and Domínguez 1986). Mannan, glucan and chitin are the main cell wall polymers of *Y. lipolytica*, *S. cerevisiae* and *C. albicans* (Vega and Domínguez 1986; Cole and Nozawa 1981).

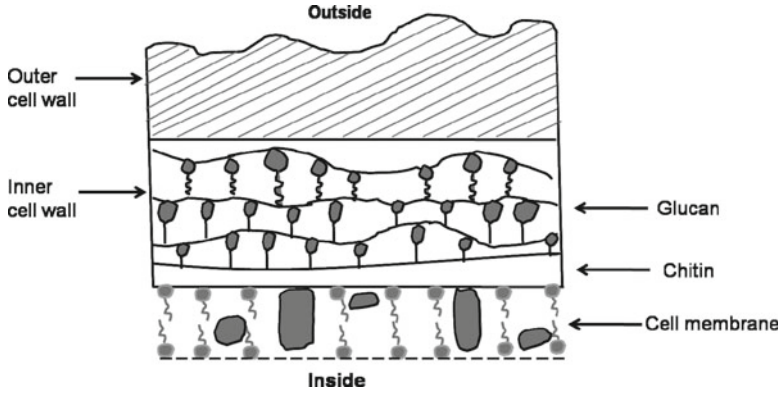


Fig. 16.5 Diagrammatic representation of the cell wall structure of yeasts

The interactions of heavy metals with these polymers were followed by FTIR analysis (Machado et al. 2009). Studies carried out by Brady et al. (1994b) have indicated that the cell wall structures play a crucial role in heavy metal uptake. Earlier studies by Davidova and Kasparova (1992) have also suggested that the adsorptive capacity of yeast cell walls for heavy metals is determined by the structural organization of the entire protein–carbohydrate complex. The role of phosphomannans and carboxyl groups of cell wall protein of *S. cerevisiae* in metal binding have also been suggested (Strandberg et al. 1981). Studies on heavy metal accumulation by the yeast *Y. lipolytica* have implicated the involvement of cell wall in metal binding. The maximum quantity of Ni and Cd were observed in cell wall and membrane debris, possibly due to the interaction of heavy metals with carboxylic group of cell wall (Strouhal et al. 2003). Cr^{6+} sequestration on the cell surface in this yeast also revealed the possible involvement of functional groups such as amino, carboxyl and hydroxyl in metal binding process (Bankar et al. 2009a). Blocking of such functional groups of the cell wall reduced the metal biosorption. These studies clearly revealed the role of cell wall functional groups in metal uptake process (Brady and Duncan 1994). Energy dispersive X-ray microanalysis (EDX) is also a useful technique to study the chemical and elemental characteristics of the adsorbent. The biomass of *R. glutinis* was subjected to EDX analysis. Prior to Pb^{2+} sorption, the biomass of this yeast showed a peak for K^+ since it is an important constituent of cell membranes and cell walls. After the process of Pb^{2+} sorption, the EDX spectrum of the biomass displayed a peak for Pb^{2+}, and the peak for K^+ disappeared (Cho and Kim 2003). K^+ ions are also reported to be released by *S. cerevisiae* after Cd^{2+} sorption (Gadd and Mowll 1983). In addition, Chen and Wang (2008) have also reported the release of ions of Mg^{2+}, Na^+ and Ca^{2+} by *S. cerevisiae* after sorption of Zn ions. The release of K^+, Mg^{2+}, Na^+, and Ca^{2+} from biosorbents after sorption of metal ions indicates the possibility of an ion exchange mechanism being involved in the metal binding

process (Reddad et al. 2002). SEM-EDX studies on *S. cerevisiae* cells that had adsorbed Zn^{2+} showed the direct precipitation of this metal on the cell surface (Chen and Wang 2008). Precipitation of copper, lead and cadmium on the cell surface of the waste *S. cerevisiae* was also observed by Marques et al. (2000). Precipitation of released phosphate was observed in *R. glutinis* after the process of Pb^{2+} sorption (Cho and Kim 2003). This report indicates that precipitation is also a mechanism involved in the metal biosorption in yeasts. Studies on Cr^{6+} biosorption by *Y. lipolytica* suggested that the overall biosorption process may involve more than one mechanisms including sorption, ion exchange, surface complexation or electrostatic attraction (Bankar et al. 2009a).

16.4.6.3 Intracellular Accumulation

The process of intracellular accumulation by living cell biomass is an energy-driven process and is dependent on active metabolism. Metal ions enter cells, move across the cell membrane and are transformed or precipitated within the cell (Wang and Chen 2006). Studies have shown that yeasts have the ability to accumulate a broad range of metal ions such as Cr^{3+}, Cr^{6+}, Ni^{2+}, Cd^{2+}, Zn^{2+} and Co^{2+} (Wang and Chen 2006; Bankar et al. 2009a; Strouhal et al. 2003; Kaszycki et al. 2004). The different yeasts that have been studied with respect to the accumulation of heavy metal ions are listed in Table 16.7.

Strouhal et al. (2003) determined the distribution of metal ions in different cell compartments of *Y. lipolytica*. For this study, the authors have used four metals at different concentrations. Cd^{2+} at 4.8, 9.3, 18.7 and 37.5; Ni^{2+} and Co^{2+} at 75, 150, 600, 12,000 and Zn^{2+} at 300, 600, 1,200, 2,400 µmol/l were studied. Metal ions accumulated in cell compartments at different rates. Accumulation of Cd^{2+} and Ni^{2+} was higher than that of Co^{2+} and Zn^{2+}. Cd^{2+} and Ni^{2+} ions were not detected on the cell surface. These metals were mostly associated with cell wall and membrane debris probably due their interaction with carboxylic functional groups of cell wall. On the contrary, high level of Zn^{2+} and Co^{2+} ions were detected on the cell surface. Muter et al. (2002) observed that the accumulation of metal ions by *Candidia utilis* occurred in the following order: Zn > Cd > Pb > Cu > Cr. According to Strouhal et al. (2003), the entry of heavy metal ions into individual cell compartments of *Y. lipolytica* decreased in the following order: (i) cell surface : Co > Zn > Cd, Ni, (ii) cell wall and membrane debris: Cd, Ni > Zn > Co, (iii) cytoplasm : Zn, Co > Cd > Ni. Paš et al. (2004) studied the accumulation of Cr^{3+} and Cr^{6+} in the yeast *Candidia intermedia*. High levels of Cr^{3+} and Cr^{6+} were observed in the protoplast as compared to the cell wall. The value of cell-accumulated Cr^{3+} was 30 mg/g for *S. cerevisiae* (Batic and Raspor 1998) and 0.45 mg/g for *C. intermedia* (Batic and Raspor 2000). In the case of Cr^{6+}, *S. cerevisiae* accumulated 4 mg/g (Krauter et al. 1996), *Schizosaccharomyces pombe* up to 0.95 mg/g (Czako-Ver et al. 1999) and for *Candidia utilis* (Muter et al. 2001), the highest value was 7.2 mg/g. All these values were obtained under different

Table 16.7 Bioaccumulation of heavy metal ions by different yeast strains

	Metal concentration in cellular content			Metal concentration in medium			
Yeast strain	Cr^{3+} (Mg g^{-1} dry wt)	Cr^{6+} (Mg g^{-1} dry wt)	Cd (%)	$Cr3^{+}$ (mM)	Cr^{6+} (mM)	Cd (g l^{-1})	References
C. guilliermondii	18	6.7		15	02		
C. maltose	4.1	4.0		08	02		
C. macedoniensis	14	0.2		12	02		
C. membranaefaciens	10.4	5.3		04	06		Kaszycki et al. (2004)
C. pulcherrima	14	2.0		12	02		
D. kloeckeri	13.1	5.8		04	02		
K. bulgaricus	9	3.8		20	01		
K. lactis	9	5.0		20	01		
P. guilliermondii	9.7	5.0		20	06		
S. cerevisiae			94			0.006	Breierová et al. (2002)
Sp. roseus			58			0.002	
A. pullulans			49			0.034	
P. fermentans			45			0.034	
Cy. capitatum			32			0.034	
Cr. laurentii			36			0.045	
R. rubra			25			0.045	
H. anomala			09			0.056	

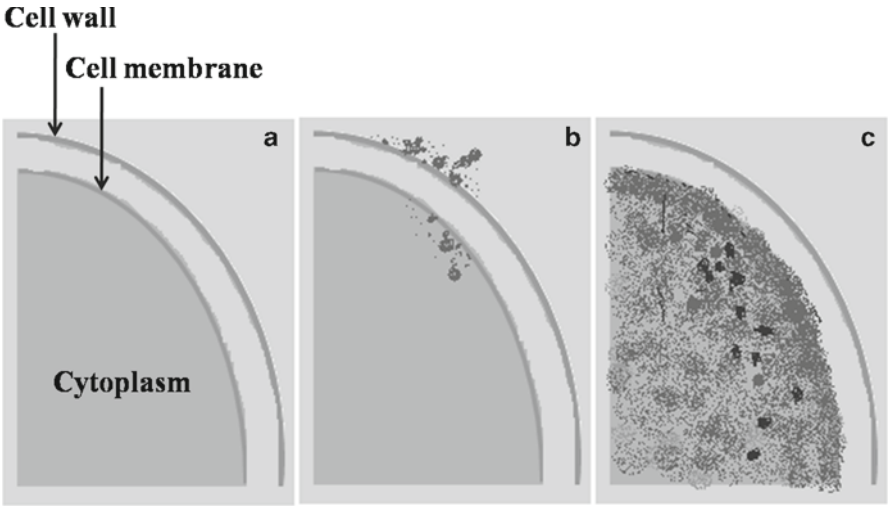


Fig. 16.6 Diagrammatic representation of Pb^{2+} accumulation in *S. cerevisiae* (**a**) initial state, (**b**) after 50 min and (**c**) after 2 h

experimental conditions with respect to metal concentrations, treatment time and biomass densities.

The processes of Pb^{2+} accumulation in *S. cerevisiae* was observed by TEM.

Figure 16.6 shows a diagrammatic representation of the distribution of Pb in these cells. Lead was not detected on the cell surface, cell membrane, and in the cytoplasm of *S. cerevisiae* before accumulation process. After 3 min of accumulation, Pb^{2+} began to bind onto the surface of the cell wall and membrane. After 50 min, the Pb^{2+} ions were observed on the cell wall and membrane and they reached to the cytoplasm within 2 h.

Yeast cells protect themselves from heavy metals ions (Mullen et al. 1989; Trevors et al. 1986) by production of some specific proteins (Li et al. 1997) that bind to the metal ions in cytoplasm or transport them into vacuoles (Li et al. 1996). For example, metallothioneins (MTs) are metal binding proteins (Winge et al. 1985; Palmiter 1998; Rodriguez 1999). The production of MTs in response to the presence of four metals (Zn, Co, Ni and Cd) has been studied in *Y. lipolytica*. There was an inverse co-relation between the toxicity of the metal and levels of metallothioneins. The intracellular concentrations of toxic metals (Ni and Cd) were lower and the cells exhibited high cellular levels of metallothioneins. On the other hand, the concentrations of the essential metals within the cell were higher (Zn and Co) and the metallothionein concentrations were lower. This study has thus signified the role of metallothioneins in the detoxification of toxic heavy metals (Strouhal et al. 2003).

16.5 Future Prospective

Removal of heavy metal ions from contaminated sites is a great challenge. Heavy metal remediation by using physico-chemical techniques is expensive and often not suitable for voluminous effluents containing complexing organic matter as well as low metal ion concentrations. Biotechnological approaches may be an alternative for such traditional methods. Biosorption can be effectively used for the removal of heavy metal ions from dilute complex solutions with high efficiency and in a rapid manner. Thus, biosorption can be an ideal method for the treatment of high volume low concentration complex waste waters. Biosorption of metal ions by using yeast biomass is of special interest due to their excellent biosorption capacity and low cost. The most well-known and commercially significant yeasts are species and strains of *S. cerevisiae*. These yeasts are obtained as waste biomass from the food and beverage industry. Thus, this potential biosorbent can be obtained at minimal cost from the respective producers who need to dispose the large quantities of biomass that are generated in an effective manner. These biosorbent will make the metal removal process highly economical and competitive for the treatment of contaminated effluents generated by metal-plating or metal-finishing operations, mining, ore processing operations, metal processing industries battery manufacturing operations, thermal power generation stations and nuclear power generation plants.

16.6 Conclusion

An extensive study on the reports on metal biosorption by yeast biomass has revealed their potential in the management of heavy metal pollution. Yeast biomass is a low cost biosorbent. This aspect will be beneficial in reducing the cost of the biosorption process. Moreover, biosorption has emerged as an attractive biological method for treatment of effluents containing low metal contamination. It is also an alternative technology for other non-biotechnology based processes such as chemical precipitation, flotation, electrochemical processes and membrane technology. However, most of the reports on biosorption have been restricted to the laboratory scale and there is a need to extrapolate this technology to commercial levels. In addition, the mechanisms involved in biosorption are still not fully understood thereby limiting applications of the biosorption process. The factors such as pH and selectivity of co-ions that influence biosorption have not been fully understood. The development of biosorption process for management of heavy metal pollution needs to be investigated further in terms of modeling, understanding biosorption mechanisms, developing methods for regeneration and immobilization of biosorbents, and treating industrial wastewaters at large scale levels.

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Chapter 17
Management of Hospital Wastes with Potential Pathogenic Microbes

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Abstract With respect to health and environmental hazards, healthcare waste has been identified as the second most hazardous waste after radioactive waste. Biomedical waste is potentially associated with pathogenic micro-organisms and various types of infections such as gastroenteric infections, respiratory infections, ocular infections, genital infections, skin infections, anthrax, meningitis, AIDS, haemorrhagic fevers, septicaemia, bacteraemia, candidaemia, hepatitis A, B and C. According to World Health Organisation (WHO), the reuse of infectious syringes contributes to about eight million Hepatitis B Virus (HBV), more than 2.3 million Hepatitis C Virus (HCV) and about 80,000 Human Immunodeficiency Virus (HIV) infections per year worldwide.

Various types of hospital wastes that cause infection are – human and animal anatomical wastes, micro-biology and bio-technology wastes, waste sharps, soiled solid wastes and liquid wastes, etc. The diseases are spread through direct human contact, contact with the blood and infectious body fluids and through air, water and soil. Healthcare professionals and the general public are equally at risk due to improper treatment and disposal of infectious wastes. Ragpickers also expose themselves to diseases like Hepatitis B, Tetanus, Septicemia, etc., while handling items like used needles, surgical gloves and blood bags disposed in municipal bins and dumpsites. To avoid the hazards caused by healthcare waste, all the infectious wastes

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are required to be segregated from the non-infectious wastes generated in a hospital and the same are required to be treated in facilities like autoclave or microwave system for disinfection or in an incinerator.

This chapter discusses various infections likely to be caused due to wastes generated in a hospital and how the waste must be managed to avoid or minimize any such infections. The state of affairs vis-a-vis biomedical waste management in few countries across the globe has been also discussed. In the end, an appropriate plan of action for hospital waste management in India and other South-East Asian countries is suggested.

Keywords Healthcare waste • Hospital waste • Biomedical waste • Waste segregation • Colour coded containers • Incinerator • Healthcare waste management plan • Infectious waste • Sharp waste

17.1 Introduction

Hospitals and other health care establishments produce enormous amount of waste. Approximately 80% of hospital waste is of domestic nature and the remaining 20%, which is technically known as bio-medical waste can have adverse effects on human health and environment. This bio-medical waste has to be managed from cradle to grave in a scientific manner to avoid potential risks. Poor management of health-care waste could potentially result in infections/diseases of health-care personnel, waste workers, patients and the general public.

It is estimated that India generates about 400 tonne of infectious biomedical waste per day about one fourth of which is being disposed without proper treatment. Unscientific disposal of healthcare waste may lead to the transmission of communicable diseases such as gastro-enteric infections, respiratory infections, infections of skin, eye and several other organs besides having the potential to spread diseases like HIV-AIDS, Hepatitis B, C, and Tuberculosis which are spread through reuse of contaminated syringes and direct human contact with the blood and infectious body fluids and also through contaminated air and water. Healthcare professionals and the general public are equally at risk that arise due to improper treatment and disposal of biomedical waste, and unhygienic hospital conditions. Rag pickers expose themselves to diseases like Hepatitis B, Tetanus, Septicaemia etc. while collecting needles, surgical gloves and blood bags disposed in municipal bins and dumpsites.

The infectious hospital waste can also be responsible for spread of several other infectious/communicable diseases like Cholera, Tuberculosis, Diphtheria etc. through injuries caused by contaminated needles or sharps and direct contact with the infectious materials. The other hospital acquired diseases include gastroenteritis, infections in respiratory tract and blood streams and skin ailments. According to WHO, the reuse of infectious syringes contributes to about eight million Hepatitis-B, more than 2.3 million Hepatitis-C and about 80,000 HIV infections every year worldwide. There is a wide range of pathogenic micro-organisms associated with

various types of infections caused due to bio-medical waste, thus management of Bio-Medical Waste (BMW) is a global issue today as its mismanagement has adverse effects on environment and human health.

Various efforts have been undertaken nationally and globally to improve upon the condition of healthcare waste management. Some countries have stringent policies pertaining to the subject while many are yet to evolve basic policies and procedures. Several countries have devised various programmes and subsidies to improve on the healthcare waste management situation. The situation of biomedical waste management is more progressive in developed countries than that in rest of the world. New procedures, guidelines and technologies are primarily experimented first in developed countries before being adopted by the developing countries.

17.2 Biomedical Waste Management in United States of America

Medical centres, hospitals and veterinary clinics in the United States generate over one million tons of bio-medical waste each year. In US, Health Care Facilities are categorized based on the amount of medical waste generated monthly and are grouped into two categories: Small Quantity Generators (SQG) and Large Quantity Generators (LQG). LQGs consist of nursing homes, clinics, health departments and laboratories and they are estimated to produce atleast 200 pounds of biomedical waste per day. Physicians, dentists and veterinarians in private practice are classified as SQGs. Although majority of the waste generated at LQGs and SQGs is as harmless as common household waste, as much as 15% of this waste poses a potential infection hazard according to the United States Environmental Protection Agency (USEPA).

17.2.1 History of Healthcare Waste Management Legislation Promulgation

In the US, during the 1980s the public became aware that used syringes and similar other wastes had been found on several East Coast beaches. In response, Congress enacted the Medical Waste Tracking Act (MWTA), which required EPA to create a 2-year medical waste demonstration program. The MWTA:

- Identified which wastes would be regulated
- Established a cradle-to-grave tracking system based on a generator-initiated tracking form (similar to RCRA manifests for hazardous waste)
- Required management standards for segregation, packaging, labelling, and storage of the waste
- Established record-keeping requirements
- Defined penalties that could be imposed for mismanagement

These standards for tracking and management of medical waste were in force in four states (New York, New Jersey, Connecticut, Rhode Island and in Puerto Rico) from June 1989 to June 1991. During this period, EPA also gathered information and performed several studies related to medical waste management. The federal Medical Waste Tracking Act (MWTA) was passed and the EPA attempted to set standards for managing the infectious waste component of medical waste which they renamed as regulated medical waste. The regulations promulgated under the MWTA were expired on June 21, 1999. The MWTA, along with EPA's associated program, served to focus attention on the medical waste issue. It also provided a model that was subsequently used by some states and by other federal agencies in developing their own medical waste management programs (Medical Waste).

17.2.2 Regulatory Authority for Healthcare Waste Management

In US, currently federal and state laws govern the disposal of medical waste, mandating specific methods to sterilize the waste so that the waste does not affect people, animals or the environment in negative ways. However the individual states can make the regulations more stringent than the Federal regulations. Unlike state hazardous waste regulations, which are all based on the federal RCRA standards, state medical waste standards vary diversely. Some state medical waste rules are fashioned after the Medical Waste Tracking Act, while others have little or no resemblance to this historical law. United States Environment Protection Agency no longer plays a central role in medical waste regulation. The states and other federal agencies have taken on that responsibility. Nearly all 50 states have enacted medical waste regulations. In some states, the department of health may play an important role or even serve as the primary regulatory agency. Where two agencies are involved, typically the department of health is responsible for on-site management and the environmental agency is responsible for transportation and disposal.

The U.S. Department of Labour Occupational Safety and Health Administration (OSHA) state program (24 states operate their own program) also regulates several aspects of medical waste, including management of sharps, requirements for containers that hold or store medical waste, labelling of medical waste bags/containers, and employees training. These standards are designed to protect healthcare workers from the risk of exposure to blood borne pathogens. However, they also help to systematically manage wastes, which benefit the public and environment.

In various states with comprehensive medical waste regulations, there are often overlaps between state environmental/health department rules and the OSHA blood borne pathogens standards. However, there are few conflicts. One set of rules may be vague or general, whereas the other set of rules are highly specific. In such cases, healthcare facilities are advised to follow the more detailed or stringent regulations. In states where comprehensive medical waste regulations do not exist, the OSHA rules fill the gap.

17.2.3 Classification of Healthcare Waste

In the US, although there is no universally accepted definition for medical waste, the definitions offered by most regulatory agencies are similar. Most federal and state agencies differentiate between general medical waste and those wastes with the potential for causing infection, for which special precautions are prudent. Depending on the state, these wastes are referred to as:

- regulated medical waste (e.g., New York, Rhode Island)
- infectious waste (e.g., Colorado, Nebraska, Nevada)
- biomedical waste (e.g., Connecticut, Florida, Georgia, Maine, Washington).

Despite the attention given to medical waste by the public and at all levels of government, the terms "hospital waste," "medical waste," "regulated medical waste" and "infectious waste" remain poorly defined. No standard universally accepted definition for these terms exists, and there appears to be as many definitions in use as there are government agencies (local, state and federal) and other groups involved in this issue. Given the diversity of interest and scientific credentials of persons, groups, and agencies (e.g., physicians, health departments, hospitals, environmentalists, trade unions, state, and federal legislators) involved in the medical waste issue, these differences are not surprising. However, because the definition adopted by a regulatory agency dictates what waste will require special handling and treatment, it has serious ramifications.

The Medical Waste Tracking Act of 1988 defines medical waste as "any solid waste that is generated in the diagnosis, treatment, or immunization of human beings or animals, in research pertaining thereto, or in the production or testing of biologicals." This definition includes, but is not limited to:

- blood-soaked bandages
- culture dishes and other glassware
- discarded surgical gloves
- discarded surgical instruments
- discarded needles used to give shots or draw blood (e.g., medical sharps)
- cultures, stocks, swabs used to inoculate cultures
- removed body organs (e.g., tonsils, appendices, limbs)
- discarded lancets

Some state regulations use a general definition, while others list specific wastes and categories of waste that are considered infectious. Some states have adopted the definition found in federal standards (e.g., Nevada adopted the DOT[1] definition). Thus "Hospital waste" (or solid waste) refers to all waste, biological or non-biological, that is discarded and not intended for further use. "Medical waste" refers to materials

[1] DOT = Dept of Transportation.

generated as a result of patient diagnosis, treatment or immunization of human beings or animals. "Infectious waste" refers to that portion of medical waste that could transmit an infectious disease. Congress and the EPA have used the term "regulated medical waste" rather than "infectious waste" in the MWTA in deference to the remote possibility of disease transmission. Thus, "medical waste" is a subset of "hospital waste," and "regulated medical waste," which is synonymous with "infectious waste" from a regulatory perspective (Regulated Medical Waste).

The following six medical wastes are commonly regulated by states:

- Pathological waste: Tissues, organs, body parts, and body fluids removed during surgery and autopsy.
- Human blood and blood products: Waste blood, serum, plasma and blood products.
- Cultures and stocks of infectious agents (microbiological waste): Specimens from medical and pathology laboratories which include culture dishes and devices used to transfer, inoculate and mix. Also it includes discarded live and attenuated vaccines.
- Contaminated sharps: Contaminated hypodermic needles, syringes, scalpel blades, Pasteur pipettes and broken glass.
- Isolation waste: Generated by hospitalized patients isolated to protect others from communicable diseases.
- Contaminated animal carcasses, body parts and beddings. From animals intentionally exposed to pathogens in research, biologicals production or in vivo pharmaceuticals testing.

17.2.4 Segregation, Packaging, Labelling and Storage of Healthcare Waste

In some states, segregation at the point of origin is required by law (e.g., Michigan, Montana, and South Carolina) while in some other states, it is highly recommended (e.g., Vermont). For packaging and labelling of regulated medical waste, specific rules vary from state to state, but in general, they are designed to safely contain the waste during storage and transport and to alert employees and the public to the potential hazard.

Labelling is typically required prior to on-site storage or off-site transport. Generally, regulated medical waste other than sharps and bulk liquids are recommended to be packaged in sealed red bags which are leak proof and rip resistant. Bulk liquids to be transported off-site in unbreakable flasks or bottles. In addition, all biomedical wastes transported off-site are recommended for packaging in rigid containers.

Some states have very specific guidelines regarding storage areas and storage time limits. There are often no maximum time limits for storage of biomedical waste. However, the waste must be maintained in a nonputrescent state, using refrigeration as and when necessary and vermin and insects must be controlled.

Where storage time limits exist, they are in the range of 7 days (New York) to 90 days (Arizona). Most states do not require permits for waste storage (Regulated Medical Waste).

17.2.5 Treatment and Disposal of Healthcare Waste

In most states, regulated medical waste must be treated before it can be disposed of. Such treatment can be performed on-site or at an approved facility. Regulated medical waste treated on-site to render it non-infectious is usually no longer considered infectious for handling and disposal purposes and in some cases, it may be mixed with and disposed off along with ordinary waste when certain rules are followed. However, many states require that treated waste remain segregated and often written notification must accompany the treated waste to its final disposal location.

All states allow healthcare facilities to treat regulated medical waste on-site when an approved method of treatment is used. Some states require permits or operating plans for the treatment units, while others only require air pollution permits for incineration units. Approved methods usually include incineration, steam sterilization (autoclaving), microwave sterilization and chemical disinfection. Most states also have a process for granting permission to use new or alternative methods of treatment. This typically involves a petition process. Some states do not approve or recommend any specific treatment methods, but leaves it up to the generator to determine what is an appropriate and effective treatment method for their wastes. Liquids, including blood but excluding chemical wastes, may be discharged into the sewer in many states, although local sanitary district approval is usually required.

In the US, the primary methods of treatment and disposal of medical waste are:

- Incineration
- Autoclaving
- Mechanical/Chemical Disinfection
- Microwaving
- Irradiation

According to the EPA, 90% of medical waste is incinerated. The incineration process can be applied to almost all medical waste types including pathological waste, and the process reduces the volume of the waste by up to 90%. While incineration provides the advantage of reducing the volume of waste into ash and the ability to dispose recognizable waste and sharps, the incinerator may contain toxic gases. The EPA has stringent requirements on emissions from medical incinerators, however the largest concern associated with incineration is pollution generated by the incineration of chemicals that are released from combusting plastics as at least 20% of medical waste is plastic. Dioxins and furans can be produced when these plastics burn. Another concern is related to the contents of incinerator ash. As incinerators are designed or retrofit with pollution prevention equipment, more of the

potentially toxic chemicals that previously ended up in emissions now remain in the ash. Incinerator ash is generally disposed of in landfills and little data is available on the effects of ash on the environment.

It is expected that as the new federal and state emission regulations are instituted having more stringent requirements, over the time, the amount of waste being incinerated will be reduced as other technologies replace on-site incinerators. As additional requirements are added to the emissions for medical waste incinerators, the cost of incinerating medical waste increases and alternative treatments have increased their market share.

For all of these treatment types, the treated waste can generally be disposed with general waste in a landfill or in some cases discharged into the sewer system. In the past, treatment of medical waste was primarily performed on-site at hospitals in dedicated medical waste facilities. Over the time, the expense and regulation of these facilities have prompted organizations to hire private companies to collect, treat and dispose of medical waste, and the percentage of medical institutions performing their own treatment and disposal is expected to drop (Basura Medical Waste Resource, http://www.wastemed.com/treatment.htm).

17.3 Biomedical Waste Management in Canada

Canada's various human and animal health care facilities generate over 40,000 tonnes of biomedical waste annually. While this is only a fraction of the six million tonnes of hazardous waste that are produced each year, concerns about the environment and the transmission of communicable diseases like AIDS have increased the need for proper handling and disposal of these wastes.

In 1989, The Canadian Council of Ministers of Environment (CCME) set out to address the significant differences in regulating and managing biomedical waste across Canada through development of the national guidelines. CCME worked on developing minimum national standards for handling, treating and disposing of biomedical waste. These guidelines relate to all aspects of biomedical waste management which includes waste reduction, segregation, collection, containment, in-house movement, storage, transportation, treatment and disposal (both on-and off-site) and occupational health and safety issues.

17.3.1 Regulatory Authority for Healthcare Waste Management

Most Canadian provinces have prepared or are considering preparing guidelines or regulations dealing with the management of biomedical waste and the local regulatory authorities are the monitoring and supervising agencies for overseeing the status of biomedical waste management in their respective jurisdictions.

Since the Canadian provinces have their respective guidelines for management of biomedical waste, diversity is observed in the guidelines prescribed at each of the province which clearly indicates a need to seek consistent national solutions to the same problems. While some regional differences may be justified, a single uniform approach to the problem would considerably benefit the Canadian health care system, both practically and in terms of regulations.

17.3.2 Classification of Healthcare Waste

Reflecting the need to have a single national definition for biomedical waste, the following definition is prescribed under the national guidelines rolled out by CCME. (This definition does not apply to microbiology laboratory waste, human blood and body fluid waste or waste sharps after these wastes have been disinfected or decontaminated.)

Biomedical waste refers to waste that is generated by:

- Human or animal health care facilities;
- Medical or veterinary research and teaching establishments;
- Health care teaching establishments;
- Clinical testing or research laboratories; and,
- Facilities involved in the production or testing of vaccines.

(a) Human Anatomical Waste
This consists of human tissues, organs and body parts, but does not include teeth, hairs and nails.

(b) Animal Waste
This consists of all animal tissues, organs, body parts, carcasses, beddings, blood and blood products, items saturated or dripping with blood, body fluids contaminated with blood and body fluids removed for diagnosis or removed during surgery, treatment or autopsy, unless a trained person has certified that the waste does not contain the viruses and agents listed in Risk Group. This excludes teeth, hair, nails, hooves and feathers.

(c) Microbiology Laboratory Waste
This consists of laboratory cultures, stocks or specimens of microorganisms, live or attenuated vaccines, human or animal cell cultures used in research and laboratory materials that has come into contact with any of these.

(d) Human Blood and Body Fluid Waste
This consists of human body fluid, blood and blood products, items saturated or dripping with blood, body fluids contaminated with blood and body fluids removed for diagnosis during surgery, treatment or autopsy. This does not include urine or faeces.

(e) Waste Sharps
Waste sharps are clinical and laboratory materials consisting of needles, syringes, blades or laboratory glass capable of causing punctures or cuts.

Biomedical waste does not include waste that is:

- from animal husbandry;
- household in origin;
- controlled in accordance with the Health of Animals Act (Canada), formerly the Animal Diseases Protection Act (Canada); or,
- Generated in the food production, general building maintenance and office administration activities of those facilities to which this definition applies.

(Guidelines for the Management of Biomedical Waste in Canada, pp 3–4).

17.3.3 Segregation, Packaging, Labelling and Storage of Healthcare Waste

The national guidelines mandate segregation of waste irrespective of method of disposal. The guidelines further state that biomedical waste must be segregated at the point of generation into the following waste categories:

- human anatomical waste;
- animal waste;
- microbiology laboratory waste;
- human blood and body fluid waste; and
- waste sharps.

Although not considered a biomedical waste, cytotoxic wastes and pharmaceutical wastes must also be segregated from the remainder of the waste stream.

For packaging, the guidelines prescribe that the packaging must remain intact throughout handling, storage, transportation and treatment. Further the guidelines mention that while selecting packaging, the following factors should be considered:

- the type of waste being contained;
- appropriate colour-coding and labelling;
- special transportation requirements;
- the method of disposal;
- local regulatory requirements; and
- Requirements of the disposal facility.

For the storage of biomedical waste, the national guidelines specify that the containers for biomedical waste must be colour-coded and labelled with the biohazard symbol. The storage areas must be totally enclosed and separate from supply rooms or food preparation areas. They must be lockable and access must be restricted to authorized personnel only. Storage areas must be identified as containing biomedical waste with the biohazard symbol clearly displayed. It is unacceptable for materials other than waste to be placed in the same storage area as biomedical waste.

Further, the guidelines specify that floors, walls and ceilings of storage areas must be thoroughly cleaned in accordance with the facility's established procedures. These procedures should be prepared in consultation with the facility's infection control committee, biosafety officer, or other appointed person(s).

Regarding storage, the guidelines prescribes that all biomedical waste must be refrigerated at 4°C or lower if stored for more than 4 days. Facilities refrigerating or freezing stored waste should use a lockable, closed cold storage facility or a lockable, domestic type freezer unit. Either type must be used only for storing biomedical waste, should visibly display the biohazard symbol and be identified as containing biomedical waste (Guidelines for the Management of Biomedical Waste in Canada, pp 6, 8–13).

17.3.4 Treatment and Disposal of Healthcare Waste

The treatment options described in the national guidelines may vary among provinces for acceptable practices based upon the principles presented in the guidelines. In Canada, the primary methods of treatment and disposal of medical waste are:

- **Steam autoclaving**: steam autoclaving is suggested for treating microbiology laboratory waste, human blood and body fluid waste (if applicable), waste sharps and non-anatomical animal wastes.
- **Chemical decontamination**: the guidelines suggest that chemical decontamination may be appropriate for treating microbiology laboratory waste, human blood and body fluid waste (if applicable) and waste sharps. Chemical decontamination is most often applied to liquid wastes before disposal and decontamining spills when they occur.
- **Incineration**: incineration has traditionally been the principal method used by health care facilities to process their anatomical and non-anatomical biomedical wastes. As per the guidelines, human anatomical waste consisting of human tissues, organs and body parts (but excluding teeth, hair and nails) must be incinerated in a biomedical waste incinerator meeting the stack discharge limits.
- **Landfilling**: as per the guidelines, landfilling is technically acceptable method to dispose of some types of decontaminated biomedical waste. Although the guidelines have recommended protocols for handling of decontaminated biomedical wastes at landfill sites, in some locations of Canada, local regulatory authorities or landfill operators specify more stringent standards.
- **Disposal in sewer**: the sanitary sewer system is an acceptable method of disposal for untreated fluid blood, suctioned fluids, excretions and secretions.

(Guidelines for the Management of Biomedical Waste in Canada, pp 14–20).

17.4 Biomedical Waste Management in Pakistan

Like rest of the world, Pakistan is also facing the problem of mismanagement of biomedical waste. In recent years, medical waste disposal has posed even more difficulties with the appearance of disposable needles, syringes and other similar items. Studies show that large hospitals generate around 2 kgs of waste per bed per day of which 0.5 kgs can be categorised as biomedical risk waste. Thus around 250,000 tonne of medical waste is annually produced from all sorts of health care facilities of Pakistan. This type of waste has a bad effect on the environment by contaminating the land, air and water resources (Hospital Waste Management Issues & Steps taken by the Government of Pakistan: Oct 2006).

To tackle the issue of hospital waste management, Govt of Pakistan have developed Hospital Waste Management Rules 2005, Guidelines on Hospital Waste Management, Specifications and Guidelines on Incinerators and Training Manuals for Paramedics.

17.4.1 History of the Legislation

Environmental Health Unit of the Ministry of Health, Government of Pakistan prepared Guidelines on Hospital Waste Management in 1998 covering all aspects of safe hospital waste management in the country including the risk associated with the waste, formation of a waste management team in hospitals, their responsibilities, plan, collection, segregation, transportation, storage, disposal methods, containers and their colour coding, waste minimisation techniques, protective clothing etc. Subsequently Hospital Waste Management Rules, 2005 were enacted in Islamabad on 3rd of August, 2005.

17.4.2 The Legislation

United States Agency for International Development (USAID) did an analysis of the hospital waste management legislation of Pakistan in the document prepared as a part of the project 'Hospital Waste Management and Environmental Assessment in Pakistan's selected facilities: Guidelines for safe and environmental management'. An assessment of these regulations identifies the following comments:

- In clause 2 "Definitions", it is written: "hospital waste" includes both risk waste and non-risk waste. It would be convenient to analyze the possibility of specifying "…risk waste and non-risk waste generated by a hospital", given that a definition of "hospital" is included.
- In the same clause 2 "Definitions": "waste management" includes waste segregation, waste collection, waste transportation, waste storage, waste disposal and

waste minimization and reuse. It would be convenient to analyze the possibility of including waste treatment also.

- In clause 14, "Duties and responsibilities of the Waste Management Officer", waste treatment is not included specifically.
- The legislation does not cope with waste treatment options. In some paragraphs incineration is mentioned, and in others autoclave is mentioned as a special treatment.
- In clause 16, "Waste segregation", there is a paragraph describing characteristics of plastic bags for risk waste: "All risk waste shall be placed in a suitable container made of metal or tough plastic, with a pedal type or swing lid, lined with a strong yellow plastic bag". "Strong" is a qualitative word. It would be better to specify the thickness of the yellow plastic bag.
- In the same clause 16, it is written: "Sharps including the cut or broken syringes and needles shall be placed in metal or high-density plastic containers resistant to penetration and leakage, designed so that items can be dropped in using one hand, and no item can be removed". It is recommended to write "…and needles shall be placed in rigid, puncture proof containers (cardboard or high-density plastic containers) resistant to penetration and leakage…" Most of rigid containers for sharps are made of cardboard or plastic materials. If plastic material is chosen, it should be other than PVC.
- Another paragraph in clause 16 specify: "Large quantities of chemical waste, and waste with a high content of mercury or cadmium shall not be incinerated, but shall be placed in chemical resistant containers and sent to specialized treatment facilities." It does not say anything related to places where there are no specialized treatment facilities.
- For non-risk waste Pakistani legislation specifies the use of a white plastic bag: "Non-risk waste shall be placed in a suitable container lined with a white plastic bag". WHO guidelines recommend black plastic bags (they are easier to find and cheaper).
- Regarding clause 19, "Waste storage", it addresses only risk waste storage: "No materials other than yellow-bagged waste shall be stored in the central storage facility". It does not say anything regarding non-risk waste storage. Non-risk waste also needs to be stored with similar precautions, but in a separated environment from hazardous waste. Furthermore, concerning distance from waste storage facility and other facilities:

The designated central storage facility shall –

(a) be located within the hospital premises close to the incinerator, if installed, but away from food storage or food preparation areas.

"Away" is a qualitative word. The statement does not specify the appropriate distance from waste storage facility and food storage, food preparation area or internal facilities. Distances could be 30 m from food storage or preparation area and 15 m from internal hospital facilities.

- Regarding waste disposal (clause 20):
  - It is written: "Yellow-bagged waste shall be disposed of by burning in an incinerator or by burial in a landfill or by any other method of disposal approved by the Federal Agency or Provincial Agency concerned". Incineration is a treatment method; it is not a final disposal method since incineration generates also some residues to deal with (ashes, gases, particulate matter, among others).
  - Another paragraph is: "Landfills shall be located at sites with minimal risk of pollution of groundwater and rivers. Access to the site shall be restricted to authorized personnel only. Risk waste shall be buried in a separate area of the landfill under a layer of earth or non-risk waste of at least one meter depth which shall then be compacted". Nothing is written about the meaning of "minimal risk". More specific parameters are needed, unless there is another Pakistani regulation about criteria for a sanitary landfill location.

(Hospital waste management & environmental assessment in Pakistani selected facilities – Guidelines for Safe and Environmental Management: Report of JSI – PAIMAN Project, with the support of USAID. December, 2006, pp 14–18).

17.5 Biomedical Waste Management in the Philippines

Philippine hospitals are estimated to generate upto 10,290 tons of waste each year. The common practice in the past was to simply dump all forms of waste together, from reception-area trash to operating-room waste, and burn them in incinerators. The study on waste management practices of hospitals in Metro Manila conducted by Victorio Molina for the Department of Health (DOH) revealed that although most of the hospitals perform waste segregation, less than 50% of the 144 hospitals studied did not have the proper mechanisms for proper waste handling and segregation. Incineration and landfill were used for final disposal of wastes (Health Care Without Harm Asia, 2007, pp 4–6).

17.5.1 History of Promulgation of Legislation

In 2001, the Department of Health (DOH), through its collaboration with different organizations and agencies, revised its Health Care Waste Management Manual to follow the policies stipulated under the Philippine Clean Air Act and the Ecological Solid Waste Management Act of 2000. The manual provides health care facilities with practical guidelines in establishing Health Care Waste Management Programs and promotes the use of alternative technologies for the disposal of health care wastes.

17.5.2 Classification of Healthcare Waste

The manual on healthcare waste management categorised healthcare waste into following:

1. *General Waste* – Comparable to domestic waste, this type of waste does not pose special handling problem or hazard to human health or to the environment. It comes mostly from the administrative and housekeeping functions of health care establishments and may also include waste generated during maintenance of health care premises. General waste should be dealt with by the municipal waste disposal system.
2. *Infectious Waste* – This type of waste is suspected to contain pathogens (bacteria, viruses, parasites or fungi) in sufficient concentration or quantity to cause disease in susceptible hosts. This includes:

    - Cultures and stocks of infectious agents from laboratory work;
    - Waste from surgery and autopsies on patients with infectious diseases (e.g. tissues, materials or equipment that have been in contact with blood or other body fluids);
    - Waste from infected patients in isolation wards (e.g. excreta, dressings from infected or surgical wounds, clothes heavily soiled with human blood or other body fluids);
    - Waste that has been in contact with infected patients undergoing haemodialysis (e.g. dialysis equipments such as tubings and filters, disposable towels, gowns, aprons, gloves and laboratory coats);
    - Infected animals from laboratories; and
    - Any other instruments or materials that have been in contact with infected persons or animals.

3. *Pathological Waste* – Pathological waste consists of tissues, organs, body parts, human foetus and animal carcasses, blood and body fluids. Within this category, recognizable human or animal body parts are also called anatomical waste. This category should be considered as a subcategory of infectious waste, even though it may also include healthy body parts.
4. *Sharps* – Include needles, syringes, scalpels, saws, blades, broken glass, infusion sets, knives, nails and any other items that can cause a cut or puncture, wounds etc. Whether or not they are infected, such items are usually considered as highly hazardous health care waste.
5. *Pharmaceutical waste* – Includes expired, unused, split and contaminated pharmaceutical products, drugs, vaccines and sera that are no longer required and need to be disposed of appropriately. This category also includes discarded items used in handling of pharmaceuticals such as bottles or boxes with residue, gloves, masks, connecting tubing and drug vials.
6. *Genotoxic Waste* – Genotoxic waste may include certain cytotoxic drugs, vomit, urine or faeces from patients treated with cytotoxic drugs, chemicals and radioactive materials. This type of waste is highly hazardous and may have mutagenic, teratogenic or carcinogenic properties.

Cytotoxic wastes are generated from several sources and include the following:

- Contaminated materials from drug preparation and administration such as syringes, needles, gauges, vials, outdated drugs, excess (left over) solutions and drugs returned from the wards;
- Urine, faeces and vomit from patients which may contain potentially hazardous amounts of the administered cytotoxic drugs or of their metabolites and which should be considered genotoxic for at least 48 hrs and sometimes up to 1 week after drug administration.

7. *Chemical Waste* – Chemical waste consists of discarded solid, liquid and gaseous chemicals for example from diagnostic and experimental work and from cleaning, housekeeping and disinfecting procedures. Chemical waste from health care may be hazardous or non-hazardous. Chemical waste is considered hazardous if it has at least one of the following properties:

   - Toxic
   - Corrosive (e.g. acids of pH < 2 and bases of pH > 12)
   - Flammable
   - Genotoxic (e.g. cytostatic drugs)

   Non-hazardous chemical waste consists of chemicals with none of the above properties such as sugars, amino acids, and certain organic and inorganic salts.
8. *Waste with high content of heavy metals* – Wastes with a high heavy-metal content represent a subcategory of hazardous chemical waste and are usually highly toxic. Mercury wastes are typically generated by spillage from broken clinical equipments (thermometers, blood pressure gauges, etc.). Residues from dentistry have high mercury content. Cadmium waste comes mainly from discarded batteries. Certain "reinforced wood panels" containing lead is still being used in radiation proofing of X-Ray and diagnostic departments. A number of drugs contain arsenic but these are treated as pharmaceutical waste.
9. *Pressurized Containers* – Many types of gases are used in health care and are often stored in pressurized cylinders, cartridges and aerosol cans. Many of these, once empty or of no further use (although they may still contain residues), are reusable but certain types (notably aerosol cans), must be disposed of. Whether inert or potentially harmful gases in pressurized containers should always be handled with care as the containers may explode if incinerated or accidentally punctured.
10. *Punctured Waste* – Includes disused sealed radiation sources, liquid and gaseous materials contaminated with radioactivity, excreta of patients who underwent radio-nuclide diagnostic and therapeutic applications, paper cups, straws, needles and syringes, test tubes and tap water washings of such paraphernalia. Radioactive health care wastes generally contain radionuclides with short half-lives, which lose their activity in a shorter time. However, certain radionuclides

Table 17.1 Colour coding for waste segregation

Color of container/bag	Type of waste
Black	Non-infectious dry waste
Green	Non-infectious wet waste (kitchen, dietary etc.)
Yellow	Infectious and pathological waste
Yellow with black band	Chemical waste including those with heavy metals
Orange	Radioactive waste
Red	Sharps and pressurized containers

Health Care Waste Management Manual, Dept of Health, Manila, pp 23–27

e.g. C-14 contaminated wastes, have much longer half-life, more than a thousand years, which need to be specially managed in a centralized treatment facility for radioactive wastes. The same is required for the management of disused sealed radiation sources used for cancer treatment.

(Health Care Waste Management Manual, Dept of Health, Manila, pp 10–12).

17.5.3 Waste Segregation and Storage

The manual mandates segregation of waste and mentions that segregation should take place as close as possible to where the waste is generated and should be maintained in storage areas and during transport. The colour coding scheme prescribed for healthcare waste is given (Table 17.1).

17.5.4 Health Care Waste Treatment Technologies and Processes

Incineration used to be the method of choice in treating health care waste. However, with the implementation of the Clean Air Act of 1999, the use of this method is no longer allowed. With this development, alternative technologies are being looked into to address the problem of health care waste management using the aforementioned selection criteria.

Most common technologies and processes used in health care waste treatment are (1) thermal, (2) chemical, (3) irradiation, (4) biological processes, (5) encapsulation, and (6) inertization.

1. *Thermal Processes*: Thermal processes rely on high heat to destroy pathogens (disease-causing microorganisms).

- Pyrolysis – Pyrolysis is the thermal decomposition of substance and materials in the absence of supplied molecular oxygen in the destruction chamber in which the said material is converted into gaseous, liquid or solid form. Pyrolysis can handle the full range of health care waste. Waste residues may be in the form of greasy aggregates or slugs, recoverable metals or carbon black. These residues are disposed off in a secured facility i.e., sanitary landfill.
- Wet and Dry Thermal Treatment – Wet thermal or steam disinfection is based on exposure of shredded infectious waste to high temperature, high pressure steam and is similar to the autoclave sterilization process. It inactivates most types of microorganisms if temperature and contact time are sufficient. For sharps, milling or crushing is recommended mainly to eliminate physical hazards from needles rendering syringes unusable and reducing the waste volume.
- Autoclave – Autoclave uses steam sterilization to render waste harmless and is an efficient wet thermal disinfection process. This technique has been used for many years in hospitals for the sterilization of reusable medical equipment.
- Microwave – This technology typically incorporates some type of size reduction device. Shredding of wastes is being done either before disinfection or after disinfection. In this process, waste is exposed to microwaves that raises the temperature to 100°C (237.6°F) for atleast 30 min. Microorganisms are destroyed by moist heat which irreversibly coagulates and denatures enzymes and structural proteins.

2. *Chemical Disinfection*: Chemical disinfection is now being applied for treatment of health care waste. Chemicals like aldehydes, chlorine compounds, phenolic compounds, etc. are added to the waste to kill or inactivate pathogens present in health care waste. Chemical disinfection is most suitable in treating blood, urine, stools and sewage. This method is also applicable in treating infectious wastes containing pathogens. However, application of this method is prescribed only when there is no available treatment facility in the area to prevent environmental problems associated with the disposal of chemical residues.

   Studies showed that chlorine-based technologies using sodium hypochlorite and chlorine dioxide as well as its by-products in wastewater may possibly have long-term environmental effects. Non-chlorine based technologies are quite varied in the way they operate and the chemical agents they employ. Others use peroxyacetic acid, ozone gas, lime-based dry powder, acid and metal catalyst or biodegradable disinfectants. Also, occupational and safety exposures should are required to be monitored when using the chemical processes.
3. *Biological Processes*: The process uses an enzyme mixture to decontaminate health care waste and the resulting by-products are put through an extruder to remove water for sewage disposal. The technology is suited for large applications

and is also being developed for possible use in agricultural sector. The technology requires regulation of temperature, pH, enzyme level and other variable.

Composting and vermiculture as biological processes for treating and disposing of placenta waste as well as food waste, yard trimmings and other organic waste is also recommended.

Radiation Technology: The disposal of biologically contaminated waste from hospitals, clinics and laboratories is of particular concern. Waste containing potentially infectious microorganisms (sewage sludge, biomedical wastes, wastewater) are treated using irradiation systems which are currently being used in waste treatment operations.

4. *Encapsulation*: Encapsulation involves filling up of containers with waste, adding and immobilizing the material and sealing the containers. The process uses either cubic boxes made of high-density polyethylene or metallic drums that are three quarters filled with sharps or chemical or pharmaceutical residues. The containers or boxes are then filled up with a medium such as plastic foam, bituminous sand and cement mortar. After the medium has dried, the containers are sealed and disposed of in landfill sites. The process is particularly appropriate for the disposal of sharps and chemical or pharmaceutical residues. The main advantage of the process is that it is very effective in reducing the risk of scavengers gaining access to the health care waste.
5. *Inertization*: It is the process that involves mixing of waste with cement and other substances before disposal. This method is specially suitable for pharmaceutical waste. This process minimizes the risks of toxic substances contained in the waste migrating into surface water or groundwater. The process is relatively inexpensive and can be performed using relatively unsophisticated equipment.

(Health Care Waste Management Manual, Dept of Health, Manila, pp 40–43).

17.6 Biomedical Waste Management in India

It is estimated that India generates about 405,702 kg s/day of biomedical waste from 129,511 Health Care Facilities (HCFs) out of which only about 291,983 kg s/day is treated and disposed of. There are 168 Common Bio-Medical Waste Treatment and Disposal Facilities (CBWTFs) in operation while 17 CBWTFs are under construction. About 20,670 HCFs are having their own treatment and disposal facilities and 95,410 HCFs are using the services of CBWTFs. (Source: CPCB Data 'Status of biomedical waste management in the country as submitted by SPCBs/PCCs[2] for the year 2009)

[2]SPCBs = State Pollution Control Boards/ PCCs = Pollution Control Committees of Union Territories.

17.6.1 History of Healthcare Waste Management Legislation Promulgation

Prior to 1990s, the waste from healthcare institutions was thrown in municipal dumps or on road sides to be disposed off along with general waste. The concept of biomedical waste management evolved in India in late 1990s when the Public Interest Litigation (PIL) was filed in the Supreme Court which led to promulgation of the Bio Medical Waste (Management and Handling) Rules, 1998 by the Union Ministry of Environment and Forests (MoEF), mandating all HCFs, irrespective of their size, to segregate, store, transport, treat and properly dispose of the Bio-Medical Waste (BMW) generated by them in accordance with the provisions of these Rules. This was done in exercise of the power conferred on the Government of India by sections 6, 8 and 25 of the Environment (Protection) Act 1986[3]. The BMW Rules along with subsequent amendments notified on 6th March 2000, 2nd June 2000 and 17th September 2003 are welcome steps towards improving the overall status of waste management in healthcare units in India. The Rules are applicable throughout India and the various guidelines published by Central Pollution Control Board (CPCB), Ministry of Health and Family Welfare (MoHFW) and State Pollution Control Boards are to be read in conjunction with the Rules.

17.6.2 The Legislation

The Biomedical Waste (Management and Handling) Rules were notified in 1998 to provide a regulatory framework on management of wastes generated by Health Care Establishments (HCEs) all over the country. Under these Rules, the wastes generated by HCEs are categorized into ten categories and disposal methods for all the categories of wastes are also specified. Standards are also prescribed for treated effluents, air emissions and the treatment technologies to be adopted under these Rules.

Apart from MoEF, the Ministry of Health and Family Welfare, Government of India (MoHFW) has also laid down the National Guidelines on Hospital Waste Management in March 2002, which apart from covering the aspects included in the Bio-Medical Waste Rules, also include guidelines for safety measures, training, management and administrative functions.

The MoHFW has also commissioned the development of a National Policy document to address the issues relating to infection control and waste management and

[3]The Environment (Protection) Act of 1986 is an umbrella legislation providing a single focus for the protection of environment and seeks to plug the loopholes of various earlier legislations relating to the environment. Several sets of rules relating to various aspects of management of hazardous waste/chemicals, municipal solid waste, micro-organisms, biomedical waste etc., have been notified under this Act. It is an Act to provide for the protection and improvement of environment and for matters related thereto.

defined a framework for implementation of an Infection Management and Environment Plan (IMEP) in healthcare facilities. The purpose of the IMEP is to ensure that all parties/stakeholders recognize that the maintenance of sanitary conditions, use of appropriate disinfection and sterilization techniques, provision of potable water and clean air in the healthcare facilities and nosocomial infection control are the basic infrastructural requirements for delivery of Reproductive and Child Health (RCH) services. The IMEP identified the key issues and has listed out the key interventions that need to be made in order to ensure safety to healthcare service providers, the patients, the general public and the environment. These interventions range from formulating composite national guidelines on infection management to building awareness and capacity at all levels to implement these guidelines. The plan contains a time bound action plan and a broad institutional framework to enable its implementation. This policy document was commissioned under the Reproductive and Child Health Programme Phase – II, with technical and financial support from DFID and the World Bank. The IMEP Guidelines are implemented and monitored under the auspices of the [4]National Rural Health Mission (NRHM) and go a long way to internalise state-of-the-art, best practices in managing health and environment risks in the healthcare institutions of India.

17.6.3 Regulatory Authority for Healthcare Waste Management

The Ministry of Environment and Forests (MoEF), Govt of India is the nodal agency in the administrative structure of the Central Government for planning, promotion, co-ordination and overseeing the implementation of India's environmental and forestry policies and programmes. The Hazardous Substances Management Division (HSMD) of the MoEF is the nodal point within the Ministry for management of chemical emergencies and hazardous substances. The major functions of the HSMD include regulatory activities for framing necessary Rules relating to environmentally sound management of hazardous wastes/chemicals, plastics, bio-medical waste and

[4]Recognizing the importance of Health in the process of economic and social development and improving the quality of life of citizens, the Government of India has resolved to launch the National Rural Health Mission to carry out necessary architectural correction in the basic health care delivery system. The Mission adopts a synergistic approach by relating health to determinants of good health viz. segments of nutrition, sanitation, hygiene and safe drinking water. It also aims at mainstreaming the Indian systems of medicine to facilitate health care. The Plan of Action includes increasing public expenditure on health, reducing regional imbalance in health infrastructure, pooling resources, integration of organizational structures, optimization of health manpower, decentralization and district management of health programmes, community participation and ownership of assets, induction of management and financial personnel into district health system, and operationalizing community health centres into functional hospitals meeting Indian Public Health Standards in each Block of the Country.

municipal solid wastes under the Environment (Protection) Act, 1986 and promotional activities by providing necessary financial assistance to the concerned agencies for their implementation.

As per the BMW Rules, 1998, the prescribed authorities for enforcement of the provisions of the Rules are State Pollution Control Boards (SPCBs) in respect of States and the Pollution Control Committees (PCCs) in respect of the Union Territories. The prescribed authority functions under the supervision and control of the respective state government and union territory administration. The status of implementation of these Rules is regularly monitored by the concerned SPCBs/PCCs.

17.6.4 Classification of Healthcare Waste

The Bio Medical Waste (Management and Handling) Rules, 1998 describes biomedical waste as any waste, which is generated during the diagnosis, treatment or immunisation of human beings or animals or in research activities pertaining thereto or in the production or testing of biologicals. The biomedical waste is classified into ten categories namely:

- Human Anatomical Waste: Human tissues, organs, body parts
- Animal Waste: Animal tissues, organs, body parts, carcasses, bleeding pars, fluids, blood, experimental animals used in research, waste generated by veterinary hospitals/colleges and animal houses.
- Microbiology and Biotechnology waste: Waste from laboratory cultures, stocks or specimens of micro-organisms, live or attenuated vaccines, human and animal cell cultures used in research and infectious agents from research and industrial laboratories, waste from production of biologicals, toxins, dishes and devices used for transfer of cultures.
- Waste sharps: Needles, syringes, scalpels, blades, glass [broken and unbroken], etc. that may cause punctures and cuts. This includes both used and unused sharps.
- Discarded medicines and cytotoxic drugs: Wastes comprising of outdated, contaminated drugs and discarded medicines.
- Soiled waste: Items contaminated with blood, body fluids including cotton, dressing, soiled plaster casts, linen, beddings, other materials contaminated with blood.
- Infectious Solid Waste: Waste generated from disposable items other than waste sharps such as catheters, intravenous sets, etc.
- Liquid Waste: Waste generated from laboratory, washing, cleaning, housekeeping and disinfecting activities.
- Incineration Ash: Ash from incineration of biomedical waste.
- Chemical Waste: Chemicals used in production of biologicals, chemicals used in disinfection and as insecticides, etc.

17.6.5 Segregation, Packaging, Labeling and Storage of Healthcare Waste in India

The BMW Rules 1998 focuses on segregation of waste at source and hence provides detailed segregation mechanism.

For packaging, the Rules specify 4 different types of colour codes for the ten categories of biomedical waste. The Rules further specifies use of non-washable and prominently visible label for waste containers/bags. Rules also imply that the liquid waste generated from laboratory, washing, cleaning, house-keeping and disinfecting activities at the healthcare facility do not require storage in containers/bags and should be disinfected by chemical treatment and if necessary after treatment meet the prescribed standards may be in an effluent treatment plant and discharged into drains.

As per the BMWM Rules 1998 and the guidelines provided by Central Pollution Control Board (CPCB), the biomedical waste shall not be stored for more than 48 h and every healthcare unit has to designate a temporary storage area. The treatment facility should also have sufficient ventilated storage space for untreated and treated bio-medical waste.

17.6.6 Treatment and Disposal of Healthcare Waste

BMW Rules 1998 stipulate that every HCF has to set up the requisite biomedical waste treatment facilities like incinerator, autoclave or microwave system for the treatment of waste, or should ensure requisite treatment of waste at a common waste treatment facility or any other waste treatment facility. Installation of individual treatment facilities by every healthcare unit requires comparatively high capital investment and thus is not a viable option. In addition, it requires separate trained personnel and infrastructure for proper operation and maintenance of treatment systems. The concept of Common Bio-medical Waste Treatment Facility (CBWTF) not only addresses such problems but also prevents proliferation of treatment facilities including incinerators in a city, which are a source of pollution and infection if not maintained properly. Further, the CBWTFs reduce the monitoring pressure on regulatory agencies.

The Bio-Medical Waste (Management and Handling) Rules, 1998 has elaborately provided the recommended treatment and disposal options for the ten different categories of wastes generated in health care establishments in Schedule I of the Rules. Standards for the treatment technologies are given in Schedule V of the Rules, which must be complied with. The environmental regulations actually mandate the treatment of infectious medical waste within 48 h of its generation. A number of treatment methods are available and it is recommended that the final choice of a suitable treatment method must be made carefully, on the basis of various factors, many of which depend on local conditions including the quantity

and composition of waste generated, available space, regulatory approval, public acceptance, cost, etc. A review of the different technologies for treatment of bio-medical waste as prescribed in the Rules show that there is no single technology, which can take care of all categories of bio-medical waste. A judicious package has to be evolved for this purpose. For example, small and medium hospitals can opt for local (in house) disinfection, mutilation/shredding and dedicated autoclaving plus off-site incineration at a common treatment/disposal facility followed by disposal in sanitary and secured landfills.

As per the provisions of Bio-medical Waste (Management and Handling) Rules, waste falling in most of the categories can be treated in systems based on non-burn technologies. Such waste account for about 90% of the total waste streams in a healthcare unit. It is mandatory to impart incineration/deep burial (depending upon the population of town or rural or urban area) to anatomical and other types of wastes falling under categories 1 and 2. Therefore, an incinerator of adequate capacity to cater only to categories 1 and 2 waste may be installed. The wastes falling under category 5 i.e. discarded medicines, cytotoxic drugs and category 10 i.e. chemical wastes (solids) can be disposed in a secured landfill. (If secured landfill is not available, category 5 may also be incinerated.)

As per the BMWM Rules and CPCB Guidelines, a Common Bio-medical Waste Treatment Facility (CBWTF) shall have following treatment facilities:

- Incinerator
- Autoclave/Microwave/Hydroclave
- Shredder
- Sharp pit/Encapsulation
- Effluent Treatment Plant
- Secured Landfill

17.7 Salient Features of Bmwm Rules, 1998

The Bio Medical Waste (Management and Handling) Rules, 1998 is a comprehensive legislation detailing each and every aspect of hospital waste management. The Rules prescribe a simple four colour bin system for segregation of the BMW, as follows:

(i) Yellow bin with yellow bag shall be used for collecting dressings, plaster, bandages and all the non-plastic materials contaminated with blood, body fluids, body parts, human organs and culture plates including pathological and bacteriological wastes, and the same shall be sent for incineration.
(ii) Red bin with red bag shall be used for collecting catheters, IV bottles, tubes, blood/urine bags and all other plastic materials and disposables contaminated with body fluids, and the same shall be sent for microwaving/autoclaving and then shredded for sending the same for recycling purpose.

(iii) Blue/white puncture-proof containers shall be used for collecting needles, syringes, glass, blades and other sharps and the same shall be immersed in a double bin system, the inner bin having perforated walls and bottom of it immersed in 1% hypo chlorite solution. These shall be used to collect the sharps and they shall be sent for disposal in concrete pits constructed as per the prescribed standards.
(iv) Black bins with black bags shall be used for discarded medicines, cytotoxic drugs, incineration ash and chemical waste which are to be disposed of in secured landfill.

The Rules further describes roles and responsibilities of all the agencies involved in healthcare waste management including that of local authorities. Rules have also stipulated methods for transportation of biomedical waste and treatment technologies to be employed for its proper management. The salient features of BMWM Rules, 1998 are:

- The Rules regulate proper treatment and disposal of bio-medical waste with the objective to ensure their safe disposal in order to avoid their adverse impact on human health and environment.
- The Rules have provisions for proper handling and management of bio-medical wastes. These Rules apply to all persons and institutions that generate, collect, receive, store, transport, treat and dispose or handle bio-medical waste in any form.
- Section 3 deals with the definitions of animal house, authorisation, authorised person, bio-medical waste treatment facilities, occupier, operator of a bio-medical waste facility etc.
- Section 4 specifies duty of occupier (generator of waste) to take all steps to ensure that such waste is handled without any adverse effect on human health and the environment.
- Section 5 specifies treatment methods to be adopted for each category of wastes. It also specifically deals with the limits upto which wastes have to be treated.
- Section 6 deals with segregation, packaging, transport and storage of bio-medical waste. According to this, infectious waste has to be segregated and not to be mixed with any other waste. It also specifies the transport procedure. Maximum storage time permitted is 48 h.
- Section 7 is regarding prescribed authority that shall implement these rules. Pollution Control Boards/Pollution Control Committees have been designated as prescribed authorities.
- Section 8 is about obtaining authorisation for every occupier except such occupier of clinics, dispensaries, and pathological laboratories, blood banks, who provide treatment/services to less than 1,000 patients per month. But all have to adhere to the Rules, irrespective of the number of patients treated in a month.
- A form has been specified for submitting an application for obtaining authorisation. An advisory committee, as required under Section 9, has to be constituted by the State Government.

- Section 10 is about the submission of an annual report to the prescribed authority in prescribed proforma, by the occupier/operator of any health care facility.
- Section 11 deals with the maintenance of proper records relating to BMW, handling and management.
- Section 12 deals with the procedure for reporting of an accident in the prescribed proforma.
- Section 13 deals with the constitution of an appellate committee to hear the aggrieved party of any order made by the prescribed authority.

Thus the Rules imply and mandate that:

- Waste should be segregated and clearly labelled at the source of generation. It is mandatory that the biohazard symbol must be clearly shown on the bins collecting biomedical waste. This is the most important step in handling waste, especially from the patient care areas.
- A system of colour codes for waste storage containers and types of waste to be put in these containers should be followed. Appropriate containers or bag holders (foot-lifting/spring type) should be placed in all locations where particular categories of waste would be generated. Posters giving instructions on waste segregation and their identification should be posted at each waste collection point to remind the healthcare personnel handling waste regarding the procedures. Containers should be removed when they are three-quarters full. Ideally, the bags (biodegradable, non-halogenated) should be tied when they are three-quarters full, placed in trollies and transported to the waste storage/treatment areas. Healthcare personnel should never attempt to correct errors of segregation by removing items from a bag or container after disposal or by placing one bag inside another bag of a different colour.
- If general and infectious wastes are accidentally mixed, the mixture should be treated as biomedical waste.
- Waste should be collected daily (or as frequently as required) and transported to designated central storage area. Nursing and other clinical staff should ensure that waste bags are tightly closed or sealed when they are about three-quarters full. Bags should not be closed by stapling. The bags or containers should be replaced immediately with new ones of the same type and colour. The containers shall be regularly washed.
- A storage location for the healthcare waste should be designated inside the healthcare establishment or research facility. The waste, in bags or containers, should be stored in a separate area, room or building of a size appropriate to the quantities of waste produced and the frequency of collection. The storage areas should be cleaned and washed daily.
- Cytotoxic waste should be stored separately from other healthcare waste in a designated secure location. Radioactive waste should be stored in containers that prevent dispersion, i.e., behind lead shielding. Waste that is to be stored during radioactive decay should be labelled with the type of radionuclide, the date and details of required storage conditions.

- It is obligatory for the occupier to label the containers for transportation as per the provisions contained in Schedules III and IV of BMW Rules'98.
- Internal transfer of hospital waste should be done in tightly tied bags, in covered containers by means of wheeled trolleys. These containers or carts should not be used for any other purpose.
- The vehicles should be regularly cleaned and disinfected with hot water/ appropriate disinfectant. All waste-bag seals should be in place and intact till the end of transportation.
- The transport vehicles carrying untreated biomedical waste (cart/barrow) must be designated and must not be used for any other purpose.
- Waste must be transported away from the areas of generation at regular intervals or every morning. Breakdowns in transport will play wide-spread havoc on the entire system and therefore back-up staff and equipment must be planned for, and shall be made available.
- Transport of waste from areas of generation must be done only by designated staff, who should be aware of the hazards of the material they handle and also about the protective measures to be taken. They should be provided with adequate personal protective equipments and should be instructed to report in case of any injury to the medical authorities.
- No untreated biomedical waste shall be stored beyond a period of 48 h. If, for any reasons, it becomes necessary to store the waste beyond a period of 48 h, permission from the prescribed authority (Pollution Control Board or Pollution Control Committee) must be taken, and it must be ensured that it does not adversely affect human health and environment. For instance, it can be stored in cold storage rooms maintained at 0 C in case of breakdown of transport or maintenance of treatment equipment.
- Regular medical check-up for the staff is mandatory. Immunisation against Tetanus, Hepatitis B, Tuberculosis and other blood borne diseases is mandatory.
- The operator of a common treatment facility shall not open the yellow bags meant for incineration.

17.8 Draft Bio-Medical Waste (Management and Handling) Rules, 2011

While implementing the 1998 Rules, the implementing agencies and other stakeholders encountered several practical problems. Therefore, there was a need to address the issues in implementation of the rules hence new comprehensive draft rules are proposed to enable the prescribed authorities to implement the rules more effectively. The revised rules will be called as the Bio-Medical Waste (Management and Handling) Rules, 2011. The draft rules have been notified for public consultation and the finalized rules shall soon come into force on the date of their publication in the Official Gazette.

The proposed new draft Rules of 2011 are comprehensive which contain important features of the Bio-Medical Waste (Management and Handling) Rules, 1998, including the three amendments issued. Several new provisions have been added in the new Rules.

(1) In the new Rules, it has been clearly mentioned that these rules are applicable only to the bio-medical wastes and shall not apply to other wastes such as radio active wastes, hazardous chemicals, municipal solid waste, hazardous wastes and batteries waste, which are covered under the respective rules.
(2) In the new rules, it has been stipulated that every occupier shall set up requisite biomedical waste treatment equipments prior to commencement of its operation or shall make necessary arrangements in order to ensure requisite treatment of bio-medical waste through an authorised common bio medical waste treatment facility.
(3) As per the earlier Rules, obtaining authorisation from prescribed authority was not required by an occupier of an institution which was providing service to less than 1000 (one thousand) patients per month. Under the new Rules every occupier or operator, irrespective of the number of patients being serviced or the quantum of bio-medical waste generation, is required to obtain authorisation.
(4) Under the existing rules, there was overlapping with regard to colour coding and segregation of waste. For instance, wastes under category-3 and 6 can be collected either in Yellow or Red bags. Similarly, wastes under category-7 may be collected either in Red or Blue bags. This caused confusion in segregation. In the new Rules, colour coding for containers or bags (Yellow, Red, Blue and Black) for collection of various categories of bio-medical wastes including the treatment options has been clearly specified to avoid overlapping and confusion.
(5) In the new Rules, duties for operator of a Common Bio-Medical Waste Treatment Facility as well as other concerned Authorities have been stipulated, in addition to the duties of occupier of a health care establishment.
(6) In the new Rules, the number of categories of wastes has been reduced from ten to eight. Colour coding for collection of non-infectious waste (general waste) has also been prescribed.
(7) The guidelines issued by the CPCB and the Central Government have been now made part of the Rules.

(The draft rules are notified for public consultation and may be seen in the website of the Ministry of Environment and Forests (www.envfor.nic.in)

17.9 Challenges of Biomedical Waste Management in India

Biomedical waste management signifies activities which aim at:

- Reduction in quantity of waste generation
- Segregation of infectious waste at the point of generation,
- Scientific handling and storage of waste generated

- Collection and transportation in carriers/vehicles exclusively dedicated for the purpose.
- Treatment and disposal in such a way so as to cause minimum environmental impacts.

Inspite of all the regulations and guidelines, the pace of biomedical waste management is not upto the level of satisfaction due to lack of effective waste management systems in many healthcare facilities, which is a key prerequisite to improving efficiency and effectiveness. For many of the HCFs, biomedical waste management is the least priority area. The various issues and challenges involved in management of healthcare waste are:

- Segregation of infectious waste at the source of bio-medical waste generation.
- Proper treatment and disposal of biomedical waste.
- Disposal of sharps including disposal of Auto-Disable (AD) syringe waste.
- Treatment and disposal of waste water.
- Lack of information, awareness and skills of hospital staff for handling biomedical waste.
- Not providing adequate budget/funds for waste management and non-compliance to BMWM Rules.
- Inadequate enforcement of BMWM Rules by regulatory authorities.
- Inadequate common treatment facilities.

In 2007, Comptroller of Auditor General (CAG) conducted Performance Audit (PA) of "Management of Waste in India" which sought to examine whether the government had identified waste as risk to environment and health, accurately assessed the amount of different kinds of wastes being generated in the country and drafted a policy on waste management which focused on waste minimisation and waste reduction, as compared to waste disposal, these being the more effective ways to manage waste. In addition, the CAG also sought to examine whether all kinds of wastes had been covered under legislation for safe disposal and whether the concerned agencies had been allocated responsibility and accountability for the management of various types of wastes. The CAG further sought to check the compliance to Rules relating to implementation, monitoring and evaluation and adequacy of funding relating to municipal solid waste, bio-medical waste and plastic waste.

The compliance to bio-medical waste rules studied under the CAG, revealed that:

- Hospitals/private operators were running waste disposal facilities without authorisation and segregation of biomedical waste and the treatment/disposal was not being done according to the categories of waste under the Rules.
- Infrastructure created in the states for treatment and disposal of biomedical waste is inadequate.
- The problem of non-compliance to Rules for the management and handling of municipal solid waste, bio-medical waste and plastic waste was further compounded by lax and ineffective enforcement and monitoring.

- In the absence of effective monitoring, violation of these Rules escaped detection.
- Shortage of staff in Municipalities/SPCBs hampered the monitoring and implementation of the Rules for waste management.

Thus, the CAG brought out deficiencies in the implementation and monitoring of BMWM Rules in India and suggested the following recommendations:

- Advisory bodies should be set up in each state and it should be consulted regularly on the matters relating to implementation of the bio-medical waste rules.
- Registrations of those hospitals that do not set up their own treatment/disposal facilities or join a common treatment facility could be cancelled. New hospitals should not be allowed to commence operation without making sure that it has a facility or made arrangements for treatment/disposal of bio-medical waste.
- Segregation of bio-medical waste according to its type should be ensured in each hospital. Measures should be taken to achieve 100% segregation by each hospital.
- Based on the kind of waste being generated in the hospitals, waste treatment/disposal infrastructure should be created. Advisory bodies and CPCB can be consulted in this regard. Hospitals could join a common treatment facility for treatment/disposal and SPCBs should ensure that each common treatment facility has the requisite and complete infrastructure to handle waste safely.

17.10 Comparative Analysis of Situation of Biomedical Waste Management in India with Other Countries

On analysing the situation in other countries, Indian policies, regulations and implementation found to lack in many of the aspects. Some of them could be summarised below:

1. As the Rule notifies *notwithstanding* anything contained in the Motor Vehicles Act, 1989, the untreated bio-medical waste shall be transported only in such vehicle as may be authorized for the purpose by the component authority as specified by the Government. The Rules do not specify what type of transport vehicle needs to be used in which the untreated Bio-medical waste can be transferred outside the hospital campus.
2. The Rules do not prohibit recapping/resheathing of needles. Furthermore chemical disinfections using 1% hypochlorite for needles and other sharps has been found ineffective, hence these need to be autoclaved or sterilized by dry heat.
3. The Rules do not specify any treatment or disposal technology for the waste (infected/biomedical) generated from home patients (e.g. diabetes patient, infected cotton or gauge, bandage cloth generated from households). They must and some guidelines should be prescribed for them too.

4. The Rules strictly prohibit the usage of chlorinated plastics for incineration. But there have been no guidelines to demonstrate the distinction between chlorinated and non chlorinated plastics. Since 70% of medical plastics are chlorinated, there is no need to make any distinction and it is better to say that plastics should not be incinerated.

17.11 Requirement for Environmentally Sound Healthcare Waste Management System in a Healthcare Facility

It is important to adopt a holistic approach for setting up a technologically and environmentally sound system for healthcare waste management which is also not very cost intensive and hence sustainable. All the aspects involved in healthcare waste management need to be studied and put in order for establishing such a system:

17.11.1 Legislative and Policy Aspects

National legislation is the basic requirement for improving health-care waste management practices in any country. It establishes legal controls and provides necessary legal powers to the national and other regulatory agency responsible for disposal of health-care waste to apply pressure for their implementation. The WHO Handbook 'safe management of wastes from healthcare activities' recommends "The law should be complemented by a policy document, and by technical guidelines developed for implementation of the law. This legal 'package' should specify regulations on treatment for different waste categories, segregation, collection, storage, handling, disposal, and transport of waste, responsibilities, and training requirements; it should take into account the resources and facilities available in the country concerned and any cultural aspects of waste-handling. A national law on healthcare waste management may stand alone or may be part of more comprehensive legislation".

17.11.2 Segregation, Handling, Storage and Transportation of Waste

The key to minimization and effective management of healthcare waste is segregation (separation) and identification of the waste. Segregation at source is necessary, as different types of waste need different treatment and disposal. It is a safe method by which the generator and the handler help in:

(i) Reducing the total treatment cost.
(ii) Ensuring that general waste does not become infectious and
(iii) Reducing the chances of infection in healthcare personnel and the general community.

Segregation is the prime responsibility of the waste producer and should take place as close as possible to where the waste is generated. With regard to handling and storage of generated healthcare waste, responsibilities should be fixed distinctively and the prescribed national standards should be adhered to. Further, the HCF administration should dedicate resources (both financial and human resource) to ensure safe and technically sound handling and storage of the waste generated. Training (induction and regular) of the staff is integral to ensure compliance to handling and storage protocol. Evaluation and Monitoring mechanisms need to be devised to assess the efficacy of the protocols followed.

Transportation of generated waste is as important as segregation. Transportation should be done in dedicated vehicles having separate compartments as per the waste categories. The vehicles or containers used for the transportation of health-care waste should not be used for the transportation of any other material. They should be kept locked at all times, except when loading and unloading. Articulated or demountable trailers (temperature-controlled if required) are particularly suitable, as they can easily be left at the site of waste production. Other systems may be used, such as specially designed large containers or skips; however, open-topped skips or containers should never be used for transporting health-care waste. Where the use of a dedicated vehicle cannot be justified, a bulk container that can be lifted on to a vehicle chassis may be considered. The container may be used for storage at the health-care establishment and replaced with an empty one when collected. Refrigerated containers may be used if the storage time exceeds the recommendations or transportation times are long. The finish of these bulk containers should be smooth and impervious and permit easy cleansing or disinfection.

While handling the waste bags, they may be placed directly into the transportation vehicle but it is safer to place them in further containers (e.g. cardboard boxes or wheeled, rigid, lidded plastic or galvanized bins). This has the advantage of reducing the handling of filled waste bags but results in higher disposal costs. These secondary containers should be placed close to the waste source.

17.11.3 Treatment and Disposal Options

Treatment may be defined as the process that changes the character of hazardous waste to render them less hazardous or non-hazardous. Treatment renders waste unrecognizable and may or may not reduce volume. Treatment of biomedical waste depends upon the nature, volume of the waste and the selected technology (i.e. if it is technologically and economically viable and environmentally sound) and meets regulatory standards and public acceptance. Sometimes pre-treatment of waste may

be required before storage/transportation or disposal of biomedical waste. This may be required at the site of generation or at another site in the hospital, collection centre, disposal area, etc. for a number of reasons which includes:

(a) reducing the bulk in order to reduce requirements for storage and transportation;
(b) disinfecting the waste so that it is no longer a source of pathogenic microorganisms;
(c) making recyclable items unusable e.g. cutting syringes and damaging the needles;
(d) meeting the obligations of BMWM Rules etc., e.g. not using chlorine disinfectants for items that are to be incinerated.

The WHO Handbook 'safe management of wastes from healthcare activities' summarises various treatment and disposal options as mentioned in Table 17.2:

While selecting the technologies to be employed, apart from capital cost one must look into the factors like:

- Appropriateness of the technology with respect to the quality and quantum of healthcare waste generated.
- Ease of operation and maintenance.
- Effective life time.
- Level of environmental compliance (emissions/residues).
- Proven technical record.
- Operational cost.
- Human resource required for operations and maintenance.

It is expected that every HCF has to set up requisite biomedical waste treatment facilities like incinerator, autoclave, microwave system for the treatment of waste, or should ensure requisite treatment of waste at a common waste treatment facility or any other waste treatment facility. Installation of individual treatment facilities by every healthcare unit requires comparatively high capital investment and thus is not a viable option. In addition, it requires separate human power and infrastructure for proper operation and maintenance of treatment systems. The concept of Common Biomedical Waste Treatment Facility (CBWTF) not only addresses such problems but also prevents proliferation of treatment facilities in a city, which themselves can be a source of hazard and infection if not maintained well. Furthermore, having CBWTFs reduce the monitoring pressure on regulatory agencies. By running the treatment equipment at CBWTF to its full capacity, the cost per kilogram for treatment of waste reduces significantly too. Its considerable advantages have made CBWTF popular and a proven concept in many developed countries.

17.11.4 Training

Education and Training is an integral component of healthcare waste management. Studies show that education and training do help to change infection status in and

Table 17.2 Comparison of various treatment and disposal technologies

Treatment/disposal method	Advantages	Disadvantages
Rotary kiln	Adequate for all infectious waste, most chemical waste and pharmaceutical waste	High investment and operating costs
Pyrolytic incineration	Very high disinfection efficiency. Adequate for all infectious waste and most pharmaceutical and chemical waste	Incomplete destruction of cytotoxics. Relatively high investment and operating costs
Single-chamber incineration	Good disinfection efficiency. Drastic reduction of weight and volume of waste. The residues may be disposed of in landfills. No need for highly trained operators. Relatively low investment and operating costs	Significant emissions of atmospheric pollutants. Need for periodic removal of slag and soot. Inefficiency in destroying thermally resistant chemicals and drugs such as cytotoxics
Drum or brick incinerator	Drastic reduction of weight and volume of the waste. Very low investment and operating costs	Destroys only 99% of microorganisms. No destruction of many chemicals and pharmaceuticals. Massive emission of black smoke, fly ash, toxic flue gas, and odours
Chemical disinfection[a]	Highly efficient disinfection under good operating conditions. Some chemical disinfectants are relatively inexpensive. Drastic reduction in waste volume	Requires highly qualified technicians for operation of the process. Uses hazardous substances that require comprehensive safety measures. Inadequate for pharmaceutical, chemical and some types of infectious waste
Wet thermal treatment[a]	Environmentally sound. Drastic reduction in waste volume. Relatively low investment and operating costs	Shredders are subject to frequent breakdowns and poor functioning. Operation requires qualified technicians. Inadequate for anatomical, pharmaceutical and chemical waste and waste that is not readily steam permeable
Microwave irradiation	Good disinfection efficiency under appropriate operating conditions. Drastic reduction in waste volume. Environmentally sound	Relatively high investment and operating costs. Potential operation and maintenance problems
Encapsulation	Simple, low-cost and safe. May also be applied to pharmaceuticals	Not recommended for non-sharp infectious waste
Safe burying	Low costs. Relatively safe if access to site is restricted and where natural infiltration is limited	Safe only if access to site is limited and certain precautions are taken
Inertization	Relatively inexpensive	Not applicable to infectious waste

[a]May not apply to more sophisticated, self-contained, commercial methods

around HCFs leading to a better environment for healthcare workers, patients and their attendants. The training of HCF personnel needs to be done at different levels. This includes doctors, nurses, administrators, paramedical personnel, housekeeping staff, engineering, kitchen and laundry personnel, *ayahs*, ward boys, security staff, gardeners, rag pickers, sorters, reprocessors and all personnel in different depts. and areas in the hospitals which are involved in BMWM. A practical and ongoing training schedule for the staff should be evolved and conducted with several educational aids in different languages to be developed for training and reinforcement of the health-care workers.

The staff education programmes should include:

- information on and justification for all the aspects of health-care waste policy;
- information on the role and responsibilities of each hospital staff member in implementing the policy;
- Technical instructions relevant for the target group, on the application of waste management practices.

The WHO handbook emphasises on periodic repetition of courses which will provide refreshment training as well as orientation for new employees and for existing employees with new responsibilities and will also update knowledge in line with policy changes. Follow-up training is instructive for trainers, indicating how much information has been retained by course participants and the likely need for future refresher courses.

Looking into all the aspects of healthcare waste management, it is propounded that institutionalizing effective waste management systems in all healthcare facilities (HCFs) is a key prerequisite to improving efficiency and effectiveness of healthcare waste management scenario in India. To introduce such an efficient system of biomedical waste management in an HCF, it is important to make arrangements for the following activities:

1. Different categories of wastes should be segregated and put in different colour coded bins/bags in accordance with the national laws and regulations.
2. Waste quantification and assessment of the types of waste generated at each point of generation (nursing station, patients' bedside, Labs, OPD, radiology, OT etc.) in each department of the healthcare facility should be done.
3. Assess the waste management practices in each department like assessment of the waste segregation and collection pattern, use of waste transportation trolleys and proper bowls to transfer waste from patients bedside to the nursing station, placement of the bins, colour of the bins, infection control practices, facilities for hand washings, clean and hygienic conditions in each ward, etc.
4. Awareness and training of staff regarding categories of wastes generated in each department and their segregation, including various issues involved in waste management.
5. A survey of all the departments needs to be carried out and a baseline assessment of waste management is required to be conducted. On the basis of the baseline assessment thus conducted, the required items (bins and bags of

required colours, needle cutters, trolleys, treatment technologies like autoclaves, shredders, ETP etc.) for setting up of effective waste management system in the HCF, need to be procured.
6. Obtain necessary consents and authorisations from the concerned pollution control authorities for handling biomedical waste generated and to establish the identified and approved technologies for treatment of the waste.
7. Allocate required funds exclusively for hospital waste management system. This should be done on yearly basis to ensure sustainability of the healthcare waste management system established.
8. Identify a team of dedicated and interested staff to undertake various activities pertaining to hospital waste management. A nodal officer in each ward/ department needs to be designated for coordination and supervision of the waste management activities.
9. Develop standard protocols and procedures to be implemented for the healthcare waste management system in the HCF.
10. Develop posters and IEC material and training programmes for the staff of the HCF.
11. Develop waste monitoring and reporting formats.
12. Constitute a Committee for monitoring and reviewing the waste management system on a regular basis (monthly/bi-monthly).
13. On the basis of monitoring and evaluation of the system established in the departments, address any short comings observed to resolve the issues.
14. Formulate an infectious control and bio-medical waste management policy and develop a mechanism for its implementation.

17.12 Conclusions

Healthcare waste management although "not-so-new" field, it has long way to go. Lack of resources (financial and human resource), lack of know-how and appropriate treatment technology is generally attributed to such pathetic situation. But even if all the resources are in place, attitudinal change is the deciding factor for evolving a better healthcare waste management scenario. From the above analysis of the situation of biomedical waste management across the globe, following recommendations are made:

1. Comprehensive Rules and Guidelines at the national level need to be formulated and widely disseminated in the country for management of biomedical waste.
2. An inventory of the HCFs (in government and private sector) to be maintained at district, state and national level. This will help in better enforcement of Rules. A national survey of healthcare waste will provide the regulatory agencies with a basis for identifying actions on a district, regional and national basis, taking into account conditions, needs and possibilities at each level.
3. Strict penalties and punishments to be enforced for defaulting the Laws.

4. Centralised treatment facilities to be promoted for treatment of biomedical waste.
5. The charges levied by the CTF operator to the participating HCFs need to be determined in consultation with the regulatory authorities for ensuring sustainability of its business and also interests of the medical fraternity.
6. Every HCF should have well defined waste management policy integrated with its infection control policy.
7. It should be made mandatory for the HCFs to ensure adequate financial and human resources to implement and sustain waste management in their respective HCFs.
8. Aspects like record maintenance, monitoring of hospital waste management plan etc. should be well defined at national level and widely circulated to avoid any disparity.
9. Regular training of those involved in biomedical waste management is to be made mandatory.

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Chapter 18
Oleaginous Fungi: A Solution to Oil Crisis

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Abstract Oleaginous fungi are known for microbial oil as they can accumulate intracellular lipid up to 70% under stress condition. Like vegetable oils, microbial oils component is TAG, composed of C16 and C18 series long chain fatty acids, due to which microbial lipids can be subjected to transesterification process. High growth rate, short generation time and high lipid content of oleaginous microorganism, under specific conditions, open new prospects for scaling up the cultivation. Although microbial lipid content is high in such microorganism, but process optimization and isolation of organism that can grow on cheap sources of carbon, would add advantage to the production system. Oil from oleaginous fungi has given a new turn to the fuel generation and to the food industries, as their lipid is rich in TAG and the specific PUFA. With the increase in population, demand for food is also soaring, in such scenario food based fuel production will be highly expensive and unwise option, a promising alternative is to look at microbial oil technology as it doesn't compete with the farmland for crop production. The present chapter focuses on the growth, mechanism and regulation of lipid accumulation in oleaginous filamentous fungi.

Keywords Oleaginous fungi • Triacylglycerides • Biodiesel • Lipid • Microbial oils

18.1 Introduction

With the massive global demand of crude oil, and shortage supply of the petroleum, has lead world to move for the alternative energy resources for fuel production. Among the renewable energy sources biodiesel have become a promising biofuel and attaining the significant acceptability in the area of transportation due to its

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compatibility with the diesels engines and low emission profile. On the environmental aspect also biodiesel have proven to be adequate as it emits less gaseous pollutant and there is minimum release of carbon dioxide to the atmosphere.

Biodiesel, a type of secondary biofuel is produced by the processing of the oils, rich in tri-acyl glycerides (TAG). Transesterification of the TAG moieties present in the lipid obtained from substrates such as vegetable oil, animal fat and wasting oils are previously used for production of biodiesel, but on economic aspect all over production cost was much higher due to the substrate which was 70–85% of the total manufacturing cost (Vicente et al. 2009). At the same time the use of vegetable oils for production of biodiesel was questionable, because of food versus fuel controversy. Moreover, to meet the demand of fuel, oilseed/plant cultivation requires large area of land, which is not feasible. Therefore, the only alternative left for the production of biodiesel is through microbial sources, which can accumulate high concentration of lipid. Microbial oils posses many advantages over the vegetable oils which already has been discussed earlier in several reports as they didn't require the land area, no competence with the food supply and at the same time the different kind of waste material.

Exploitation of microbial system for production of biodiesel depends upon the TAG content in the organism. Basic understanding of the biosynthetic pathways and regulatory networks of lipid metabolism in these oleaginous organisms will help in better performance of these efficient microbes by manipulation of active enzyme and regulators or by down regulating the metabolic cycles. The current chapter will focus on the mechanism and regulation of the pathways utilized by oleaginous filamentous fungi for the production of TAGs.

18.2 Oleaginous Fungi

Microbes are viable feedstock for production of biofuel as they can accumulate the fuel precursor, triglycerides which are converted to fatty acid mono alkyl esters. The major microbial species are bacteria, fungi, yeasts and micro algae capable of accumulating the lipid more than 20–30% and are termed as oleaginous microbes (Ratledge 1991). Oleaginous fungi are reported to accumulate maximum intracellular lipid up to 50–70% in the form of cytosolic lipid bodies in comparison to other microbes. Lipid bodies also known as lipid droplets in oleaginous fungus are composed of neutral lipids mainly TAG which involve regulation at two key steps in biosynthetic pathway during the transition to oleagenic state (Murphy 2001). These spherical bodies accumulate the TAG moieties, but the exact distribution and diameters varies with the organism, growth phase environmental conditions. Such fungus, under high ratio of carbon and nitrogen accumulate these high amounts of lipid as storage reserves, 40–65% total cell dry weight (Lösel and Sancholle 1996; Radzhabova et al. 1990) Exploitation of oleaginous fungi for oil production on cheap feedstock of carbon like waste molasses, sludge sewage, monosodium glutamate wastewater, free-fatty acids, crude glycerol (Angerbauer et al. 2008; Fakas et al. 2008; Mlickova et al. 2004; Xue et al. 2008; Zhu et al. 2008) and agricultural byproducts (Fakas et al. 2008; Singh 1991; Huang et al. 2009) add advantages to

production system. Along with substrate compositions and supplementation of cultivation media with addition of exogenous oils or fatty acid in media has enhanced the lipid production and over all yield.

An oleaginous fungus, a eukaryotic microorganism, poses remarkable abilities to accumulate lipids during the secondary metabolic growth. The diversity of fungal species has facilitated the selection of oleaginous strains as they can compete at commercial scale with the traditional lipid production from plant and animal source on the basis of several reasons:

- Extremely high growth rate makes oleaginous fungi as acceptable feedstock for biofuel production.
- Dynamic enzymatic system for lipid production and accumulation.
- Utilization of cheap waste materials as substrate for oil production.
- Productions of microbial oils are not affected by seasonal and climatic condition.
- Under controlled environment, with the manipulation of nutrient condition the lipid yield and profile can be maximized.
- Mutation at the key steps of lipid synthesizing enzymes improves the lipid yield.
- Appropriate hosts for cloning of foreign genes ending up with the formation of lipid and other valuable products (PUFA).

Oleaginous microbes are perfect models of eukaryotic system for studying lipid biochemistry, metabolic regulation and function than complex multi-cellular systems of plants and animals (Certik and Shimizu 1999). However, the problem still exist, microbial oils are unable to meet the industry demand. Strategies employed to attain the commercial production includes fermentation (submerged and solid-state), uses of low-cost substrates, screening for more efficient strains and reduction in the processing steps. With the growing interest in biofuel, several processes are designed coupling the traditional fermentation with biotechnological methods (such as causing mutation, use of inhibitors, genetic engineering techniques, media engineering etc.) intend to attain the commercial production level. Use of all these biotechnological methods requires the fundamental knowledge of TAG biosynthesis and regulation. An understanding of the basic biochemistry and genetics of lipid accumulation in such microorganisms is therefore essential if yields are to be improved in these oleaginous microbes.

18.3 Factor Affecting the Microbial Lipid Synthesis

18.3.1 Carbon to Nitrogen Ratio

The formation of TAG is promoted in response to stress condition imposed and during imbalanced growth. One of the reasons is variation in the carbon and nitrogen ratios (C/N), by nitrogen limitation, down regulation of citric acid cycle occurs

Table 18.1 Biochemical event involved in lipid accumulation under nitrogen limiting condition

Non-oleagenic cell	Oleagenic cell
Inactive AMP deaminase,	Under depletion of N_2, fall of AMP occurs
$AMP \not\rightarrow IMP + NH_4^+$	$AMP \xrightarrow{\text{AMP deaminase}} IMP + NH_4^+$
In non oleagenic state • AMP is not required by isocitrate dehydrogenase. • Citric acid cycle normally goes on as isocitrate is converted to α- ketoglutarate. • These cell lack enzyme ATP-citrate lyase, due to which lipid accumulation is not initiated	• Inactivation of isocitrate dehydrogenase causing major decrease of citric acid cycle. • Conversion of accumulated isocitrate to citrate via aconitase • Citrates are effluxed to cytosol and are converted to acetyl CoA by specific enzyme ATP-citrate lyase, found in oleagenic organisms. • ATP-citrate lyase is crucial enzyme for lipid accumulation as acetyl CoA formed is used for fatty acid synthesis

(Wynn and Ratledge 1997). Cells remain metabolically active utilizing the excess carbon source in nitrogen exhaustion, and a result increase in cell dry weight and lipid content are noticed due to prevented cell proliferation, existing cell become obese. Lipid bodies formed are energy storage for existing cells which can no longer divide (Table 18.1).

Disadvantage of nutrient starvation is the lowered biomass, decreasing the overall lipid content, because during the nutrient-stress high lipid accumulation occurs in cells on the expense of reduced cell division (Ratledge 2002).

18.3.2 Nitrogen Source

Biochemical event which causes such diversion under nitrogen limiting condition is the decrease in the AMP concentration due to activation of the enzyme AMP deaminase (Ratledge and Wynn 2002) which hydrolyses AMP to inosine5'-monophosphate. Under normal state the fungi doesn't require the AMP for the activity of isocitrate dehydrogenase, but due to nitrogen exhaustion fungi enters the oleagenic state a way to scavenge the ammonium ions from intracellular materials and the cell dependency increases, resulting in the conditional requirement of AMP.

Nitrogen sources are also important factor for TAG accumulation. Neutral lipid composition varies with the nitrogen source used in cultivation medium because cell growth varies. Even the lipid accumulation is affected when grown in organic source rather than inorganic. In fungus *Pellicularia*, (Suzuki et al. 1984) decrease of triglyceride content (19–31%) was quantified when cells were grown using

ammonium nitrate, ammonium sulfate and ammonium chloride as nitrogen source. When *Pellicularia* grow in urea, sodium nitrate and potassium nitrate, an increase in the ratio of triglycerides (65–85% of the neutral lipids) occurs with the decrease in the ratio of other lipid components. Several reports are there which shows that nitrogen source creates significant difference in the triglycerides concentration.

18.3.3 Physiological Conditions

In combination with the other factors, incubation temperature, pH and oxygen concentration also governs the triglyceride formation. Cells under different growth temperature, produces lipid with varied composition of polar (phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine and phosphatidylinositol) and neutral lipids (Triacylglycerols (TAG) and steryl esters). Normally fungi produces lipid over wide range of pH (Jeffery et al. 1999). Brown et al. (1990) studies showed that changes in lipid content and fatty acids occurred in *Trichoderma reesei* with a change in pH and temperature. Shaking condition causes an improved dissolved oxygen tension.

18.3.4 Minerals

Lipid production is also influenced by the mineral ions. Malic enzyme, the key regulator for lipid accumulation was got effected by metal ions, and was dependent on Mg^{2+} and Mn^{2+} (Savitha et al. 1997). Inhibition of lipogenesis has been reported by Zn^{2+} by Jackson and Lanser (1993).

18.4 Metabolic Pathway of Microbial TAG Biosynthesis

Triacylglycerol (TAG) is the major storage lipid molecule that lacks the charged group. These reserved materials move into the hydrophobic core (lipid bodies) getting surrounded by mono-layer of phospholipids with few specific proteins (Fig. 18.1), and are used by the organism upon requirement during membrane development if growth resumes or synthesis of cellular metabolites during starvation. Neutral lipids are mobilized by action of TAG lipases and steryl ester hydrolyase, reenter the metabolic pathway.

These intracellular lipids (TAGs) are produced from glycerol 3 phosphate through sequential process carried by number of enzymes. In eukaryotic system, endoplasmic reticulum is the main site for lipid synthesis but many studies on TAG synthesis have reported that lipid bodies of yeast (Christiansen 1978) and plant (Gurr et al. 1974; Ichihara et al. 1990) contain some of active enzymes.

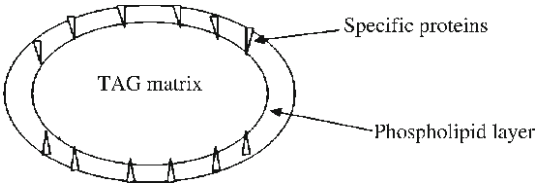

Fig. 18.1 Lipid bodies are surrounded by phospholipids monolayer, in which protein molecules are embedded

The synthetic pathway of TAG starts with the fatty acid synthesis, followed by acyl chain elongation and then TAG formation in specialized organelles i.e. endoplasmic reticulum.

18.4.1 Fatty Acid Synthesis

Fatty acid biosynthesis starts with the biotin-dependant carboxylation of cytosolic acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase (ACC), which catalyzes the important committing step (Davis et al. 2000; Li and Cronan 1993) in the synthetic pathway.

18.4.2 Fatty Acid Chain Elongation

Fatty acid synthase (FAS) is a multi-enzymatic complex (Subrahmanyam and Cronan 1998) responsible for the fatty acid chain elongation. The synthesized malonyl CoA is transferred to the thiol group containing acyl-carrier protein unit (ACP) of FAS complex by malonyl CoA-ACP transacetylase. This small protein not only forms thioesters with the malonyl moieties but also with acetyl CoA and transfer the acetyl group to other unit of FAS complex, Keto acyl-ACP synthase which causes condensation reaction. The ketoacetyl-ACP or ketobutryl-ACP formed undergoes repeated cycle of transformation and condensation to form the saturated fatty acid molecule (Courchesne et al. 2009). Long chain fatty acid with unsaturated acyl chain is produced by elongases and desaturase located in endoplasmic reticulum and mitochondria. Liberation of fatty acid is catalyzed by ACP-thioesterase which cleaves the acyl chain and fatty acid is released.

18.4.3 Triacyl Glycerol Synthesis

Glycerol-3-phosphate acyltransferase causes condensation of glycerol-3-phosphate with acyl-CoA, the first step in TAG synthesis, generating lyso-phosphatidic

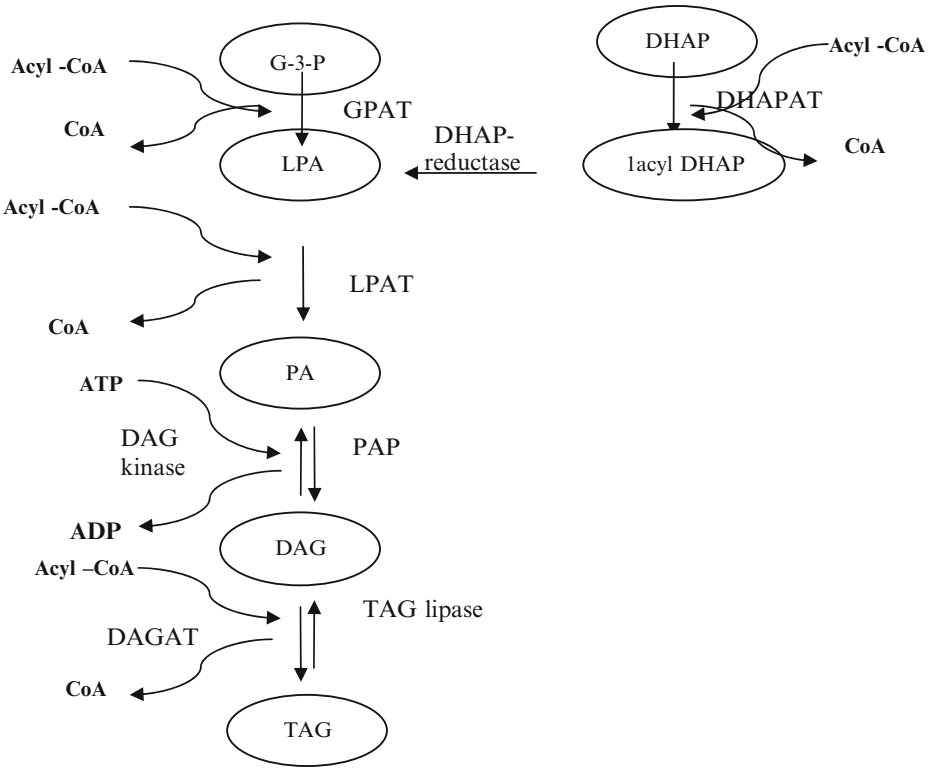

Fig. 18.2 A scheme to show the sequence of events during TAG synthesis

acid (LPA). LPA is also produced by acylation at *sn-1* position followed by reduction of dihydroxyacetone phosphate catalyzed by dihydroxyacetone phosphate acyltransferase and acyl dihydroxyacetone phosphate reductase (Gangar et al. 2001). LPA undergoes acylation to form phosphatidic acid (PA) at *sn*-2 position, which is catalyzed by LPA acyltransferase (Sorger and Daum 2002). PA phosphatase immediately dephosphorylates phosphatidic acid to diacylglycerol (DAG). In the final enzymatic step, DAG is acylated by DAG acyltransferase to produce TAG. Diacylglycerol acyltransferase (DGAT) is an integral membrane protein responsible for transferring an acyl group from acyl-CoA to the *sn*-3 position of 1,2-diacylglycerol (DAG) to form triacylglycerol. TAG formation is via the Kennedy pathways, therefore it is not involved in membrane biosynthesis (Kennedy 1961). In the case of yeast and plant acyl-CoA independent formation of TAG takes place, and reaction is catalyzed by the phospholipid: diacylglycerol acyltransferase (Dahlqvist et al. 2000; Stahl et al. 2004) (Fig. 18.2, Table 18.2).

Table 18.2 Shows the enzymes involved in the metabolic pathway

Enzymes	Substrate	Products	Mechanism
Glycerol-3-phosphate: acyltransferase, (GPAT)	Glycerol 3 phosphate, (G-3-P)	Lyso-phosphatidic acid, (LPA)	Acylation
Dihydrogenacetone phosphate acyltransferase (DHAPAT)	Dihydrogen acetone phosphate (DHAP)	1acylDihydrogen acetone phosphate (1acyl DHAP)	Acylation
Dihydrogen acetone phosphate reductase (DHAP oxidoreductase)	1acylDihydrogen acetone phosphate (1 acyl DHAP)	Lyso-phosphatidic acid, (LPA)	Reduction
Lysophosphatidic acid: acyl-CoA acyltransferase (LPAT)	Lyso-phosphatidic acid, (LPA)	Phosphatidic acid, (PA)	Acylation at sn-2 position
Phosphatidic acid phosphatase, (PAP)	Phosphatidic acid, (PA)	Diacyl glycerol, (DAG)	Dephosphorylation
Diacylglycerol acyltransferase, (DAGAT)	Diacyl glycerol, (DAG)	Triacyl glycerol, (TAG)	Acylation at position 3

18.5 Metabolic Regulation of Microbial Lipid Synthesis

18.5.1 ATP-Citrate Lyase

ATP: citrate lyase (ACL; EC 4.1.3.8) or ATP: oxaloacetate lyase is identified as a regulatory enzyme during lipogensis in most of the oleogenic organisms. For lipid synthesis and accumulation, Citrate has to be transported to cytoplasm from mitochondria. The cytosolic citrate is converted to acetyl CoA and oxaloacetate by ACL, specific for oleagenic microbes, which trigger the formation of lipid synthesis.

$$\text{Citrate} + \text{ATP} + \text{CoA} \xrightarrow{\text{ACL}} \text{Acetyl CoA} + \text{Oxaloacetate} + \text{ADP} + \text{Pi}$$

The resulting cytoplasmic acetyl CoA or malony CoA formed from acetyl CoA by action of carboxylase are the building block during the fatty acid synthesis (Fig. 18.3).

During the nitrogen exhaustion, the isocitrate is converted to citrate which is effluxed from the mitochondria to cytoplasm due to inactivation of ICDH as a result isocitrate is not metabolized to α-ketoglutarate (Sect. 18.3.1). In the oleagenic condition the ICDH enzyme activity is fully dependent on the AMP and presence of AMP which down regulates the whole cascade of event involved in citric acid cycle. ACL is then responsible for the conversion of citrate to acetyl CoA, which is utilised by the *de novo* fatty acid synthesis.

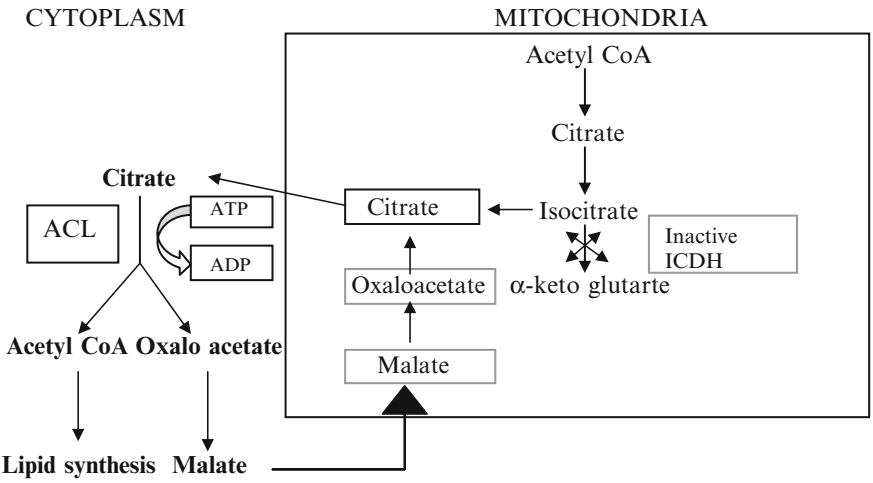

Fig. 18.3 A schematic diagram to show how the building block for fatty acid synthesis, Acteyl CoA is synthesized in cytoplasm by regulatory enzyme ATP-citrate lyase, active only in oleagenic fungi

18.5.2 Malic Enzyme: Key Regulator of Lipid Accumulation

The key enzyme that regulates the formation of triacylglycerol formation in oleaginous fungi is the malic enzyme (ME; EC 1.1.1.40) activity (Wynn and Ratledge 1997). Malic enzyme governs the process is by catalyzing the, malate and providing the supply of $NADPH^+$ needed during the lipid synthesis by FAS (Wynn et al. 1999).

$$\text{L - Malate} + \text{NADP} + \xrightarrow{\text{ME}} \text{pyruvate} + \text{CO}_2 + \text{NADPH}^+$$

Multiple isoforms of ME are identified in oleaginous fungus. In *Mortierella alpine,* seven isoforms (A-G) of ME were identified in which isoform E, was involved in lipid accumulation, in nutrient starvation condition (Zhang and Ratledge 2008). In case of *Mucor circinelloides* six isoforms are identified in which isoform IV is only associated with lipid accumulation (Song et al. 2001; Savitha et al. 1997b) found that enzyme from *Mucor circinelloides* acts only on L-malate and was unable to catalyze D-malate.

Throughout the fatty acid synthesis, the NADPH is required at various steps (Ratledge 2002).

During the elongation of fatty acid chain, with every acetyl or malnoyl moieties addition, FAS molecules require 2 mol of NADPH.

- FAS complex also requires 1 mol of NADPH during two reductive steps carried out by 3-keto acyl reductase and 2,3-enoyl reductase involved in fatty acid chain elongation.

For such surplus production of NADPH various enzymes are involved like glucose-6-dehydrogenase, phosphogluconate dehydrogenase etc. but it was found

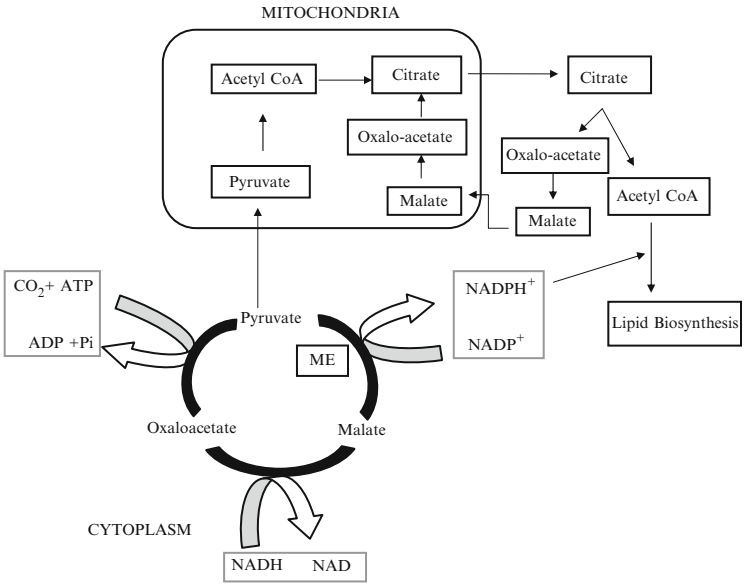

Fig. 18.4 A scheme to show the production of NADPH by Malic enzyme, responsible for surplus supply during fatty acid synthesis and elongation

that lipid accumulation and ME activity are linked in case of fungus. Wynn and Ratledge (1997) found that *Mucor circinelloides* cells, when treated with sesame oil, lost their ability to accumulate lipid, from 25% it was reduced to 2% due to inhibition of malic enzyme. Similar studies were carried to confirm the involvement of ME in lipid accumulation on mutant strain of *Aspergillus nidulans,* and mutant strain was unable to accumulate storage lipids. These studies confirmed the disappearance of ME activity results in cessation of the lipid accumulation because NADPH required by FAS complex for the fatty acid biosynthesis is provided entirely by malic enzyme (Fig. 18.4).

This potential enzyme involvement in lipogensis has become as target for those who are attempting to enhance the lipid accumulation in microbial cell. Overexpression of malic enzyme in oil accumulating fungus *Mucor circinelloides* lead to increase synthesis of fatty acid and over all 2.5-fold increase in the accumulated lipid (Zhang et al. 2007).

18.6 Strategies for Enhanced Lipid Synthesis and Accumulation

The common strategy used till date for using fungi as factories of lipid synthesis and accumulation is by controlling the nutrient content and physiological condition. Under nutrient and physiological stress such microbes has better lipid accumulation,

making this approach widely acceptable. Another engineering techniques which can be applied are the on the genetic engineering aspect, genes which are encoding for biosynthetic pathway can be over-expressed or mutation can be created to turn the metabolic flux towards the TAG synthesis. Metabolic engineering has come up with new techniques of blocking the metabolic pathway by using inhibitors for the enzymes of competing pathways; as a result the substrate is utilized by the TAG biosynthetic enzymes. However, uses of all these emerging engineering approaches in fungi are in nascent stage and would be more helpful in long term prospective in production of microbial oil.

18.7 Conclusions

Growing awareness of world in biofuel and inadequacy of plant and animal oils has made oleaginous microbes as potential candidate for the biodiesel generation. To meet the global demand for the oil, a reliable process are to be designed for maximize the production and recovery of microbial oils and it is only possible by improving the quality and decreasing the overall productivity cost. Knowledge about biosynthetic pathway of TAG, the enzymes involved and their regulation in oleaginous species will play major role in developing the industrial process. This information will open new possibilities for employing strategies at molecular level and metabolic engineering of specific step which will modulate the whole process of lipid accumulation.

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Chapter 19
Haloalkaliphilic Bacteria and Actinobacteria from the Saline Habitats: New Opportunities for Biocatalysis and Bioremediation

Satya P. Singh, Vikram H. Raval, Megha K. Purohit, Jignasha T. Thumar, Sangeeta D. Gohel, Sandip Pandey, Viral G. Akbari, and Chirantan M. Rawal

Abstract During the past decade, significant progress has been made in the application of enzymes as useful catalysts. Much of this development could be attributed to the search for new resources, molecular engineering and non-aqueous biocatalysis. The vast majority of enzymes used in biotechnology are derived from well characterized bacterial, fungal, plant, and animal sources. Though protein and medium engineering has brought novel changes into the enzymes, it is equally important, however, to explore the vast array of microbial life adapted to strikingly rigorous and punishing environments. Such environments include extremes of temperature, pressure, pH, and salt content. Nevertheless, the organisms inhabiting such habitats remain poorly characterized.

Among the various groups of extremophiles, haloalkaliphilic organisms are largely investigated from Soda lakes and Dead Seas around the globe, while other habitats for such organisms are rarely explored. During the last several years, we have been working on the diversity, molecular phylogeny and enzymatic potential of haloalkaliphilic bacteria and actinobacteria from the natural and man made saline habitats of coastal Gujarat in Western India. The organisms have displayed varied diversity with the ability to produce extracellular enzymes. The wide spread distribution and characterization of these bacteria from beyond the soda lakes clearly indicated their ecological significance, while enzymatic potential would attract several biotechnological applications under alkalinity and high salt conditions.

While major focus has been on molecular phylogeny and diversity, only limited information is available on their enzymatic potential and enzyme characterization.

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Studies on cloning, sequencing and expression of enzymes from Haloalkaliphilic bacteria, archaea and actinomycetes are quite limited. Our studies on extracellular enzymes from a number of Haloalkaliphilic bacteria has highlighted on many novel traits, such as resistance against urea denaturation and catalytic potential under multitude of extremity. The search for novel enzyme sequences through metagenomic approaches has indicated towards the diversity of alkaline proteases among Haloalkaliphilic bacteria. It is evident that the patterns of enzyme secretion and characteristics would also be quite useful in the assessment of microbial heterogeneity in a given habitat.

Keywords Haloalkaliphilic bacteria • Salt-tolerant actinobacteria • Thermostability • Alkaline proteases • Non-aqueous biocatalysis • Cloning and expression of salt-tolerant enzymes

19.1 Introduction

The microbial diversity has focused renewed emphasis and in this regard extremophiles hold great significance. Limited studies have identified a huge diversity of extremophiles. Culture-dependent and culture-independent methods have been employed for understanding the diversity of microbes in these environments. Among them, halophiles/haloalkaliphiles are an interesting class of extremophilic organisms that have adapted to harsh, hyper saline conditions and/or alkaline environments. The microorganisms being able to grow optimally or very well at pH values at or above 9 along with high salinity (up to 33% (w/v) NaCl) are collectively known as haloalkaliphiles, (Horikoshi 2008). The organisms living in such dual extreme environments possess special adaptation strategies that make them interesting not only for fundamental research but also towards exploration of their applications (Horikoshi 2008). These organisms may hold secret, for the origin of life and unfold many basic questions about the stability of the macromolecules, under extreme conditions. Therefore, their studies would provide important clues for adaptation under salinity and alkaline pH.

In persuasion of above theme, we have focused attention on the haloalkaliphilic bacteria from the natural and man made saline environments along the Coastal Gujarat (India), beyond the boundaries of the Soda Lakes, Dead Seas and Carbonate springs. Gujarat having long costal region with intensive industrial activities, may provide remarkable diversity within the natural microbial flora. Our work on these bacteria over the last several years has indicated their wide occurrence in moderately saline environment of Coastal Gujarat.

India has enormous and unique coastline of the two oceans, a couple of gulfs and a bay. Among the various parts, Gujarat (Western India) accounts for 1,600 km long shore, with industrial activities of mega projects, representing the remarkable diversity within the natural microbial flora. Of this, the Saurashtra region under Kathiawar peninsula occupies a total stretch of 865 km. Existence of halotolerant,

haloalkalitolerant and haloalkaliphilic bacteria and actinomycetes clearly indicated the wide spread distribution of these organisms in saline environment (Gupta et al. 2005; Patel et al. 2005, 2006a, b; Dodia et al. 2006, 2008a, b; Joshi et al. 2008; Thumar and Singh 2007a, b, 2009).

19.2 Critical Review

19.2.1 Halophilic and Haloalkaliphilic Bacteria

Besides the extreme halophiles, the moderate halophiles are also important group of microorganisms adapted to live in hyper saline habitats and constitute a heterogeneous group, which includes a great variety of bacteria (Manikandan et al. 2009; Ramesh et al. 2009). Moderately halophilic bacteria have the capabilities for exciting and promising applications and hence, could be among the most potential candidates, compared with other extremophiles.

During the past decades, the studies on ecology, physiology, and taxonomy of halophilic organisms revealed an impressive diversity. However, till now haloalkaliphiles are studied for the microbiological classification and phylogeny, while only limited attempts have been made to explore molecular basis of adaptation, enzymatic potential and their other biotechnological implications. The diversity of the halophilic, haloalkaliphilic and alkaliphilic microbes has been studied from the hyper saline and hyper alkaline environments.

19.2.2 Actinobacteria from the Saline Habitats

Many alkaliphilic bacteria and few archaea live under the extreme range of alkaline pH, 9–12 (Grant et al. 1992). They rely on sophisticated transport mechanisms to maintain their intracellular pH near neutrality by pumping or exerting protons. Among the bacteria, a mycelial growth is the characteristic among the actinomycetes, a diverse group of Gram-positive bacteria. Actinomycetes have attracted greater attention due to their various natural products and specific mechanisms of adapting extreme environments. While actinomycetes from normal habitats have focused considerable attention during the last many decades, exploration of such organisms from extreme habitats, particularly saline and alkaline, is a relatively new horizon. There are only few reports in literature about these microbes (Al-Zarban et al. 2002, b; Hozzein et al. 2004; Kim et al. 2005; Stach et al. 2005; Montalvo et al. 2005; Dodia et al. 2008a, b; Joshi et al. 2008; Thumar and Singh 2009).

This has been mainly due to the difficulty associated with their isolation and maintenance under laboratory conditions. Recent culture independent studies have shown that marine environments contain a high diversity of actinobacterial species,

if rediscovered by cultivation-based methods (Maldonado et al. 2005; Stach et al. 2005). Approximately 90% of the actinomycetes, cultured from saline and alkaline environments by using the unique techniques, represented the prospective new genera, a result indicative of its high selectivity. It is, therefore, relevant to pay further attention to extreme actinomycetes, as a possible way to discover novel taxa and, consequently, new secondary metabolites and other value added products. Besides, they may also provide unique systems to investigate adaptive strategies and stability of macromolecules. The diversity displayed by them in terms of morphology, biochemical and molecular characters provides a greater insight into the microbial heterogeneity under extreme environments.

19.2.3 Enzymatic Potential of Organism

19.2.3.1 Biocatalysts from Haloalkaliphilic/Halophilic Bacteria

Considerable efforts have been made to study extracellular salt-tolerant enzymes of the moderately halophilic and haloalkaliphilic bacteria, towards developing a new era in biotechnological processes. These enzymes include hydrolases (proteases, nucleases, lipases, phosphatases) and many polymer-degrading enzymes (amylases, cellulases and chitinases), viewed as important candidates for various industries such as food, detergent, chemical, pharmaceutical, paper and pulp or waste-treatment (Betrand et al. 1990; Burg et al. 1991; Patel et al. 2006; Thumar and Singh 2007a, b; Arikan 2008; Carvalho R.V. et al. 2008a; Carvalho A.F. et al. 2008b; Dodia et al. 2008a, b; Ghorbel et al. 2008; Joshi et al. 2008; Boominadhan et al. 2009).

Biopolymer degrading enzymes from haophilic/halotolerant/haloalakliphilic organisms offer new opportunities for the treatment of oilfield waste containing high salinity with elevated temperatures. Under these conditions, the solubility and, consequently, the accessibility of the substrate are improved. Exploitation of reversed micelles in combination with halophilic enzymes is likely to result in the development of novel applications using these enzymes (Carala and Babu 2004). While the biotechnological applications of enzymes from extremophiles offer great horizons, it's still long way to go to capture the opportunities. Nevertheless, in view of the great potential of biocatalysis, it is quite likely that new concepts will be developed resulting in the application of enzymes from extremophiles.

Bacteria secrete variety of enzymes, many of them being commercially significant. Beside, the patterns of enzyme secretion and characteristics may also suggest on the population heterogeneity in a particular extreme habitat. Enzymes from extreme microbes have great potential for biocatalysis and biotransformation, due to their stability under number of extreme conditions.

Several microbes have been investigated for their ability to secrete these enzymes and over the years, *Bacillus* species have emerged as the key producers of extracellular proteases having potential applications in detergent, food, pharmaceutical, leather and chemical industries, (Berg et al. 1991; Singh 2006; Patel et al. 2005, 2006a, b;

Dodia et al. 2008a, b). During recent years, there has been increasing emphasis on the search and development of enzymes with capabilities to function and maintain stability under multitude of extreme conditions.

The results indicated that different proteolytic bacteria release different amounts or activities of proteases (Dodia et al. 2006, 2008a, b; Joshi et al. 2008; Siddhapura et al. 2010). The proteolytic bacterial communities may play a major role in determining the population dynamics in context with the available nutrition.

19.2.3.2 Biocatalyst from Salt-Tolerant Alkaliphiic Actinomycetes

During the last few years, some extracellular enzymes from halophilic and alkaliphilic bacteria have been studied. However, only limited is known about the enzymatic potential of extremophilic actinomycetes, in general and salt-tolerant alkaliphilic actinomycetes in particular. They have been investigated to secrete a range of extracellular enzymes such as alkaline protease, amylase, lipase and cellulase. The optimum activity of these enzymes usually occurs at high salinity and alkaline pH, making them suitable for many harsh industrial processes. In addition, many of these enzymes are also thermotolerant and can maintain their stability at room temperature for a long period of time.

During the last few years, some extracellular enzymes from salt-tolerant alkaliphilic actinomycetes have been studied (Thumar and Singh 2007a, b, 2009; Kazan et al. 2009).

However, it is evident from the literature that the exploration of enzymatic potential of these microbes is just the beginning and till date only few enzymes are investigated in detail. Salt-tolerant alkaliphilic actinomycetes from the saline habitats along the Coastal Gujarat in India, secreted range of extracellular enzymes including proteases, amylases, cellulases and lipase. Overall, more then 60% of these isolates produced extracellular alkaline proteases, amylases and cellulases at higher salt and pH (Mehta et al. 2006; Thumar and Singh 2007a, b). In addition, they displayed significant production and tolerance in the presence of organic solvents, (Thumar and Singh 2009). An alkaliphilic actinomycete, *Nocardiopsis* sp. TOA-1, has been reported to produce alkaline protease optimally at pH 9–10, (Mitsuiki et al. 2002).

A serine type alkaline protease from *Streptomyces clavuligerus* strain Mit-1 was active and stable in a broad range of pH (Thumar and Singh 2007a). High temperature optimum for enzyme activity was unique for actinomycetes alkaline proteases. The greater thermal tolerance of the protease would favor their applications in temperature sensitive industrial processes. Similarly, enzyme stability in the presence of various surfactants and oxidizing-reducing agent would add to its commercial value. Extreme resistance of alkaline proteases from Mit-1 against chemical denaturation by urea was a unique feature (Thumar and Singh 2007a). This finding is rather unique and restricted to only few proteins. Relatively higher temperature optima for catalysis and significantly higher thermostability of the alkaline proteases also reflect unique features of the enzyme among the salt tolerant alkaliphilic actinomycetes from saline habitats. Since only few enzymes are purified and

characterized from extremophilic actinomycetes, the work on the enzymatic characteristics and stability assumes significance (Esin and Atac 2007). A novel haloalkaliphilic, thermostable serine protease was purified from the extreme halophilic archaeon, *Halogeometricum borinquense* strain TSS101 (Vidyasagar et al. 2006).

With the recent advent of biotechnology, there has been a growing interest and demand for enzymes with novel properties. Considerable efforts have been devoted to the selection of microorganisms via sophisticated screening techniques and process methodology for the production of enzymes with new physiological/physical properties and tolerance to extreme conditions required for the industrial processes.

19.2.4 Molecular Biology and Recombinant Enzymes

Recombinant DNA Technology in conjunction with other molecular techniques is being used to improve and evolve enzymes and opening new opportunities for the construction of genetically modified microbial strains with the selected enzyme. Many newer preparations, such as Durazym, Maxapem and Purafect, have been produced, using techniques of site-directed mutagenesis and/or random mutagenesis. Directed evolution has also paved the way to a great variety of subtilisin variants with better specificities and stability. Knowledge of full nucleotide sequences of the enzyme genes has facilitated the deduction of the primary structure of the encoded enzymes and, in many cases, identification of various functional regions. These sequences also serve as the basis for phylogenetic analysis of proteins and assist in predicting the secondary structure of proteins, leading to the understanding of structure and function relationship of the enzymes.

Maintenance of stability and activity in high salt is major challenge for halophilic and haloalkaliphilic proteins. These enzymes from extremely halophilic archaea and bacteria require high concentrations of salt for their activity and stability and are inactivated in *Escherichia coli* unless refolded in the presence of salts under *in-vitro* conditions.

19.2.4.1 Phylogenetic Diversity of Actinomycetes by Molecular Fingerprinting Technique

The 16S rRNA gene has been widely used for phylogenetic and diversity studies for several reasons. It consists of conserved and variable regions, which allows the development of primers and probes with variable levels of specificity; (Mehling et al. 1995), used 16S rDNA sequencing to determine those regions suitable for detection of streptomycetes, and proposed a genus-specific probe and primers targeting the 16S rRNA gene. 16S rRNA region of our isolates from Coastal Gujarat was amplified with Universal primers in majority, while some of the isolates were amplified by specific primer sets, (Dalsaniya 2009). The amplification with different

primers at different annealing temperatures revealed that the amplification was highly temperature specific. Many reports in literature have highlighted amplification of 16S rRNA gene of actinomycetes with U1 and U2 primer sets, (Suchita et al. 2006; Xue-Chang et al. 2007). Similarly, reports are also indicative of the amplification by StrepB/StrepE, StrepB/StrepF and NF/R specific primer sets, (Gohel et al. 2009; Dalsaniya et al. 2009).

The search for sequence variations in genomic DNA has becomes quite important in the studies related to inherited diseases and genes related to development of cancer. Different methods to detect DNA sequence variations have been developed during the past few years and one of these approaches are denaturing gradient gel electrophoresis (DGGE). This has been shown as quite sensitive method and thus has emerged as a choice in studying mutations in large genes. With respect to its application in the assessment of molecular diversity among the microbial populations, DGGE was carried out with five different primer sets targeting 16S rRNA gene of salt-tolerant alkaliphilic actinomycetes isolated from Coastal Gujarat (Western India), (Dalsaniya et al. 2009). The amplicon size of the isolates amplified with the same primer was quite similar, while it differed in size with other primer sets. It's quite apparent from these findings that DGGE generated valuable information on the group specificity and phylogenetic relatedness of salt-tolerant actinomycetes.

19.2.4.2 Cloning and Over-Expression of Enzymes

A number of alkaline protease-encoding bacterial genes have been cloned and expressed in new hosts, the two major organisms of choice for cloning and over-expression being *E. coli* and *B. subtilis*. Different molecular properties of starch hydrolyzing thermostable archaeal and bacterial enzymes including α-amylase, α-glucosidase, glucoamylase, pullulanase, and cyclodextrin glycosyltransferase have also been studied.

In addition, Nep gene encoding protease from haloalkaliphilic archaeon, *Natrialba magadii* was cloned and sequenced. The similarity search identified that the enzyme was related to serine proteases of the subtilisin family from archaea and bacteria (50–85% similarity). The *nep* gene was expressed in *Escherichia coli* and resulted in production of active Nep protease, although the level of expression was very low. It appears as the first description of a recombinant system that facilitates high level secretion of a haloarchaeal protease.

A new strain of *Bacillus pumuilus*, c172-14 (pBX96) was engineered by introducing the pBX 96 plasmid, carrying the α-amylase *amy* gene, into the host strain of alkalophilic *Bacillus pumuilus* c172 through transformation. The level of alkaline protease production improved by 43% in new strain compared to the parent strain, (Zhang et al. 2008). Structure and function relationship has been elucidated towards the improved enzymatic traits among the enzymes from extremophilic groups, employing protein engineering approaches, such as gene shuffling and chimeric genes, Effect of induction, growth temperature and molecular chaperone has been

assessed on the expression and solubilization of recombinant enzyme, cellobiose phosphorylase from *Cellvibrio gilvus* under *in-vivo* conditions into *E. coli,* (Singh et al. 2010).

Developments in molecular approaches to improve the cloning and expression of suitable genes leading to enhanced solubilization of the expressed proteins from halophilic and other extremophilic organisms in heterologous hosts will certainly lead to number of enzyme-driven catalysis in chemical, food, pharmaceutical and other industrial applications, (Machida et al. 1998; Hayashi 1998; Kim et al. 2005; Machida et al. 2000; Singh et al. 2002; Sato et al. 2010).

19.2.5 Effect of Organic Solvents on Growth and Enzyme Catalysis

Organic solvents are toxic to microorganism, as they disorganize the cytoplasmic membranes and impair vital functions. Index of biological toxicity is estimated by log P_{ow} (common logarithm of partition coefficient of given solvent in a mixture of n-octanol and water). A lower value of log P_{ow} signifies higher toxicity (Inoue and Horikoshi 1991). Tolerance of microorganism and their enzymes to organic solvent focused attention ever since the discovery of toluene-tolerant *Pseudomonas putida* strain IH-2000 (Inoue and Horikoshi 1991). A halophilic archaeal strain EH4, (Betrand et al. 1990; Ward and Brock 1978; Oren et al. 1992; Carala and Babu 2004) was capable of degrading a wide range of n-alkanes and aromatic hydrocarbon in the presence of high salt.

A halophilic archaeal strain, (Betrand et al. 1990; Ward and Brock 1978; Oren et al. 1992; Carala and Babu 2004) was capable of degrading and maintaining growth at wide range of n-alkanes and aromatic hydrocarbon in the presence of high salt, (Kulichevskaya et al. 1991; Zvyagintseva et al. 1995). Few halophilic strains were isolated from a plot of Kalamkass oil field which was saturated with brine and oil, (Fukushima et al. 2005). Further, *Marrinobacter hydrocarbonclasticus* degraded a variety of aliphatic and aromatic hydrocarbons. A halotolerant *Streptomyces* sp. isolated from oil field in Russia, was capable of degrading crude petroleum, (Kuznetnov et al. 1992). With 10–25% (w/v) salt concentration in growth medium, these isolates exhibited their normal growth with the range of alkanes, alcohols, aliphatic and aromatic solvents. Related to these studies, majority of our Haloalkaliphilic isolates from Coastal Gujarat were capable to grow in the presence of solvents with log P_{ow} above 2.5. In the presence of organic solvents, two growth patterns were observed; in pattern A, the growth rate at exponential phase was considerably lower in the presence of organic solvents, but final cell density reached above 80% of control, while in pattern B; both, growth rate and the final cell density were low.

Use of organic solvent as sole carbon source by microbes is efficient approach for bioremediation of solvent polluted land. In this context, halophilic bacteria isolated from Seminol County in Oklahoma, USA, referred as Sem-2, used benzene as sole carbon source, (Carala and Babu 2004). It also degraded toluene, ethylbenzene

and xylenes as sole carbon source. Among all tested compounds, toluene was most favored. A Haloalkaliphilic bacterial strain, isolated in our laboratory, *Halomonas venusta* Kh-10-9_1 was capable to use n-hexane up to 20% (w/v) as a sole carbon source.

With comparatively more negatively charged amino acid residues on their surface, halophilic and haloalkaliphilic enzymes have greater possibility to maintain stability in the presence of organic solvents. Solvent tolerance of a halophilic extracellular α-amylase has been reported. The enzyme was produced by Haloarchaeon, *Haloarcula* sp. strain S-1. This amylase exhibited maximal activity at 50°C in 4.3 M NaCl, pH 7. Similarly, an extra cellular protease produced by haloalkaliphilic archaeon *Natrialba magadii* (Diego et al. 2006) was active and stable in aqueous-organic solvent mixture containing 1.5 M NaCl and glycerol, Dimethylsulhooxide (DMSO), N,N-dimethyl formamide, propylenglycerol. Amylase from *Halomonas aquamarina* Ve_1-10-8_3 isolated from Okha, Gujarat exhibited its maximal activity at 50°C with 10% (w/v) salt and pH 9, our unpublished work. A metalo-protease from moderate halophilic bacterium *Salinivibrio* sp. strain AF-2004 also exhibited tolerance against organic solvents. The optimum temperature and salinity of the enzyme were at 55°C and 0–0.5 M NaCl, although at salinities up to 4 M NaCl, the enzyme was still active. The protease was stable and had a broad pH profile (5.0–10.0) with an optimum 8.5 for casein hydrolysis. Attempts are being made in our laboratory to search for solvent tolerant proteases and other enzymes from the haloalkaliphilic bacteria and large number of potent isolates have been screened and identified. These enzymes tolerated 10–20% (w/v) of alkanes, alcohol and aliphatic solvents at 8–10 pH and 10% (w/v) salt.

The application of alkaline proteases and other enzymes is well documented and its operation in organic media is an interesting area for research and applications in biotechnology. Generally, alkaline proteases are thermally stable in the range of 37–70°C. However, alkaline proteases from haloalkaliphilic organisms in biphasic medium have been studied in only limited sense.

Large number of Haloalkaliphilic bacterial isolates obtained from saline habitats of coastal Gujarat in western India, were able to grow and secret enzymes in the presence of different organic solvents; such as butanol, methanol, n-hexane and propanal. The findings are indicative of future applications of these biocatalysts. The stability of the alkaline proteases in the presence of organic solvent would be an attractive feature of the biocatalysis. Peptide synthesis by proteases under non-aqueous conditions projects one of the interesting aspects of biocatalysis.

19.2.6 New Opportunities for Bioremediation

As all other ecosystems, the saline environments are affected by pollution. However, only limited information is available on the biodegradation of hydrocarbons in saline environments (Ward and Brock 1978; Diaz et al. 2000; Kargi and Dincer 2000). In addition, it is estimated that 5% of industrial effluents are saline and hypersaline

in nature. Therefore, conventional non-extremophilic microorganisms are unable to efficiently perform the removal of organic pollutants at high salt concentrations.

Halophilic microorganisms, such as *Bacillus naphthovorans strain MN-003, Staphylococcus sp. MN-005, Micrococcus sp. MN-006, Neptunomonas naphthovoransBoth NAG-2 N-126; Mycobacterium vanbaleenii PYR-1* are metabolically different and are adapted to extreme salinity (Hedlund et al. 1999; Zhuang et al. 2002, 2003). These microorganisms are good candidates for the bioremediation of hypersaline environments and treatment of saline effluents. Moderately halophilic/halotolerant bacteria and the extremely halophilic bacteria have a broader catabolic versatility and capability than previously thought, (Kuznetsov et al. 1992; Huu et al. 1999; Nicholson and Fathepure 2004, 2005).

The work in our own laboratory has generated profile of various PAH degrading bacteria able to grow in naphthalene, anthracene as well as pyrene as the sole source of carbon and energy. The isolates, OM-4, detected as *Exiguobacterium aestuarii* (Gene Bank Accession Number: AY594265), PM-8 identified as *Acinetobacter junii* (GeneBank Accession Number: X81664) and PAH-8, detected as *Micrococcus luteus* (GeneBank Accession Number: AY881238) were shown to be potential PAH degrader and were isolated from Okha-madhi, Porbandar-Madhavpur and Rajkot, respectively. Isolates were accessed for their PAH degradation, biochemical properties, salt tolerance, effect of carbon sources and antibiotic resistance, (Akbari and Singh 2008, 2009).

19.2.7 Metagenomics

19.2.7.1 Concept

It's estimated that approximately 99% of the microorganisms in nature are not cultivable by standard cultivation techniques. Isolation of total genomic DNA (metagenomic DNA) from environment is a recent approach in molecular biology (Bach et al. 2001; Acevedo et al. 2008; Purohit and Singh 2009a; Siddhapura et al. 2010). The metagenomic approaches highlight the population heterogeneity and phylogenetic status of a habitat in totality (Gabor et al. 2003; Desai and Madamwar 2007). To this novel approach, bioinformatics based softwares and tools have added impetus (Parks and Beiko 2010).

19.2.7.2 Approaches and Techniques

From the DNA extracted from an environmental sample, a genomic library containing pieces of the genomes of all the microbes is constructed. The metagenomics has two approaches to proceed with:

Sequence-Based Metagenomics
In the late 1980s, the direct analysis of rRNA gene sequences had shown that the vast majority of microorganisms present in the environment had not been captured

by culture-dependent methods. Even with the recent success of novel and high throughput culturing strategies; we are still unable to mimic most microbial environments sufficiently to induce growth of many environmentally relevant microbes. It focuses on the genomic resource of a dynamic microbial community, rather than on individual strains of microbes or individual genes and their functions.

Function-Based Metagenomics
It explores and aims at the specific products from the microbes in a community and researchers screen metagenomic libraries for various molecules; such as biocatalysts, vitamins or antibiotics. Through this method, the functions that are largely unknown so far, can be explored. Moreover, metagenomics focuses on microbial community as a system that has functional properties beyond individual genes or individual microbes. Metabolic cascades, for example, can be distributed over different members of multi-taxa communities.

19.3 Analysis

Haloalkaliphilic bacteria, a group of organisms with twin extremities of pH and salinity, have traditionally been investigated from variety of saline habitats. During the last several years, we have focused on the diversity and enzymatic potential of haloalkaliphilic bacteria from the natural and man made saline habitats along the Coastal Gujarat in India. Our studies revealed wide occurrence of these organisms reflecting significant diversity in these ecological niches. While haloalkaliphilic bacteria have focused considerable attention during the last several years, majority of such studies relate to diversity and phylogenetic analysis and only limited attention has focused on other aspects, such as adaptive features and biotechnological potential. Our studies over the last few years, have indicated wide occurrence of the extracellular enzymes, alkaline proteases being in particular (Thumar and Singh 2007a, b; Dodia et al. 2008a, b; Joshi et al. 2008). The occurrence and diversity of extracellular enzymes revealed that they could also be useful as marker to judge the microbial heterogeneity among the haloalkaliphilic bacteria. Majority of these enzymes are also thermostable in nature where salt acted as positive effectors.

The application of halotolerant-alkaliphilic actinomycetes and their metabolites in industrial processes has opened a new era in biotechnology. Further, the studies on alkaliphiles have led to the discovery of many enzymes that exhibit interesting properties. The advances in the application of alkaliphilic- or alkalitolerant-based biomolecules during the past 20 years are in the center stage due to the introduction of proteolytic enzymes in the detergent industry. Industrial applications of alkaliphiles have been investigated and some enzymes have been commercialized. Of the enzymes now available to industry; proteases, cellulases, lipases and pullulanases are by far the most widely employed. The combination of extreme conditions under which the biocatalysts are able to function would be of particular interest for developing novel processes and investigating structure and function relationship.

For non-cultivable microbes, during the last 10 years, numbers of protocols for DNA extraction from environmental sample have been reported, and commercial soil DNA extraction kits are also available. Many protocols for isolating total DNA from environmental sample have been developed, which are broadly classified as direct and indirect methods. The suitability of the methods is judged on the basis of shearing, purity and quantity of the extracted DNA. The methods described for the saline soil reflected that although it would be applicable for variety of saline soils, no specific treatment would require. Further, the extracted DNA would be suitable for the applications in molecular biology (Purohit and Singh 2009b; Siddhapura et al. 2010).

19.4 Future Prospects

New tools and techniques for exploring new microbial resources through culture dependent conventional approaches and culture-independent metagenomic tools provide great opportunities to bring biomolecules for varied applications. The objectives, however, vary from directed product discovery to ecosystem analysis. Metagenomics has redefined the concept of a genome and has added enormously to the gene discovery. Although, the field of microbial biotechnology is quite diverse, only limited attention has been paid to haloalkaliphiles particularly from moderately saline habitats. To explore such habitats, we studied haloalkaliphilic bacteria with respect to diversity, distribution and production of extracellular enzymes. However, the larger umbrella of genomics and proteomics is yet to come up with respect to the microbial diversity of saline habitats. Work on these microbes is the beginning to long journey and further studies would provide information and deeper insights into the adaptation to extremity as well potential avenues for biotechnology. The future studies would focus on the molecular phylogeny and population dynamics, structural basis of protein stability under extreme conditions, development of expression systems, over-expression and protein folding.

19.5 Conclusions

Advances in recombinant DNA tools, high-throughput technologies, genomics and proteomics have fuelled the development of new catalysts. In particular, gene cloning and directed evolution have emerged as powerful tools for biocatalyst engineering in order to develop enzymes with novel properties, even without requiring the knowledge of enzyme structure and catalytic mechanisms. Extremozymes from halophilic/haloalkaliphilic bacteria and actinomycetes will definitely offer new opportunities for biocatalysis and biotransformation. Both, the discovery of new extremophilic organisms from varied habitats and the determination of genome sequences from soil and other sources via metagenomics will provide a route to new enzymes and metabolites, with the possibilities towards novel applications, including

non-aqueous catalysis and poly aromatic hydrocarbon (PAH) degradation. Gene cloning, over-expression of proteins and directed evolution would improve enzyme stability to suit harsh application conditions.

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Chapter 20
Environmental Impact from the Use of Bt Toxin

Sandeep B. Gaudana and Tamishraha Bagchi

Abstract Use of chemical pesticides was one of the key factors in the success of the green revolution. The wide spread and unregulated use of chemical pesticides however, has led to harmful effects on both agricultural land and humans. Compared to chemical pesticides, the potential benefits such as specificity, biodegradability and safety, associated with biopesticides had projected them as a viable alternative to chemical pesticides. The Cry toxins of *Bacillus thuringiensis* (Bt) are the most popular and widely used biopesticide covering more than 90% of the biopesticide market. Their use has afforded positive economic benefits to the producers and has also led to a huge reduction in the use of chemical pesticides. Recombinant DNA technology has also allowed us to develop transgenic Bt crops. However, the problems associated with the use of cry proteins remain to be fully addressed, understood and tackled. The effect on non-target insects and the resulting imbalance in the natural food chains, development of resistance in the target insects and adverse health effects including allergy and immunogenicity are the key phenomena which need immediate attention. This chapter will attempt to bring out the various problems associated with the use of Bt toxin; with respect to effect on the target as well as non-target insects, and what is known about its impact on human health and other associated issues.

Keywords *Bacillus thuringiensis* • Environmental impact • Immunomodulation • Non target organisms • Cry toxin • Development of resistance

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20.1 Introduction

What has been a technological development for mankind, has in some or the other way invariably been a disturbance to Nature. So, in the process of satiating our ever growing need for growth, we have caused severe damages to our natural resources and then the quest to possibly repair the same arises. The utilization of land for agriculture and its alteration for increased yields is one of the major human interventions in the natural environment. Between 1700 and 1980, the total area of cultivated land worldwide had increased by 466% (Meyer and Turner 1992). The employment of chemical fertilizers, pesticides, irrigation and mechanization jointly lead to remarkable and unprecedented intensification of agriculture, leading to magnificent augmentation of agricultural produce. The process of Green Revolution began in the 1960s, with the development and dissemination of high-yielding seeds. But for the green revolution, it would have been practically impossible to overcome the challenge of meeting the food demands of the rapidly exploding global human population. Because of the green revolution, agriculture has met the food needs of most of the world's population even as the population doubled during the last four decades of the twentieth century.

Agricultural advancement has been palpable both in the developing as well as developed nations. But then the realization prevailed that the price paid in terms of damage to environment and human health was heavier than the benefits reaped from the used of the chemical pesticides. An alternate to this was sought from biopesticides; the predominant candidate being *Bacillus thuringiensis* (Bt). This chapter aims at addressing various issue related to the utilization of Bt toxin.

20.2 Sustainability of Agricultural Intensification

On one hand when the success of agricultural intensification is undoubtedly immense, the shortcomings of the same haven't been far behind. The need for progression in agricultural yields is far from over as it has to constantly keep pace with the increasing human population. At the same time, the questions started to be raised on the long term sustainability of the agricultural intensification practices as the negative impacts associated with them started to surface. The sustainability of high-intensity agriculture becomes questionable because of the loss of soil fertility, the erosion of soil, the increased incidence of crop and livestock diseases, and the high energy and chemical inputs associated with it. The major concerns raised from advanced agricultural practices that were followed in the latter half of the twentieth century are increased erosion, lower soil fertility, and reduced biodiversity; negative regional consequences, such as pollution of ground water and eutrophication of rivers and lakes; and negative global consequences, including impacts on atmospheric constituents and climate. The use of chemical pesticides had been one of the key

factors positively affecting the success of the green revolution. With the wide spread and unregulated use of chemical pesticides, the earlier unforeseen hazards resulting from their use started to surface. Killing of non-target insects, toxicity to humans, recalcitrance and resultant presence and persistence in environment are the major harmful outcomes of the chemical pesticides. Various technologies that have been developed and applied for genetically improving the quality of crops include the artificial manipulation of chromosome number, the development of addition and substitution lines for specific chromosomes, chemical and radiation treatments to induce mutations and chromosome rearrangements, as well as cell and tissue culture approaches such as embryo rescue, in vitro fertilisation and protoplast fusion to allow the recovery of interspecific and generic hybrids (Conner et al. 2003). These techniques have in turn enabled the improvement in yields and the incorporation of desired traits in the cultivars. The genetic gains from the integration of these technologies into mainstream plant breeding have substantially improved the performance of the resulting cultivars. However, the conventional techniques of genetic manipulations presented limitations due to the fact that the process of generating the crop of desired character using the conventional techniques lasted for an uncertain period, since the crosses have to be carried out till the generation showing desired character is attained. Moreover, the specificity of the outcome of the conventional techniques also remained uncertain. In addition the transfer of traits was restricted within the chromosome of the same species and hence it was not possible to transfer the characters to and from different and distant species. The advent of recombinant DNA technology provided the opportunity to overcome these shortcomings allowing the plant breeders to respond more quickly to increasing consumer demands. The advancement of transgenics opened up immense possibilities as it provided with the possibilities of transfer of traits between virtually any life forms.

Although there are many applications of genetic engineering in agriculture, the major focus of biotechnology has remained in generating transgenic crops such as herbicide resistant crops and pest and disease resistant crops. According to an estimate there are 67,000 pest species that damage crops out of which 9,000 species are insects and mites (Ross and Lembi 1985). The pest incurred loss in major crops amounts to 52% in wheat, 58% in soybean, 59% in maize, 74% in potato, 83% in rice and 84% in cotton (Oerke et al. 1994). In addition to this direct loss the indirect loss occurs due to impaired quality of produce, increased cost of production resulting from additional costs of pesticides and their application. Transgenic plants expressing biopesticide presented a solution to the above drawback associated with the use of chemical insecticides. Expression of gene coding for *Bacillus thuringiensis* (Bt) in tobacco and tomato were the pioneering examples of genetically engineered plants for insect resistance (Barton et al. 1987; Vaeck et al. 1987). Subsequently, many Bt genes have been expressed leading to development of transgenic tobacco, potato, tomato, cotton, brinjal and rice to name a few (Kumar et al. 2008).

20.3 Critical Review of the Impact of the Use of Bt Toxin

20.3.1 History and Recent Status of Bacillus Thuringiensis as Biopesticide

Bt is an aerobic, spore-forming, Gram positive bacterium which produces a number of toxins, including insecticidal crystal proteins (Cry proteins), vegetative insecticidal proteins (Vips), and β-exotoxins (Schnepf et al. 1998). Berliner (1911) had isolated a *Bacillus* species from the Mediterranean flour moth, *Anagasta kuehniella*, and named it after the province Thuringia in Germany where the infected moth was found and thereby became the first one to describe *Bacillus thuringiensis* (*Bt*). However, the credit for the first isolation of *Bt* lies with a Japanese biologist, Ishiwata Shigetane who way back in 1901 discovered a previously undescribed bacterium as the causative agent of a disease of silkworms. The first commercial *Bt* products were simply fermentation cultures of isolates having similar host specificity and potency as the original isolates. The earliest commercial use of *Bt* began in France in the year 1938 under the name Sporeine. The first commercial product in the United States, Thuricide, appeared in 1957. In the early days of its use *Bt* was known to be pathogenic only to very specific species in the Order Lepidoptera (moths and butterflies) and that too only to young larvae. In 1970, a new isolate of *Bt* was discovered that was up to 200 times more active against pests targeted by the existing *Bt* products. This new isolate, which represented a new subspecies, was called *kurstaki* and was designated as HD-1. *Bt kurstaki* HD1 became the benchmark for comparing the potency of all future *Bt* isolates. Further *Bt* isolates were discovered and found to be variably pathogenic to different species of the Order *Lepidoptera*. *Bt* was not considered a general insect pathogen, but during the 1970s a strain toxic to primitive flies of the Order Diptera (mosquitoes and blackflies) was isolated and named subspecies *israelensis*. By 1980, a commercial product was being sold for control of mosquito and blackfly larvae; aquatic invertebrates and fish were unaffected by this new strain (Becker and Margalit 1993). In 1982, a new *Bt* strain named subspecies *tenebrionsis* was isolated from a dead pupa of the yellow mealworm beetle, *Tenebrio molitor* (Order Coleoptera) (Keller and Langenbruch 1993). *Bt tenebrionsis* was particularly pathogenic to beetles in the Family Chrysomelidae (a.k.a. leaf beetles, which includes the Colorado potato beetle, *Leptinotarsa decemlineata*). Today approximately 280 unique *Bt* strains have been isolated from insects, soils, foliage, and grain dust (Crickmore et al. 2010). New strains are differentiated by the characteristics of their crystalline protein, its gene sequence, and its spectrum of insecticidal activity. All the strains have been organized into major groupings depending on their spectrum of insecticidal activity. Schnepf and Whiteley (1981) were the first to clone and characterize the gene coding for the crystal proteins from the plasmid DNA of *Bt kurstaki* HD1. Many other cry genes were cloned in immediate succession. The accomplishments followed with the successful demonstration that plants can be genetically engineered, which finally lead to Bt cotton reaching the market in 1996.

20.3.2 Structure and Nomenclature of Cry Toxins

The general structure of Cry toxins can be understood with reference to the amino acid sequence alignment of Cry toxins reported by Crickmore et al. 2010 (Fig. 20.1) and the crystal structure of Cry1Aa toxin reported by Li et al. 1991 (Fig. 20.2). According to the crystal structure, Cry toxins are globular molecules comprising of three distinct domains; the consecutive domains connected by a single linker. In Cry1Aa domain I extends from residues 33–253 amino acids and is a seven α-helix bundle in which a central helix (helix α-5) is completely surrounded by six outer helices. This domain is understood to be involved in channel formation in the membrane of gut epithelial cells of the target insect. The six α-helices are amphipathic and are long enough to span the 30 Å-thick hydrophobic region of a membrane bilayer. Domain II is formed by 265–461 amino acid residues in Cry1Aa. This domain consists of three anti-parallel β-sheets with similar topologies packed around a hydrophobic core. Domain II is understood to play a key role in specificity determination, since this domain being swapped between related toxins Cry1Aa and Cry1Ac, resulted in the chimeric proteins with altered specificity (Schnepf et al. 1990). Domain III comprises of 501–644 amino acid residues and is a β-sandwich of two anti-parallel β-sheets. This domain is understood to be involved in various functions like structural stability, ion channel gating, binding to brush border membrane vesicles (BBMV) and determination of insecticidal specificity (Schnepf et al. 1998; Masson et al. 2002).

Hofte and Whiteley (1989) made the first attempt to systematically organize the genetic nomenclature that relied on the insecticidal activities of crystal proteins for the primary ranking of their corresponding genes. According to this system of classification, the *cryI* genes encoded proteins toxic to lepidopterans; *cryII* genes encoded proteins toxic to both lepidopterans and dipterans; *cryIII* genes encoded proteins toxic to coleopterans; and *cryIV* genes encoded proteins toxic to dipterans alone. A revised system of classification was proposed by Crickmore et al. (1998); the system is based upon the model used for cytochrome P-450 superfamily nomenclature, the underlying basis for which is to assign names to members of gene superfamilies according to their degree of evolutionary divergence as estimated by phylogenetic tree algorithms. In the revised nomenclature, the primary rank of the toxin is denoted by Arabic numerals in place of the Roman numerals in the preceding system. They used the freely available software applications CLUSTAL W and PHYLIP. The deduced amino acid sequences of the full-length toxins were aligned using CLUSTAL W and a distance matrix was produced, quantitating the sequence similarities among the set of toxins.

20.3.3 Receptors for Cry Toxins

The presence of a specific Cry toxin binding receptor(s) on the epithelial cells of insect midgut is an important determinant for susceptibility of a particular insect to

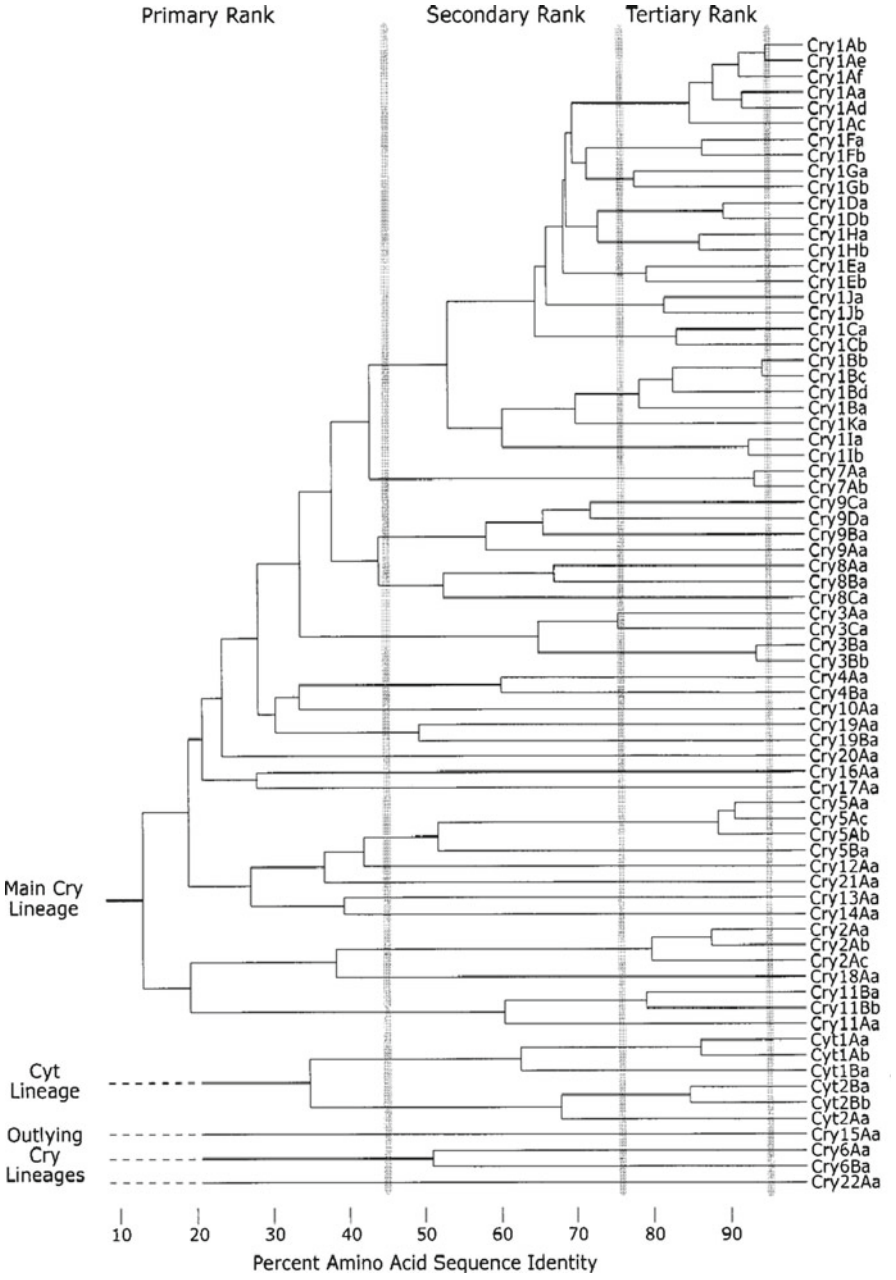

Fig. 20.1 Phylogram demonstrating amino acid sequence identity among Crystal (*Cry*) and Cytolytic (*Cyt*) Bt toxins (Crickmore et al. 2010)

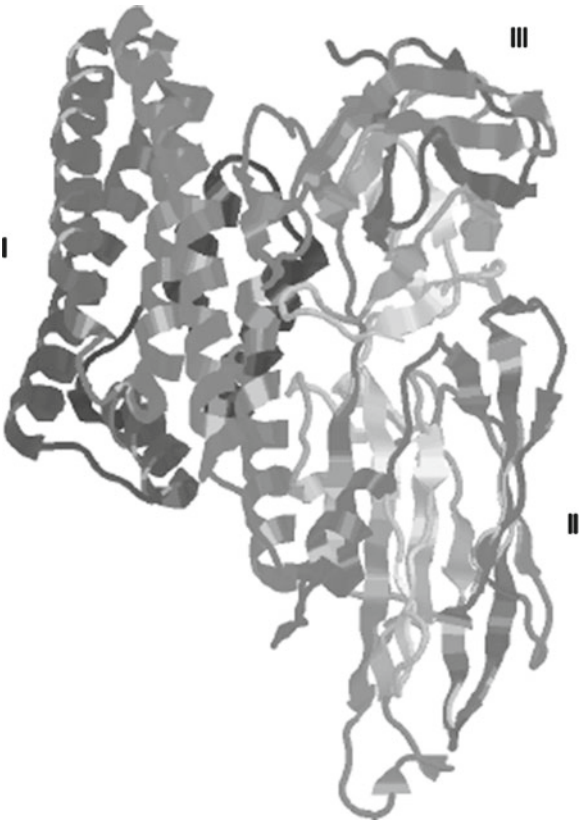

Fig. 20.2 Three dimensional structure of an activated toxin, Cry1Aa (Li et al. 1991). The toxin has three structural domains: Domain I, Domain II and domain III

the respective toxin. The correlation between binding and toxicity was first demonstrated using BBMV prepared from microvilli by use of a technique developed by Wolfersberger et al. (1987).

The two principal classes of receptors widely accepted to a play key role in the function of Cry toxins are aminopeptidase N (APN) and cadherins.

20.3.3.1 APN

The APN family is a class of enzymes that cleaves neutral amino acids from the N terminus of polypeptides. They serve a variety of functions in a wide range of species, but in the lepidopteran larval midgut, they work in cooperation with endopeptidases and carboxypeptidases to digest proteins derived from the insect's diet. In addition to being studied for their role in digestion, APNs have been extensively studied as putative Cry toxin receptors (Pigott and Ellar 2007). Lorence et al. (1997) demonstrated that cleavage of APN by phosphatidyl-inositol specific phospholipase C treatment which cleaves out the GPI anchored proteins, drastically reduced the pore forming activity of the toxin assayed in BBMV from *Trichoplusia ni* (cabbage looper).

Similar treatment significantly decreased the levels of Cry1Ab incorporation into insoluble lipid raft membranes (Zhuang et al. 2002). Jenkins et al. (2000) proposed a sequential binding model for interaction of Cry1Ac toxin with APN. In this model, APN is first recognized by domain III of Cry1Ac through the GalNAc moiety, followed by a protein-protein contact of the domain II loop region of Cry1Ac. The first contact is fast and reversible, and mutations close to a domain III cavity affect this initial binding, while mutations in domain II affect the rate constants of the second interaction step which is slower and tighter (Jenkins et al. 2000). APNs belong to the zinc binding metalloprotease/ peptidase superfamily and to a subfamily called the gluczincins. Members of this family are characterized by the short zincin motif HEXXH, where X stands for any amino acid, followed by a conserved glutamic acid residue 24 amino acids downstream from the first histidine.

The histidines and the last glutamic acid residue serve as zinc ligands, while the first glutamic acid residue is important for enzyme catalysis. A highly conserved GAMEN motif is also believed to form part of the active site (Pigott and Ellar 2007). Cry1 proteins including Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ba, Cry1C,a and Cry1Fa which are toxic to lepidopterans, have been shown to bind to APNs.

20.3.3.2 Cadherin

The cadherin superfamily of proteins is highly diverse and serves a variety of functions, including cell adhesion, migration, cytoskeletal organization, and morphogenesis. Classical cadherins have 5 cadherin repeats but as many as 34 repeats have been reported (Pigott and Ellar 2007). Some cadherins also have mucin, laminin, or epidermal growth factor-like repeats. The proteins are glycosylated and are usually anchored to the membrane by a single transmembrane domain, although more transmembrane or GPI-anchored variants have also been identified (Vestal and Ranscht 1992). The first cadherin-like protein shown to interact with Cry toxins, BT-R1, was a 210-kDa glycoprotein identified in *M. sexta* BBMV (Vadlamudi et al. 1993). Later, they were shown to be involved in Cry toxin binding and toxicity in several other lepidopteran species such as *Bombyx mori*, *H. virescens*, *H. armigera*, *Pectinophora gossypiella*,*Ostrinia nubilalis* and more recently in the dipteran *An. gambiae* (Soberon et al. 2009). All of these have been shown to have a similar domain organization: an ectodomain formed by 9–12 cadherin repeats, a membrane proximal extracellular domain, a transmembrane domain, and a cytoplasmic domain (Pigott and Ellar 2007). BT-R1 cloned and expressed on the surface of COS-7 cells rendered the cells susceptible to Cry1Ab (Derbyshire et al. 2001).

20.3.4 Impact of the Use of Transgenic Bt Crops on Farmers

The transgenic crops expressing Bt toxin have a direct bearing in terms of its impact on economy as well as health of the farmers. The economic benefits of the use of Bt

have been due to the increased yields resulting from better protection of the crops from pests and hence reduced loss of the agricultural produce. Another influencing factor is the reduction in the amount of chemical pesticides required to be sprayed on the Bt crops as compared to the non-Bt crops. Apart from the direct benefit from the reduced use of chemical pesticide, the economic benefit also results out of the savings from lesser fuel and labour required for the pesticide application. The positive impact on the health of the farmers is reflected in the significantly reduced instances of poisoning from chemical pesticides in case of the farmers who adopted the Bt varieties of crops in comparison to the non-adopters. Most of the studies involving the impact of the transgenic Bt crops on the farmers have shown a favourable effect of these crops on the economic state and health of the farmers. Bennett et al. (2004) analysed the impact of the use of Bt cotton on the farmers of India and found that in the first 2 years after the introduction of Bt cotton in India, there was a 72% and 83% reduction in the use of chemical insecticide against bollworm in the year 2002 and 2003 respectively. In 2002, the average increase in yield for Bt over non-Bt was about 45%, while in 2003 this increased to 63%. The overall economic gain by the Bt cotton growers in reflected from the fact that the average gross gain for the Bt adopters was higher by 49% in 2002 and 74% in 2003 than the non-adopters (Bennett et al. 2004). Pray et al. (2002) have opined that the farmers in China have found the Bt cotton hugely beneficial since it has enabled them to increase their yield per ha and reduce pesticide costs, the time spent spraying dangerous pesticides, and the number of incidences of pesticide poisoning. Bennett et al. (2006) found in a large scale survey on the impact of Bt cotton adoption by resource-poor smallholder cotton farmers in South Africa, that the adopters of this technology benefitted in terms of higher yields, lower pesticide use, less labour for pesticide application and substantially higher gross margins per hectare.

20.3.5 Presence of Bt in Soil

There is a concern regarding the exudation of the Bt toxin from the roots of the transgenic plants expressing Bt toxin and regarding the persistence of the same in the soil. There are no two opinions about the fact that the Bt toxin does get released from the roots of the transgenic Bt plants and is detectable in the soil (Saxena and Stotzky 2000). Wang et al. (2007) studied the degradation of Cry1Ab protein from transgenic rice in five Paddy soils and in aqueous solution, so as to mimic the aerobic and anaerobic conditions respectively. They observed that degradation of the Cry1Ab protein was significantly prolonged under flooded conditions compared with aerobic conditions, with half-lives extended to 45.9–141 days. They further observed that the pH was one of the prime factors when degradation was studied in aqueous conditions; however the degradation in soil was not related to any specific soil property. In contrast to this, the degradation of Cry3Bb1 released into the soil from transgenic Bt corn was significantly influenced by the mineral content and the pH of the soil (Icoz and Stotzky 2008). However, the authors concluded that

irrespective of the conditions, the toxin did not persist for a long period in the soil and got rapidly degraded. The authors therefore concluded that the Cry1Ab protein, when introduced into a paddy field upon harvest, will probably undergo rapid removal after the field is drained and exposed to aerobic conditions. In contrast to this, Saxena and Stotzky (2000) noted that the Bt toxin remains active in the soil, where it binds rapidly and tightly to clays and humic acids. The bound toxin does retain its insecticidal properties and is protected against microbial degradation by being bound to soil particles, persisting in the soil for as long as 234 days. The authors also cautioned that such persistence of the toxin in its bound form may escape detection, but could play an adverse role by selecting the resistant target insect population and also by harming the non-target organisms.

20.3.6 Development of Resistance in Target Insects

Development of resistance against Bt toxin in the target insects is one of the major challenges for sustainable use of this technology as it would render a toxin ineffective against that insect pest. Evolution of resistance is defined as a genetically based decrease in a population's susceptibility to a toxin (Tabashnik et al. 1994). To understand the mechanisms of insect resistance to Cry toxins, the general mode of action of these toxins needs to be understood. Although until recently there was a general acceptance about Cry toxins acting by pore-forming mechanism, recently however, a signal transduction based model has been proposed as an alternative (Soberon et al. 2009). Cry toxins have been understood to form pores following insertion into the membrane, causing osmotic lysis of the midgut epithelial cells in their target insect. However, Zhang et al. (2006) proposed that Cry toxins activate a cascade of G protein mediated signal pathway. Both models include identical initial steps. In these steps, the protoxin is solubilized in the insect gut lumen followed by activation of solubilised toxin by the midgut proteases and finally binding of the toxin to the cadherin receptor, which is understood to be the primary receptor for Cry toxins. In the pore forming model, the interaction with cadherin initiates the cleavage of helix α-1, leading to toxin oligomerization. The oligomeric Cry toxin binds to GPI anchored receptors which help in toxin insertion into the membrane. The resultant pore that is formed is important for cell death. Rausell et al. (2004) hypothesized that the oligomer, in contrast to the monomer, is able to interact efficiently with phospholipid membranes and to form stable pores. The schematic representation of the process of oligomerization is depicted in Fig. 20.4. In the signal transduction model, toxin interaction with cadherin activates a G protein that increases the activity of adenylyl cyclase (AC), resulting in increased cAMP levels which in turn activate protein kinaseA(PKA) that is responsible for the activation of an intracellular pathway resulting in cell death. A schematic representation of the mode of action of Cry toxins in describing both the pore formation as well as the signal transduction models and the possible modes of development of resistance, is depicted in Fig. 20.3. In theory, disruption of any step in the pathway for the mode of action of the toxin

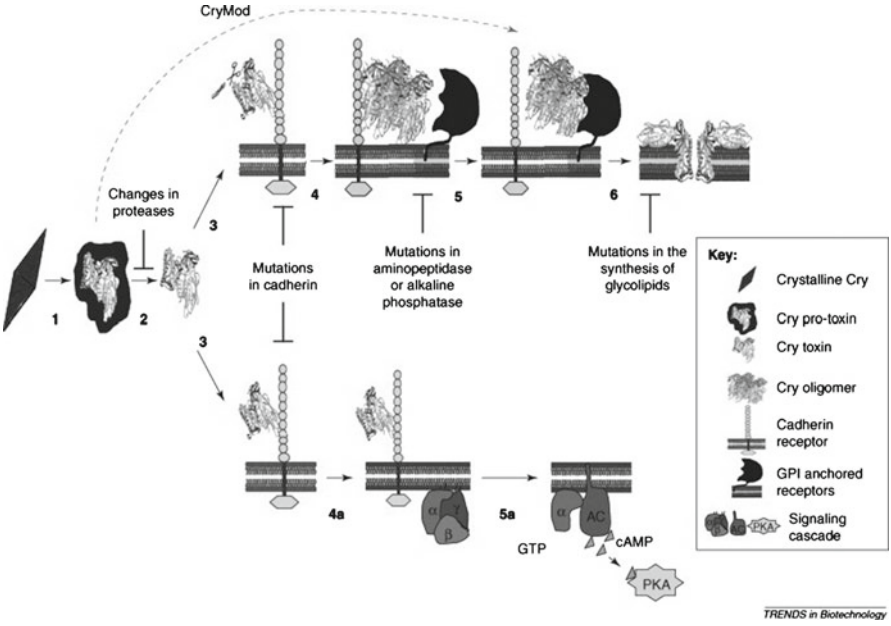

Fig. 20.3 Schematic representation of the models of the mode of action of 3 domain Cry toxins and resulting mechanism of resistance (Bravo and Soberon 2008)

could cause insect resistance. In general, the most frequently observed mechanism of Cry toxin resistance involves defects in receptor binding, followed by resistance owing to defects in protease production, elevated immune response or enhanced esterase production. Ma et al. (2005) demonstrated that feeding a sub-lethal concentration of Cry1Ac toxin to *H. armigera* led to tolerance to Cry1Ac toxin. This could be correlated with an elevated immune response associated with the production of pro-coagulant hexamerin that recognizes and forms specific aggregates around the toxin molecules. The various insect pests known to have developed resistance against the Bt toxins and the mechanism underlining the development of the resistance have been listed in Table 20.1. Evolution of resistance is a genetically based decrease in a population's susceptibility to a toxin. Because of concerns that insects would evolve resistance to Bt crops, a resistance management tactic, the 'high dose plus refuge strategy', was mandated in several countries including the USA. This strategy entails the use of Bt crops that express high concentrations of Cry toxins and the planting of refuges of non-Bt crops near Bt crops. Global monitoring data of six lepidopteran pests has indicated that during the first decade of Bt crops, resistance evolved in some populations of *Helicoverpa zea* but not in any of the other five species analyzed viz. *H. armigera, H. virescens, O. nubilalis, P. gossypiella* or *S. nonagrioides* (Bravo and Soberon 2008). The patterns observed in the field are consistent with projections based on refuge strategy theory and suggest that refuges have helped to delay resistance. With the increasing use of Bt crops, particularly in

Table 20.1 Description of some insect pests and nematodes resistant to Cry toxins (Bravo and Soberon 2008)

Scientific name	Common name	Main affected crops	Resistance to cry toxins	Mechanism of resistance
Caenorhabditis elegans	Nematode worm	No effect	Cry5B	Defects in glycolipid synthesis
Culex quinquefasciatus	Mosquito	No effect	Cry4A, Cry4B, Cry11Aa	Unknown
Diatraea saccharalis	Sugarcane borer	Corn, sorghum, sugar	Cry1Ab	Unknown recessive
Ephestia kuehniella	Mediterranean flour moth	Stored flours	Tolerance to Cry1A, Cry2A	Tolerance owing to overproduction of lipophorin
Helicoverpa armigera	American bollworm	Cotton, beans, corn, sorghum	Cry1Ac	Lack of cadherin receptor. Overproduction of esterases and hexamerin
Helicoverpa zea	Corn earworm	Corn, cotton, tobacco, tomato	Cry1Ac	Unknown
Heliothis virescens	Tobacco budworm	Cotton, corn, tomato	Cry1Ac, Cry2Aa	Lack of cadherin and alkaline-phosphatase receptors. Defects in proteases
Pectinophora gossypiella	Pink bollworm	Cotton	Cry1Ac, Cry1Ab	Lack of cadherin receptor
Plodia interpunctella	Indianmeal moth	Meals, flours, nuts	Bt subsp. *entomocidus*	Defects in midgut proteases
Plutella xylostella	Diamond-back moth	Brassicae, crucifereae	Cry1Ac, Cry1Ab	Unknown, recessive
Spodoptera exigua	Beet armyworm	Rice, sugar beet, cotton, tomato	Cry1C	Lack of aminopeptidase 1
Trichoplusia ni	Cabbage looper	Brassicae, crucifereae	Cry1Ac	Unknown, recessive

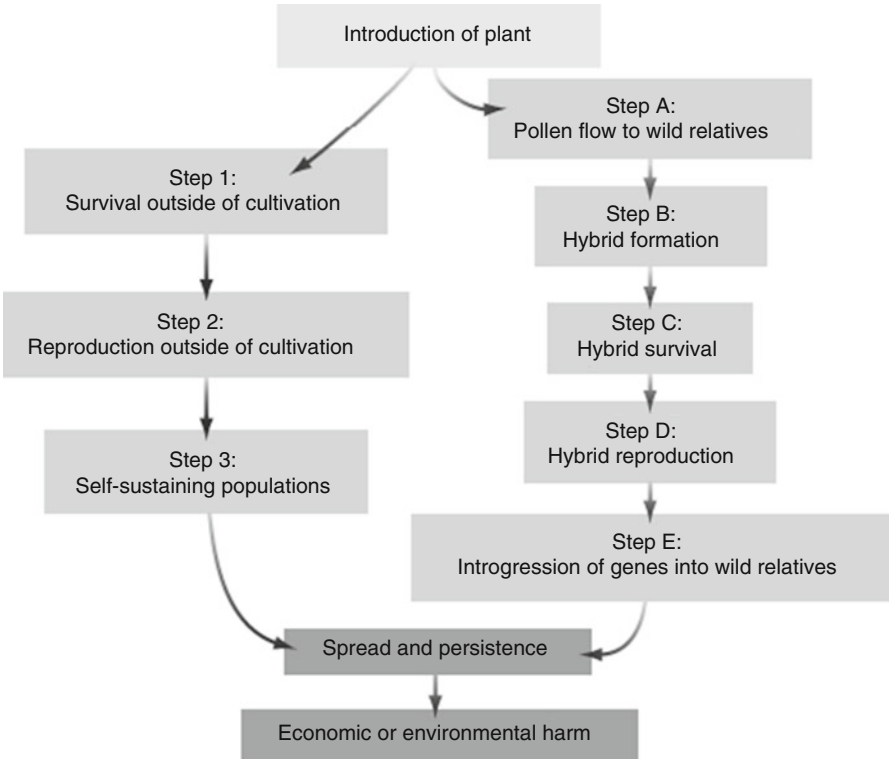

Fig. 20.4 Flow chart showing steps for possible invasion of genes from transgenic plants (Wolfenbarger and Phifer 2000)

less regulated crop systems where refuges are not mandatory, insect resistance is likely to become an increasing problem. Therefore, ways to counter insect resistance need to be developed and put in place to ensure a sustainable application of this technology for tackling agronomically important insect pests.

20.3.7 Effect of Bt Toxin on Non-target Organisms

Any deleterious effect of the Bt toxin on any life forms, directly or indirectly, other than the target pest is of primary concern. The persistence of the toxin in the natural resources including land and water or the transfer of the genes coding for the toxin from a transgenic plant to a wild variety needs to be tightly kept under a check. In Fig. 20.4, the flow chart illustrates two main pathways (self-sustaining populations or introgression of genes) for how an introduced organism, such as a genetically engineered organism (GEO), or its genes could have negative impacts on natural ecosystems. These stepwise factors are necessary for an invasion, but not sufficient

to cause one (Wolfenbarger and Phifer 2000). Figure 20.3 is a flow chart explaining a possible stepwise process of invasion of the genes from transgenic plants. The outcomes of selected studies regarding effect of transgenic plants on non-target organisms have been summarised in Table 20.2.

20.3.7.1 Effects on Microorganisms

The reports on effects of Bt on soil microflora have varied from neutral as well as positive. Brasil et al. (2006) assessed the effect of *Bacillus thuringiensis* HD1 on sorghum rhizospheric microorganisms and concluded that the strain did not have any influence on the microbial population. Previously, Visser et al. (1994) made similar observations from their experiments with a commercialized preparation of Bt, DiPel 176 in simple microcosms and extrapolated the observations concluding that Bt would have no notable influence on non-target microbiota under field conditions. Pruett et al. (1980) and Petras and Casida (1985) reported an increase in overall microbial numbers in soil 2–4 weeks after the use of formulated product containing Bt spores. Castaldini et al. (2005) evaluated the impact of transgenic corn plants Bt11 and Bt 176 as well as their residues on bulk soil and rhizospheric eubacterial communities on the arbuscular mychorrhizal fungus *Glomus mosseae* and also on soil respiration. They observed a significantly lower level of mychorrhizal colonization in Bt 176 corn roots. In addition, lower rhizospheric eubacterial communities and lower rhizohspheric heterotrophic bacteria was reported for both Bt corn plants compared to that for the non-Bt control corn plants. In conclusion, Castaldini et al. (2005) recommended thorough investigation to assess and cautioned against the hazardous effects of GM plants to non-target beneficial soil microbes whose variation would produce long term negative effects on crops successively cultivated in the same soil year after years. Dunfield and Germida (2004) reviewed the effects of GM crops on soil and plant associated microorganisms. They noted that the changes in microbial communities associated with growing transgenic crops are relatively variable and transient as compared to other well accepted agricultural practices such as crop rotation, tillage, herbicide usage and irrigation.

There is a lack of consensus among the different studies regarding an overall effect of the use of Bt toxin on the soil microorganisms. Even minor alterations in the fine balance amongst the soil microorganisms which are involved in varied functions ranging from plant growth promotion, phytopathogenecity to nutrient cycling processes would have disturbing effects on plant growth as well ecosystem sustainability. Hence, detailed and in depth analysis of long term effects of any novel agricultural practices including introduction of a transgenic crop became absolutely essential.

20.3.7.2 Effects on Non-target Insects

Wipfli and Merritt (1994) studied direct and indirect (food-chain) effects of *Bacillus thuringiensis* var. israelensis (Bti) on 16 taxa of various benthic insects from

Table 20.2 Summary of studies addressing whether transgenic plant tissue could harm nontarget organisms through direct ingestion or indirectly by ingesting prey that have fed on transgenic plants (Adopted from Wolfenbarger and Phifer (2000))

Crop	Transgenic trait(s)	Potential invasive characteristic examined	Habitat studied	Conclusion
Oilseed rape (*Brassica napus*)	Two lines used: 1. Glufosinate tolerance 2. Kanamycin resistance	Persistence in natural habitats	12 UH	Step 1 unlikely
Oilseed rape	Same as above	Seed survival Overwinter	12 UH	Step 1 unlikely
Oilseed rape	Three lines used: 1. Male sterility, glufosinate tolerance, and kanamycin Resistance 2. Fertility restorer, glufosinate tolerance, and kanamycin resistance 3. Hybrid between #1 and #2	Yield densities used to assess intraspecific competition and interspecific competition with *Sinapsis alba*	AP	No enhanced risk of step 1; likelihood of step 1 not addressed
Oilseed rape	Glufosinate tolerance	Intraspecific gene flow: hybrid formation	AP	Step A, B possible. No enhanced risk.
Oilseed rape	Glufosinate tolerance, kanamycin resistance, and male fertility restorer	Gene flow with *B. rapa*: Hybrid Reproductive characteristics	FE, GH	Step D possible; no enhanced risk; no information on steps A to C.
Oilseed rape	High stearate	Seed survivorship	AF	Step 1 possible
Oilseed rape	1. High laureate 2. High stearate 3. High laureate 3 *B. rapa* hybrid	Seed germination; seedling vigor	AF	Step 1, steps B, C possible; no information on step A
Oilseed rape	Glufosinate tolerance	Gene flow with *B. campestris* (5*rapa*)	FE	Steps A to D possible
Oilseed rape	Glufosinate tolerance	Gene flow with wild mustard (*S. arvensis*)	FP, GH	Steps A, B unlikely
Potato (*Solanum tuberosum*)	Phosphoresence and kanamycin resistance	Gene flow with *S. tuberosum*		Steps A, B possible
Sugar beet (*Beta vulgaris*)	Glufosinate tolerance	Overwinter survival; hybrid survival	FP	Step 1, Step C possible; no enhanced risk.

L laboratory; *F* field; *GNA Galanthus nivalis* agglutin

Ephemeroptera, Plecoptera, Trichoptera, and Diptera. They found that the Bti exposure had no lethal or sublethal effects on most taxa excepting two species *Tipula abdominalis* (Diptera:Tipulidae) and *Arthroplea bipunctata* (Ephemeroptera: Heptageniidae), for which mortality was recorded although at considerably high dosages. From their study, the authors concluded that Bti appeared to be harmless to non target benthic insects. In contrast to conventional insecticides, which usually have a negative impact on biological control organisms, the abundance and activity of parasitoids and predators are similar in Bt and non-Bt crops (Romeis et al. 2006). Since, Bt-transgenic varieties can lead to substantial reductions in insecticide use in some crops; they can contribute to integrated pest management systems with a strong biological control component. In contradiction to this there are reports describing negative impacts of Bt on non target insects. Johnson et al. (1995) reported that early instar swallowtail butterflies belonging to genus *Papilio* were sensitive to Bt-treated foliage upto 30 days after application. Vandenberg (1990) reported that very high concentrations (10^8 spores/ml) of Bt subsp. *tenebrionis* though reduced the longevity of bee adults but did not cause disease. Lovei et al. (2009) highlighted in their review that the negative effects of Bt toxin on important indicator organisms such as the natural enemies of the insect pests was more prevalent as compared to the positive or neutral effects. This invited a prompt and staunch rebuttal from a consortium of specialists on research of non-target insects related to Bt crops authored by Shelton et al. (2009). The rebuttal lavishly criticized the observations and conclusion of Lovei et al. and questioned their methodology and the approach. They also voiced their concern regarding polarized opinions on transgenic crops pertaining to the possible influence that it may have on the policy building process by the governments of different nations.

20.3.7.3 Effects on Mammals

The primary requirement for the action of Cry toxins is the processing of the protoxin into its activated form, which occurs in an alkaline pH and by specific proteases which are present in the gut of the target insects. More importantly, the toxins identify specific receptors on the gut epithelial cells of susceptible insect larvae. So, the mammals including humans were thought to be safe from the effects of this class of proteins. Earlier studies on the effect of Bt toxin on mammals were generally confined to studying their toxicity to cultured cell lines to which they were found to be safe. The other point of concern was if the Bt toxin lead to allergies especially in farm workers and the population residing in close vicinity of the farms in which Bt toxin was used. Excepting the study by Bernstein et al. (1999), the other reports have denied any allergenic reaction in human population from external contact to the Bt toxin. Vendomois et al. (2009) carried out an analysis of blood and organ systems of rats fed on three commercialized varieties of genetically modified maize: NK 603, MON 810 and MON 863. In the three GM maize varieties investigated, new side effects linked to their consumption were revealed. The authors noted that the effects were mostly concentrated in kidney and liver function however they

reported that some effects on heart, adrenal, spleen and blood cells were also frequently noted. In contrast, Hu et al. (2010) have pointed out a potential benefit of Bt toxin Cry5B, an effective anthelmintic agent in different mammalian hosts. Shimada et al. (2003) reported Cry1Ab being safe for mammalian cells following their study regarding effect of Cry1Ab on primary cultured bovine hepatocytes. They reported the safety of the toxin from the observations that following the exposure of Cry1Ab to the bovine hepatocytes, there was no damage to morphology, albumin synthesis and integrity to the cell membrane that was ascertained employing LDH release assay. Levin et al. (2009) reviewed the data from studies conducted in the United States of America (USA), New Zealand and Canada regarding effects of exposure to *Bacillus thuringiensis kurstaki* on human health. They concluded that no adverse short term health effects of the toxin had come to the notice from the studies that were carried out in that regard.

20.3.7.4 Immunogenicity of Cry Toxins

Vanquez-Padron et al. (1999) demonstrated the induction of systemic and mucosal antibody responses on administration of Cry1Ac by intraperitoneal and intragastric route. They also demonstrated the ability of Cry1Ac to bind to the surface proteins in the mouse small intestine. Cry proteins are known to be specific in their action against target insects and have been generally regarded as safe to non target insects as well as to birds and mammals, including humans. The studies regarding the immunogenicity of this class of proteins have been undertaken in mice models. The findings of all those studies indicate the immunogenic potential of Cry proteins. Rojas-Hermandez et al. (2004) demonstrated that administration of Cry1Ac increases the protection against meningoencephalitis mediated by *Naegleria fowleri*. Intragastric and intraparetoneal administration of Cry1Ac induced systemic and mucosal antibody responses in mice. Vanquez-Padron et al. (1999) studied the serum antibody levels induced by Cry1Ac in comparison to that in response to the cholera toxin, the later being a highly potent immunogen. In that study by Vanquez-Padron et al. (1999) the serum antibody levels induced by Cry1Ac were even higher than those induced by cholera toxin and hence hinted towards the indication of this protein being a potent systemic and mucosal immunogen. The administration of Cry1Ac induced protection against the malaria parasite in CBA/Ca mice when it was administered before infecting the mice with *Plasmodium chabaudi* As and *Plasmodium berghei* ANKA which are non-lethal and lethal *Plasmodium* strains respectively. In the same study the authors reported an alteration in levels of pro-inflamatory cytokines such as IFN-γ and TGF-β following exposure to Cry1Ac in both *Plasmodium* infected mice (Legorreta-Herrera et al. 2010). In a different approach, studies were also conducted to see the effect on immunological parameters following ingestion of the transgenic food expressing Cry toxin. Finnamore et al. (2008) reported that in addition to an alteration in percentage of T and B cells and of $CD4^+$, $CD8^+$, γδT and αβT subpopulations, the mice fed on transgenic maize expressing the bioactive form of Cry1Ab showed an increase in serum IL-6, IL-13, IL-12p70 and MIP-1β.

In our laboratory, we studied the immunomodulatory effect of Cry1Ac on human adenocarcinoma cell line (Caco-2). Caco-2 monolayers were incubated with 1 μg/ml of partially purified activated Cry1Ac sample. Thereafter, the change in transcript levels of select cytokines was analyzed. Cry1Ac stimulation of Caco-2 resulted in upregulation of transcripts for chemokine/cytokines IL-8, TNF-α and IL-12p35 which indicates that indicates that Cry1Ac has potential to act as pro inflamatory agent. Further, to see if these effects could be reversed by employing probiotic organism, the response of Caco-2 when exposed to Cry1Ac and further incubated with a potential probiotic *Lactobacillus plantarum* CS 24.2 was also studied. In case of IL-8 and TNF-α, the upregulation was maintained even after the cells were exposed to *L. plantarum* CS24.2. Interestingly, Cry1Ac mediated increase in transcript levels of IL-12p35 is seen to be reversed by the exposure to *L. plantarum* CS 24.2. The immunomodulating ability of several lactobacillus strains have been reported in previous reports (Choi et al. 2008; Gaudana et al. 2010). The findings of our study hence are in line of the caution expressed in exposing the human population to food bearing potent antigen like Cry1Ac. The study also indicated the promise held in administering probiotics for countering the possible deleterious antigenic effects by foreign agents such as Cry toxins expressed in food for human consumption.

20.4 Future Perspectives

The detailed and clear understanding on the mechanism of action of Bt toxins and the role of various receptors is still lacking. This in turn holds the key to addressing the issue of resistance against Bt toxins in target insects, which could hamper the sustainability of this technology. The long term adverse effects of Bt toxins on various life forms including microorganisms, various non-target insects and animals needs to be closely monitored. Recent studies have established the immunogenic potential of Cry toxins in animal models and hence the same needs to be elaborately studied in human systems before they are approved for being incorporated in transgenic foods for humans.

20.5 Conclusions

Given the enormous deleterious effects of chemical pesticides, biopesticides in the form of Bt toxins have extended several economic, health and ecosystem related benefits. The exudation of the toxin from the roots of transgenic plant does take place, however its possibility to cause any harm to the environment does depend upon its persistence in the soil. The reports on the degradation of various Bt toxins have been variable and the phenomenon needs to be studied over longer periods and in different prevalent conditions. Studies on the adverse effects of the Bt toxin on

various non-target life forms have been inconclusive and need to be addressed in detail and over a longer period of time. Though not toxic to mammals, the Cry toxins have been found to be immunogenic in the studies carried out on mice models and is hence a point of concern and thorough investigation.

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Chapter 21
Biodegradation of Mono-aromatic Compounds by Bacteria

Rahul Shrivastava and Prashant S. Phale

Abstract Aromatic hydrocarbons represent a group of ring structured hydrophobic and reduced compounds which are industrially significant and toxic in nature. Due to their presence in nature through anthropogenic and biological routes, microorganisms have evolved/adapted to degrade these compounds. Aerobic bacteria initialize their degradation by incorporation of oxygen atoms by a group of enzymes 'oxygenases' which leads to the formation of *cis*-dihydrodiols. Subsequent formation of catechol intermediates followed by *ortho*- or *meta*-ring cleavage leads to intermediates which enter Kreb's cycle. The reaction sequences are repeated for additional rings in case of polycyclic aromatic compounds. A large array of metabolic diversity exists within these strains and several parameters regulate the efficient utilization of such compounds. Hence, an ideal microbial culture should have following features for its effective application in sustainable environmental development strategies: (i) ability to metabolize wide range of aromatic compounds even at high concentrations with novel pathways and enzymes, (ii) complete degradation of toxic pollutants without formation of toxic by-products, (iii) production of biosurfactants for solubilization and uptake of hydrophobic xenobiotic compounds and (iv) absence of carbon catabolite repression.

Keywords Bioremediation • Biodegradation • Monoaromatic compounds • Metabolism • Oxygenases • Catabolic pathways

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21.1 Introduction

Aromatic compounds are a class of organic pollutants which enter the environment mainly through anthropogenic activities. Industrialization and human activities lead to further increase and accumulation of these compounds in nature (Foght 2008). Lignin, humus and non-degradable tannins are the prime natural sources of aromatic hydrocarbons due to their poor biodegradability (Field et al. 1995). Aromatic hydrocarbons are hydrophobic compounds consisting of one or more aromatic rings arranged in a linear, angular or clustered arrangement. Benzene, being the simplest aromatic hydrocarbon, has the elemental composition of C_6H_6 and contains three carbon-carbon double bonds. π-Electrons associated with these double bonds are delocalized and results in large negative resonance energy of benzene and related compounds, which in turn results in providing thermodynamic stability to these compounds (Waack 1962; Gibson and Subramanian 1984; Phale et al. 2007).

The carcinogenic and mutagenic effects of aromatic hydrocarbons on various life forms make their study important. Hill first reported the carcinogenic effect of hydrocarbons from snuff in 1761 (Redmond 1970; Blummer and Youngblood 1975). Later Dr. Pott observed the incidence of scrotal skin cancer in chimney sweeps (Gibson and Subramanian 1984). Hundred years later the relation between skin cancer and skin contact with tars and oil was established. Studies on chemical carcinogenesis estimates that 60–90% of human cancers are caused by exposure to environmental chemicals (Miller 1978; Gelboin 1980). Besides human, aromatic compounds and their derivatives are harmful to the animals and plants. The degree of deleterious effect on the plants depends upon their solubility and subsequent process of distribution over long distances *via* water bodies and affecting the agricultural fields (Bamforth and Singelton 2005). Hence the economic removal of these toxic compounds from the environment has been the focus of immense research in last 50 years.

21.2 Mono-aromatic Hydrocarbons, Their Sources and Properties

Mono-aromatic hydrocarbons are compounds consisting of one aromatic ring and atleast 6 π-electrons according to the Huckel's rule. Simplest mono-aromatic hydrocarbon is benzene and all the other compounds are substituted derivatives of benzene. Major environmentally significant mono-aromatic compounds are described in this section.

21.2.1 BTEX (Benzene, Toluene, Ethyl Benzene, Xylene)

These compounds are considered to be primarily responsible for ground water pollution and causes significant environmental risk due to features like high volatility,

solubility and toxicity (Coates et al. 2002). Benzene and toluene are released into the environment through gasoline, petroleum fuels, and industrial effluents of metal, paint, textile manufacture, wood processing, chemical production, and tobacco products. On the other hand, ethylbenzene and xylene contamination has been associated with the manufacture of pesticides, chemicals, detergents, varnishes and paints (Coates et al. 2002; Chakraborty and Coates 2004).

As they are suspected as being carcinogens, their release in the environment is strictly controlled and they are classified as priority environmental pollutants (An 2004). BTEX compounds do not strongly adsorb to the soil; therefore, they can reach groundwater and contaminate it (Langwaldt and Puhakka 2000). Important sources of water contamination are industrial wastewater and released petroleum products from storage tank, gas work sites, airports, paint manufacturer, chemical industries, and railway yard (Andreoni and Gianfreda 2007). BTEX compounds may comprise more than 50–60% by weight of the solubilized compounds when gasoline is introduced into water (Kermanshahi et al. 2005; Farhadian et al. 2008). Permissible limits of BTX given by EPA (Environmental Protection Agency, USA) in potable water are 0.005, 1, and 10 ppm for benzene, toluene, and mixed xylenes, respectively (Farhadian et al. 2008).

21.2.2 Chlorobenzenes

Chlorinated benzenes are a group of cyclic aromatic compounds in which one or more hydrogen atoms of the benzene ring have been replaced by a chlorine atom. The generic molecular formula is C_6H_6–nCln, where $n = 1$–6. Chlorobenzenes are used mainly as intermediates in the synthesis of pesticides and other chemicals. 1,4-Dichlorobenzene (1,4-DCB) is used in space deodorants and as a moth repellent. The higher chlorinated benzenes (trichlorobenzenes, 1,2,3,4-tetrachlorobenzene [1,2,3,4-TeCB], and pentachlorobenzene [PeCB]) have been used as components of dielectric fluids. Potential hazardous chlorinated benzenes responsible for pollution are: monochlorobenzene (MCB), dichlorobenzene (DCB) (three isomers), trichlorobenzene (TCB) (three isomers), tetrachlorobenzene (TeCB) (three isomers), pentachlorobenzene (PeCB) and hexachlorobenzene (HeCB) (Wang and Jones 1994).

21.2.3 Phenol

A hydroxyl group attached to the benzene skeleton represents a large group of aromatic compounds, generally termed as phenols. Natural as well as industrial processes are source of phenol and its higher derivatives in the environment. Industrial waste water plants, coal mines and refineries are some of the major source of its pollution (van Schie and Young 1998). Forest fire and decay of lignocellulosic material are natural source of phenol in the nature. Phenols are toxic to flora and fauna and affect several biochemical functions. Phenol is also a priority pollutant and is included in the list of EPA (Nair et al. 2008).

21.2.4 Nitroaromatic and Its Derivatives

Nitroaromatic compounds constitute a large and industrially important group of compounds which are used in the processing and manufacturing of dyes, explosives and pesticides. These compounds are also detected in industrial waste as well as in the atmosphere (Cao et al. 2004). Photochemical reactions in the atmosphere also lead to the generation of nitroaromatic compounds in the environment.

As nitroaromatic compounds pose a serious threat to the human health and produce a public concern, its behavior and fate in the environment have received great attention for human health (Lessner et al. 2002) and it is listed as a priority pollutant by the EPA.

21.2.5 Phthalates and Derivatives

Phthalates (*o*-, *m*-, *p*-) are esters of phthalic acid which contains two carboxyl group attached to the benzene ring. They are primarily used in plastic industry (Latini 2005). Phthalates find their widespread use in the plastic industry because of their properties like chemical stability, flexibility, softening, ease of fabrication and low cost. Some of these compounds are also metabolically produced as intermediates in the degradation of polyaromatic hydrocarbons, for example, in several bacterial species *o*-phthalate was shown to be an intermediate in phenanthrene, pyrene, fluorine and fluoranthrene catabolism (Phale et al. 2007; Vamsee-Krishna and Phale 2008).

21.2.6 Phenylacetic Acid and Derivatives

Phenylacetic acid (PA) and hydroxylated derivatives of PA are produced as catabolic intermediates by both bacteria and fungi growing on a wide variety of different naturally occurring aromatic compounds such as aromatic amino acids or lignin as well as various synthetic aromatic compounds (O'Connor et al. 2005).

21.3 Aerobic Metabolism of Mono-aromatic Compounds

21.3.1 Bioremediation and Biodegradation

The ability of bacteria to utilize aromatics as the sole source of carbon and energy for growth was first demonstrated in 1908 by Stromer, who isolated *Bacillus hexacarbovorum*, which could grow on toluene and xylene. In 1913 Sohngen reported the utilization of benzene by microorganisms. Later, Wagner isolated two organisms

Bacterium benzoli 'a' and 'b', both of which were capable of growing on benzene, xylene and toluene (Gibson and Subramanian 1984). Since then, the microbial interaction with aromatic hydrocarbons has been the focus of study. Further interest was developed in the isolation and study of microorganisms capable of mineralizing aromatic compounds and restoration of natural environment in a process called as 'Bioremediation' which is defined as "the process whereby organic wastes are biologically degraded under controlled conditions to an innocuous state, or to levels below concentration limits established by regulatory authorities" (Mueller et al. 1996). Utilization of aromatic hydrocarbons as a source of carbon and energy by microorganisms is generally regarded as biodegradation. This results in the formation of small molecules, which yield energy on further oxidation e.g., aromatic rings are cleaved to form aliphatic compounds, which are then metabolized and subsequently oxidized to CO_2.

Aromatic compounds are highly reduced and inert in nature and hence insertion of molecular oxygen (O_2) is required to initiate their degradation. Since molecular oxygen (O_2) is chemically inert, a group of enzymes termed as "Oxygenases" performs this function (Mason et al. 1955; Hayaishi et al. 1955). After initial oxidation of reduced aromatic compounds, molecular oxygen is generally not required for their further degradation. However, oxygen is required as a reactant during biodegradation of aromatic rings for ring activation and its cleavage. Thus, oxygen can be utilized by microorganisms as both, electron acceptor and as a reacting co-substrate during initial steps of metabolic reactions.

21.3.2 Oxidation of Aromatic Hydrocarbons via Oxygenases

'Oxygenases', an oxidoreductase class of enzymes, participate in the incorporation of molecular oxygen into the aromatic compounds (Mason et al. 1955; Hayaishi et al. 1955). Based on the number of atoms incorporated during the reaction, they are classified into two distinct groups (Fig. 21.1): (a) monooxygenases, also known as 'hydroxylase' or 'mixed-function oxidase', these enzymes incorporate a single atom of molecular oxygen and results in the formation of mono-hydroxy products; (b) dioxygenases incorporate both atoms of molecular oxygen in a single substrate molecule and results in either ring-hydroxylation yielding dihydroxylated products or ring-cleavage of aromatic diols, thus breaking the aromaticity of the nucleus. The resulting unsaturated aliphatic acid enters the central carbon pathway. The overall classification of oxygenases is shown in Fig. 21.1.

In monooxygenase reactions, one atom of the dioxygen is incorporated into the substrate and the other atom is reduced to water. The term 'hydroxylase' is also used for some monooxygenases, which catalyze the insertion of a hydroxyl group at *ortho-, meta-* and *para*-position. Toluene 2-monoxygenase (1.14.13.-) (Shields et al. 1991), toluene 3-monoxygenase (1.14.13.-) (Olsen et al. 1994) and toluene 4-monoxygenase (1.14.13.-) (Whited and Gibson 1991) catalyses the incorporation of hydroxyl group at the *ortho-, meta-* and *para*-position to yield 2-hydroxy, 3-hydroxy

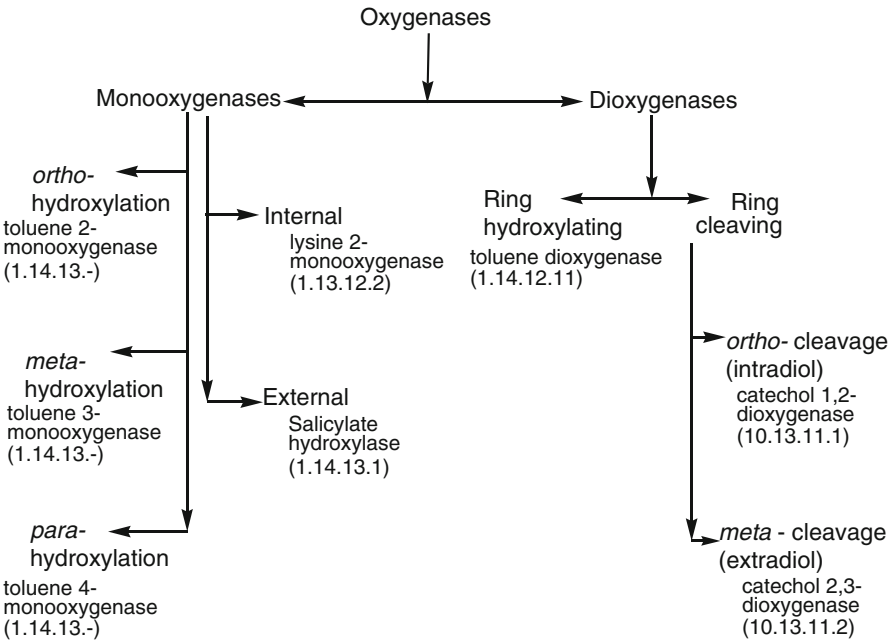

Fig. 21.1 Classification of oxygenases on the basis of atoms of oxygen incorporated and their position in the aromatic substrate (EC numbers of representative enzymes)

and 4-hydroxy toluene, respectively (Fig. 21.2). Salicylate 1-monooxygenase (EC number 1.14.13.1) is another example of hydroxylation where salicylic acid is converted into catechol in NADPH dependent manner (Yamamoto et al. 1965) (Fig. 21.2).

Dioxygenases incorporate both atoms of molecular oxygen into the substrates and are further grouped into two major types of dioxygenases: (i) 'ring-hydroxylating dioxygenase', responsible for double hydroxylation of the substrates and require NADH or NADPH as co-factors and (ii) 'ring-cleaving dioxygenase', responsible for aromatic ring cleavage with simultaneous incorporation of molecular O_2 into the substrate and requires metal ions as co-factor. Ring hydroxylating dioxygenases are responsible for the initial double hydroxylation of an aromatic compound to form a *cis*-dihydrodiol. This reaction removes the aromaticity of the product, making it more susceptible to further oxidations e.g. benzene dioxygenase (Axcell and Geary 1975), toluene dioxygenase (Yeh et al. 1977; Subramanian et al. 1981) etc. The ring-cleavage of catecholic compounds is performed by one of the two distinct classes of dioxygenases: intradiol- and extradiol-dioxygenases (Fig. 21.2). Intradiol dioxygenase cleaves the aromatic ring between two hydroxyl groups (also referred to as the *ortho* pathway) whereas extradiol-dioxygenase cleaves it adjacent to hydroxyl group (the *meta* pathway). The biochemical features of these two reaction sequences are found to be similar in all genera of eubacteria. Intradiol cleavage of catechol by catechol-1,2-dioxygenase results in the formation of *cis,cis*-muconic

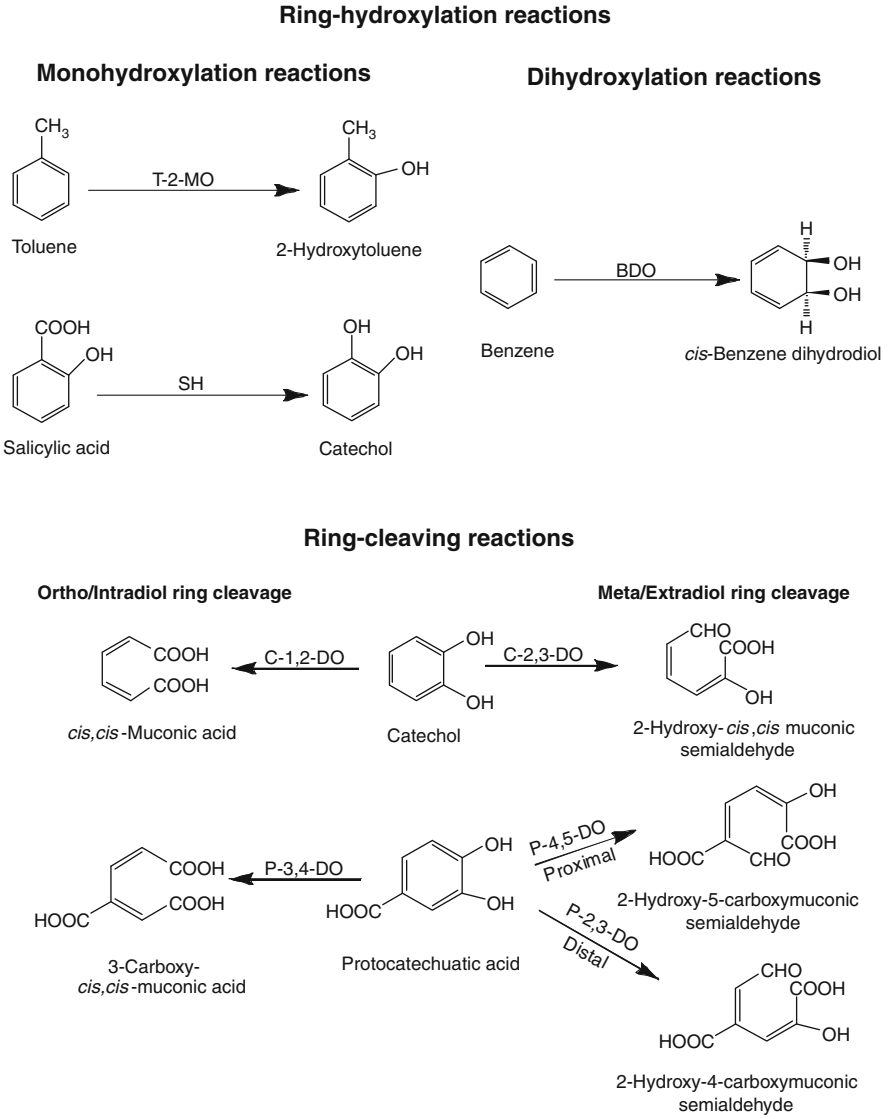

Fig. 21.2 Hydroxylation and ring cleaving reactions involved in initial steps of metabolism of aromatic compounds. T-2-MO, Toluene-2-monooxygenase; SH, Salicylate hydroxylase; BDO, Benzene dioxygenase; C-1,2-DO, Catechol-1,2-dioxygenase; C-2,3-DO, Catechol-2,3-dioxygenase; P-3,4-DO, Protocatechuate-3,4-dioxygenase; P-4,5-DO, Protocatechuate-4,5-dioxygenase; P-2,3-DO, Protocatechuate-2,3-dioxygenase

acid (Hayaish and Hashimoto 1950), which is further metabolized to acetyl-CoA and succinyl-CoA via the β-ketoadipate pathway and enters the central carbon pathway. Extradiol cleavage of catechol by catechol-2,3-dioxygenase results in the formation of 2-hydroxymuconic semialdehyde (Kojima et al. 1961) (Fig. 21.2). Protocatechuate is subjected to three different modes of cleavage: (1) intradiol

cleavage by protocatechuate 3,4-dioxygenase, yielding 3-carboxy-*cis,cis*-muconic acid (Stanier and Ingraham 1954); (2) proximal extradiol cleavage by protocatechuate 4,5-dioxygenase, yielding 2-hydroxy-5-carboxymuconic semialdehyde (Ono et al. 1970); and (3) distal extradiol cleavage by protocatechuate 2,3-dioxygenase giving rise to 2-hydroxy-4-carboxymuconic semialdehyde (Wolgel and Lipscomb 1990) (Fig. 21.2). Compared to catechol, aromatic compounds with hydroxyl groups at *para* positions and/or with a carboxylate or an amino group in the place of second hydroxyl group (e.g., 2-aminophenol, gentisate, 1-hydroxy-2-naphthoic acid, hydroquinone, and salicylate) are subjected to an extradiol type of cleavage by a unique group of ring-cleaving dioxygenase (Gaal and Neujahr 1979; Harpel and Lipscomb 1990; Iwabuchi and Harayama 1998; Xu et al. 1999; Phale et al. 2007). Ring cleaved compounds generated in the metabolism were finally converted into small aliphatic molecules which can enter central metabolism (Williams and Sayers 1994; Phale et al. 2007). Diagrammatic representation of metabolic routes of various monoaromatic compounds is shown in Fig. 21.3.

21.3.3 Benzene

Detailed studies on benzene degradation in several microorganisms has resulted in the elucidation of its metabolic routes and different metabolic intermediates involved in the pathway (Smith 1990). In the initial steps, benzene is oxidized to form *cis*-benzene dihydrodiol by the action of the enzyme 'benzene dioxygenase'. Subsequent steps result in the formation of catechol which is further catabolized by either catechol 1,2-dioxygenase (*ortho*- or intradiol-cleavage) and or catechol 2,3-dioxygenase (*meta*- or extradiol-cleavage) (Fig. 21.3). Further metabolism of benzene *via* 2-hydroxymuconic semialdehyde leads to the formation of pyruvate and acetaldehyde while *ortho*-cleavage product *cis-cis* muconic acid enters β-ketoadipate pathway for further metabolism. Plasmid encoded *ortho*-pathway for the utilization of benzene was first reported from *Acinetobacter calcoaceticus* RJE74, which carries a large plasmid (pWW174) encoding the enzymes for the catabolism of benzene *via* the β-ketoadipate pathway (Winstanley et al. 1987). Biological production of *cis*-benzene dihydrodiol was studied and used as a chiral building block for polymers and pharmaceuticals (Ley et al. 1987; Smith 1990).

21.3.4 Arenes and Aromatic Alcohols

The presence of an alkyl group on the aromatic ring as in toluene or xylene presents two alternative routes of metabolism. The oxidative attack could be on the aromatic ring, giving rise to alkyl catechol, or the alkyl group could be oxidized to aromatic carboxylic acids, which are further oxidized to dihydroxylated ring fission substrates (Fig. 21.3). Five unique bacterial pathways that result in the oxygenase-catalyzed

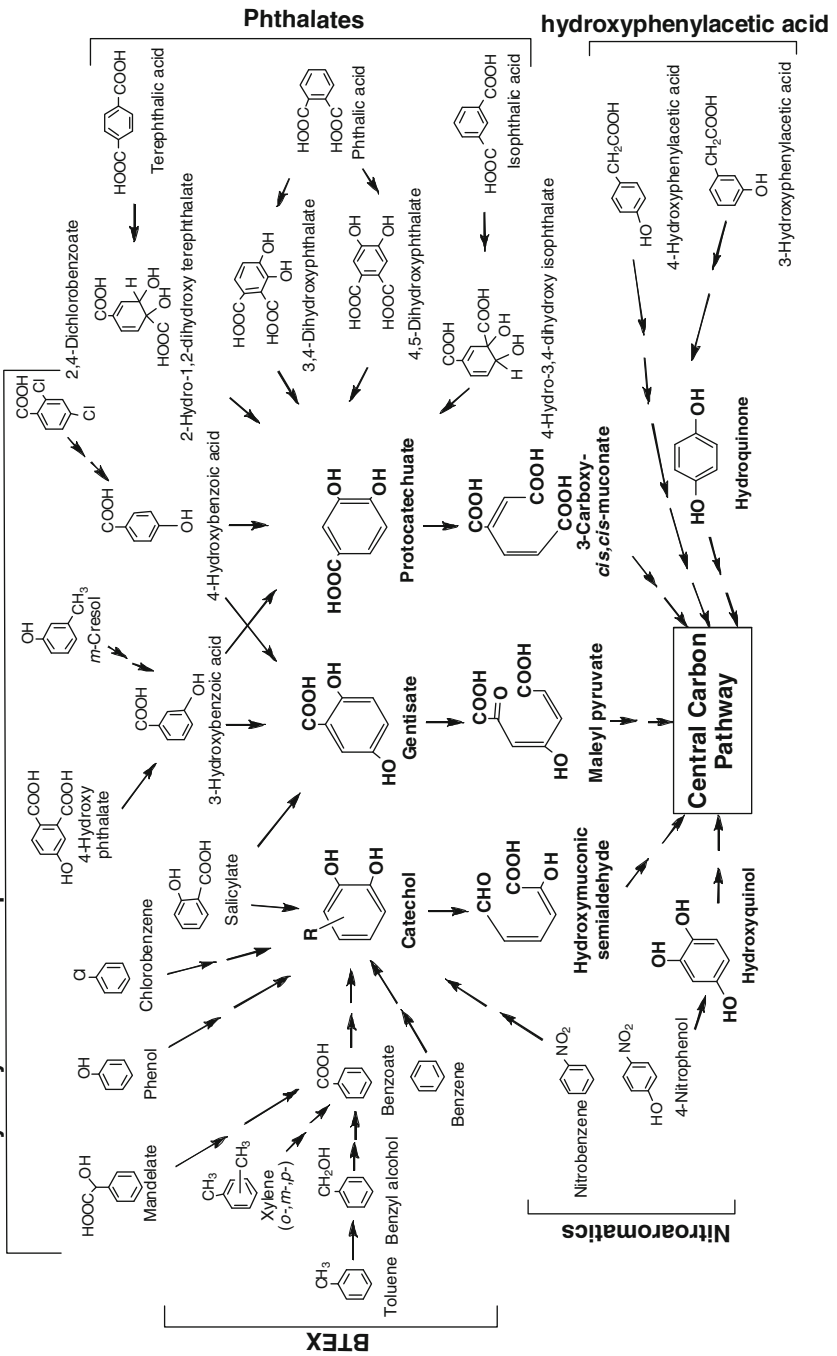

Fig. 21.3 Representation of aerobic metabolic fate of various monoaromatic compounds in bacteria. *Central structures* represent common intermediates (may be substituted depending on the nature of side groups) formed during metabolism of aromatic compounds

hydroxylation of toluene have been described. One involves the oxidation of toluene through benzyl alcohol, benzaldehyde and benzoate known to be encoded by the TOL plasmid (Worsey and Williams 1975). The plasmids contain two catabolic operons (Nakazawa et al. 1980; Franklin et al. 1981). The 'upper' pathway operon encodes enzymes for the successive oxidation of the hydrocarbons to the corresponding alcohol, aldehyde and carboxylic acid derivatives. The 'lower' or *meta*-cleavage pathway operon encodes enzymes for the conversion of the carboxylic acids to catechols, whose aromatic rings are then cleaved (*meta*-cleavage) to produce corresponding semialdehydes, which are then further catabolized through the TCA cycle (Ramos et al. 1987). The remaining pathways initiate toluene oxidation through the hydroxylation of the aromatic ring *via* either mono- or dioxygenases. Only one toluene dioxygenase, the toluene-2,3-dioxygenase of *Pseudomonas putida* F1 has been described. The enzyme produces a *cis*-toluene-2,3-dihydrodiol from toluene with the insertion of diatomic oxygen (Gibson et al. 1970). Toluene monooxygenases that hydroxylate the aromatic nucleus at all the three possible positions producing *ortho*-, *meta*- and *para*- cresols have been described. These include toluene *ortho*-monooxygenase of *Burkholderia cepacia* G4 (Gibson et al. 1970; Shields et al. 1989, 1995), the toluene *meta*-monooxygenase of *Pseudomonas pickettii* PK01 (Olsen et al. 1994) and the toluene *para*-monooxygenase of *Pseudomonas mendocina* KR1 Figure (Whited and Gibson 1991).

Xylene monooxygenase (EC no. 1.14.15.-) encoded by the TOL plasmid pWW0 of *Pseudomonas putida* catalyzes the hydroxylation of the methyl side-chain of toluene and xylenes. Genetic studies have suggested that the monooxygenase consists of two different proteins, products of xylA and xylM genes, which function as an electron-transfer protein and a terminal hydroxylase, respectively (Harayama et al. 1989). The electron transfer component of xylene monooxygenase, the gene product of xylA has been purified (Shaw and Harayama 1992). Benzyl alcohol degradation was also carried out by variety of toluene degrading microorganisms. In *P. putida* CSV86, toluene utilization was not reported, however, the strain can utilize benzyl alcohol as a carbon source and degrades it by *ortho*-cleavage pathway (Basu et al. 2003).

Benzyl alcohol dehydrogenase (EC no. 1.1.1.90) catalyzes the oxidation of benzyl alcohol to benzaldehyde with the concomitant reduction of NAD^+; the reaction is reversible. Benzaldehyde dehydrogenase (EC no. 1.2.1.28) catalyzes the oxidation of benzaldehyde to benzoic acid with the concomitant reduction of NAD^+; the reaction is irreversible (Shaw and Harayama 1990). Benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase also catalyze the oxidation of many substituted aromatic alcohols and aldehydes but failed to oxidize aliphatic alcohols and aldehydes (Shaw and Harayama 1990; Shrivastava et al. 2011).

Isomers of xylene have been shown to be biodegraded by bacteria. Both compounds are degraded by certain strains of *Pseudomonas* by initial oxidation of one of the methyl groups to the corresponding methyl benzylalcohols, tolualdehydes, toluic acids and methyl catechol (Davis et al. 1968; Davey and Gibson 1974). The resultant catechols then undergo *meta*-cleavage. The ring-cleavage products of the two different methyl catechols (3-methylcatechol from *meta*-xylene; 4-methylcatechol from *para*-xylene) are catabolized by different enzyme systems (Duggleby and Williams

1986). The product from 3-methylcatechol cleavage is further degraded by a single hydrolase type enzyme (Duggleby and Williams 1986; Smith and Ratledge 1989), whereas the product from 4-methylcatechol, an aldehyde, is converted *via* the enzymes of the 4-oxocrotonate branch (Sala-Trepat et al. 1972; Wigmore et al. 1974). An alternative mode of attack of *para*- and *meta*-xylenes is *via* direct dioxygenase attack on the aromatic moiety yielding the corresponding *cis*-dihydrodiol with subsequent conversion to substituted catechols (3,6-dimethylcatechol from *para*-xylene; 3,5-dimethylcatechol from *meta*-xylene) by dehydrogenase type enzymes (Gibson et al. 1974). In these alternative pathways, the resultant catechols are not further degraded and this route is regarded as a biotransformation reaction. Complete biodegradation of *ortho*-xylene were first reported in *Pseudomonas stutzeri* and *Cornebacterium* sp. O*rtho*-xylene was catabolized *via* 3,4-dimethylcatechol with subsequent *meta*-cleavage (Baggi et al. 1987) with 2-dihydroxy-5-methyl-6-oxo-2,4-heptadienoate as the ring-fission product (Schraa et al. 1987).

Since, waste water and underground fuel storage generally contain a mixture of BTEX compounds; the aerobic metabolism of BTEX compounds by microorganisms has been studied since last 70 years. Due to their highly volatile nature and toxic effects, significant amount of research on aerobic and anaerobic BTEX degradation has been performed to remediate the contaminated sites. A variety of bacterial strains of genus *Rhodococcus, Pseudomonas* and *Alcaligenes* were isolated and found to utilize BTEX compounds (Deeb and varez-Cohen 1999). However, it has been realized that for the complete removal of BTEX from the contaminated sites, mixture of bacterial cultures were required as some of the compounds like *o*-xylene are less easily degraded as compared to other BTEX compounds. Several studies were conducted using consortium for the degradation of BTEX compounds (Chen and Taylor 1995). A consortium model and kinetics of BTEX degradation was studied and cometabolism of *o*-xylene was observed with toluene (Littlejohns and Daugulis 2008). Biodegradation of BTEX mixtures is known to be affected by substrate interactions such as inhibition and competition. Hence, studies were carried out to understand the underlying interactions between substrates and optimize the BTEX usage in biological treatment applications (Bitzi et al. 1991; Burback and Perry 1993; Oh et al. 1994; Lee et al. 2002).

21.3.5 Chlorobenzene and Substituted Derivatives

Under aerobic conditions, several studies have suggested the co-metabolism of chlorinated benzenes. Chlorobenzene grown cells of *Pseudomonas* sp. strain JS150 were able to oxidize disubstituted chlorobenzenes although these were not the growth substrates (Haigler et al. 1992). Similarly, the cometabolism of several chlorinated benzenes was reported in *Pseudomonas aeruginosa* strain RHO1, *Methylosinus trichosporium*, *Pseudomonas putida* MST and a strain of *Pseudomonas putida* (Bestetti et al. 1992; Brunsbach and Reineke 1994).

The first report of utilization of chlorinated benzene was by an unidentified bacterial strain WR1306 (Reineke and Knackmuss 1984). Since then a number of bacterial

strains have been isolated and shown to utilize chlorobenzene and higher homologues like di, tri and tetra-chlorobenzenes (Marinucci and Bartha 1979; Schraa et al. 1986; de Bont et al. 1986; Spain and Nishino 1987; Haigler et al. 1988; Sander et al. 1991; Nishino et al. 1992; Spiess et al. 1995; Sommer and Gorisch 1997, Potrawfke et al. 1998; Muller et al. 2003; Adebusoye et al. 2007; Kunze et al. 2009; Lu et al. 2010). The degradative attack on chlorinated benzenes is initiated by dioxygenases to produce chlorinated dihydrodiol intermediates which are subsequently converted to chlorocatechols intermediate by dihydrodiol dehydroganeses (Spain and Nishino 1987; Haigler et al. 1988; Sander et al. 1991; Spiess et al. 1995; Mars et al. 1997; Beil et al. 1997, 1998; Van der Meer et al. 1998; Potrawfke et al. 1998).

A chlorobenzene dioxygenase from *Burkholderia* sp. strain PS12 was cloned into *Escherichia coli*, which could express an active form of the enzyme (Beil et al. 1997). The heterologous recombinant chlorobenzene dioxygenase converted 1,2,4,5-tetrachlorobenzene to an unstable tetrachlorodihydrodiol intermediate, which spontaneously rearomatizes with concomitant chloride elimination to the corresponding 3,4,6-trichlorocatechol. In most other cases, the initial dioxygenation results in the formation of stable dihydrodiol intermediates with the same number of chlorine groups as the original substrate (de Bont et al. 1986; Haigler et al. 1988; Sander et al. 1991; Spiess et al. 1995). The heterologous recombinant chlorobenzene dioxygenase e.g., converted 1,2,4-trichlorobenzene to the corresponding stable trichlorodihydrodiol (Beil et al. 1997). These *cis*-dihydrodiols are oxidized to the corresponding chlorocatechols by the action of dehydrogenases. The chlorocatechols are subsequently oxidized by either one of two types of chlorocatechol dioxygenases, causing either *ortho*-cleavage (catechol 1,2-dioxygenase) to chloromuconic acids (Spain and Nishino 1987; Haigler et al. 1988; Sander et al. 1991; Spiess et al. 1995; Spiess and Gorisch 1996; Sommer and Gorisch 1997; Potrawfke et al. 1998) or *meta*-cleavage (catechol 2,3-dioxygenase) to 2-hydroxy-6-chlorocarbonyl muconic acid (Klecka and Gibson 1981; Pettigrew et al. 1991; Mars et al. 1997). These chloromuconic acids are metabolized further to intermediates of the Kreb's cycle.

A prevalent organochlorine insecticide, Lindane, is a chlorinated mixture of benzene which is termed as hexachlorocyclohexane (HCH). Lindane typically constitutes α, β, γ and δ-isomer of HCH. They have been regarded as carcinogenic and listed in EPA priority list of pollutants (Phillips et al. 2005). Several bacterial strains were isolated for their ability to utilize HCHs as carbon and energy source (Senoo and Wada 1989; Sahu et al. 1990; Phillips et al. 2005). Lin pathway is regarded as major pathway for the aerobic metabolism of HCHs (Lal et al. 2010) in which HCHs are converted to maleylacetate and metabolized *via* β-ketoadipate pathway.

21.3.6 Phenols

Aerobic utilization of phenol has been widely reported and several micro-organisms have been isolated like *Pseudomonas* sp., *Alcaligenes*, *Acinetobacter sp.*, *Bacillus*, *Ralstonia* and *Arthrobacter* sp. (Feist and Hegeman 1969; Hill and Robinson 1975;

Buswell 1975; Bettmann and Rehm 1984; Sokol 1987; Dikshitulu et al. 1993; Paller et al. 1995; Schroder et al. 1997; Götz and Reuss 1997; Leonard and Lindley 1998; Leonard et al. 1999; Reardon et al. 2000; Oboirien et al. 2005; Agarry et al. 2008).

A mono-oxygenase, phenol hydroxylase, initiates the degradation of phenol under aerobic conditions by hydroxylating the aromatic ring at *ortho*-position to the pre-existing hydroxyl group to form catechol (Fig. 21.3). This is the main intermediate resulting from metabolism of phenol by different microbial strains. Depending on the type of strain, the catechol then undergoes a ring-cleavage that can occur either at the *ortho*- position (Gaal and Neujahr 1979; Paller et al. 1995) which leads to the formation of succinyl Co-A and acetyl Co-A or at the *meta*- position (Feist and Hegeman 1969; Hill and Robinson 1975; Kukor and Olsen 1990; Ghadi and Sangodkar 1994; Leonard and Lindley 1998) leading to the formation of pyruvate and acetaldehyde.

Phenol hydroxylase is composed of either one or more than one component depending on the organism. The mono-oxygenase, phenol hydroxylase of the *Trichosporon cutaneum*, *Pseudomonas pickett*, *Bacillus stearothermophylus* BR219 and some species of *Acinetobacter* and *Alcaligenes* are mono-component flavoproteins (Neujahr and Gaal 1973; Kim and Oriel 1995; Divari et al. 2003; Agarry et al. 2008), while phenol hydroxylase from *Pseudomonas* CF600 (Shingler et al. 1989; Nordlund et al. 1990; Powlowski and Shingler 1990; Pessione et al. 1999) and *Acinetobacter radioresistens* (Shingler et al. 1989) are multi-component proteins. Multi-component aromatic mono-oxygenases contain at least two components. The former is an oligomeric protein while the latter is a monomeric iron transfer flavoprotein (Powlowski and Shingler 1990).

21.3.7 Nitroaromatic Compounds

It has been widely evident that the microbial degradation of nitroaromatic compounds can occur by four different mechanisms (Smith 1990). These are: (a) nitrite forming oxygenative reaction; (b) amines generating reduction process; (c) nitrite forming reduction of nitro group; (d) hydroxylamine forming partial reduction. The aromatic compounds resulting from the reactions described above are further degraded *via* the general degradation patterns of homocyclic aromatics (Smith 1990; Marvin-Sikkema and de Bont 1994; Ju and Parales 2010).

Oxygenative pathway involves the utilization of the nitroaromatic compounds as a nitrogen source by using nitro-group and leads to the formation of nitrite in the process (Bruhn et al. 1987; Dickel and Knackmuss 1991; Marvin-Sikkema and de Bont 1994). The oxygenases involved in such pathway remove nitrite from the substrate, e.g. 2-nitrophenol oxygenase, 4-nitrophenol oxygenase etc. (Zeyer and Kocher 1988; Spain et al. 1979; Spain and Gibson 1991), The substrate specificity of 2-nitrophenol oxygenase was reported to be broad and it act on several halogen- and alkyl-substituted 2-nitrophenols. This enzyme also catalyzes the conversion of 2-nitrophenol to catechol and nitrite (Zeyer and Kearney 1984;

Zeyer et al. 1986; Zeyer and Kocher 1988). In contrast to the pathway described above, several microorganisms convert nitroaromatics to nitrocatechols (Fig. 21.3) before removing the nitro group as nitrite (Raymond and Alexander 1971; Haigler and Spain 1993).

The degradation pathway of nitrobenzoates, nitrotoluenes and nitrophenols involve the reduction of nitro group to an amino group in several microorganisms. Initial reduction generates a nitroso and a hydroxyamino group. Subsequent oxygenase action leads to the formation of ammonium and catechol, which is further metabolized by ring-cleavage reactions. The presence of aminoaromatic intermediates are not conclusively determined and some bacteria are not capable of mineralizing nitroaromatics completely after reduction of the nitro group, but use the liberated ammonium as a nitrogen source (Zeyer and Kearney 1984; Boopathy and Kulpa 1992).

Third pathway of nitro group elimination involves the formation of a hydride-Meisenheimer complex which has been detected in several bacteria. The reaction involves the complete reduction of nitro group to nitrite and generates a complex. This hydride-Meisenheimer complex indicates that the degradation starts with a nucleophilic attack on the aromatic ring (Lenke and Knackmuss 1992).

Some of the nitroaromatic compounds like 4-nitrobenzoate and 2-nitrobenzoate are frequently metabolized *via* pathway that involves hydroxylamine formation. The end product in the reaction sequence is ammonia (Groenewegen et al. 1992).

In addition to the four pathways described above, in some bacteria like *Alcaligenes eutrophus and Pseudomonas sp.* (Ecker et al. 1992; Haigler and Spain 1993) the metabolism of nitroaromatic compounds involves addition of two hydroxyl groups with the removal of a nitro group as nitrite from the aromatic ring.

21.3.8 Phthalate and Its Derivatives

The metabolic pathway and various enzymes that participate in the degradation of *o*-phthalate are extensively characterized (Keyser et al. 1976; Eaton 2001). On the other hand, the reports on the degradation of terephthalate (Schlafli et al. 1994; Shigematsu et al. 2003a) are not abundant. Lastly, there are only three reports of bacterial strains that can utilize isophthalate (Wang et al. 1995; Vamsee-Krishna et al. 2006; Fukuhara et al. 2010) as sole source of carbon and energy. The degradation of phthalate isomers is initiated by inducible dioxygenase enzymes which are different for different isomers (Vamsee-Krishna et al. 2006). Additionally, the initiation of hydroxylation reaction is different in gram-positive and gram-negative bacteria. The metabolism in gram-positive bacteria involves hydroxylation at the 3 and 4 positions (3,4- route) while in gram-negative bacteria hydroxylation is at the 4 and 5 positions (4,5- route) (Stingley et al. 2004).

The enzyme which catalyzes the initial reaction of phthalate degradation, phthalate dioxygenase, is a two component enzyme possessing a reductase, a flavoprotein (ferridoxin-type [2Fe-2S] center) and an oxygenase (PDO; a non-haeme iron protein with one Rieske-type [2Fe-2S] center). The oxygenase component is comprised

of large and small subunits (Batie et al. 1987; Correll et al. 1992; Gassner and Ballou 1995; Tarasev and Ballou 2005). Terephthalate dioxygenase is also a two component enzyme consisting of oxygenase and reductase activity (Schlafli et al. 1994; Shigematsu et al. 2003a). Initial hydroxylation of phthalate isomers results in the formation of dihydroxy-derivatives which then undergo dehydrogenation and decarboxylation to form protocatechuate (Choi et al. 2005; Patil et al. 2006; Vamsee-Krishna et al. 2006) and are further metabolized *via* the *ortho*- (Zylstra et al. 1989; Vamsee-Krishna et al. 2006) or *meta*-ring cleavage pathway (Eaton 2001). Terephthalate in the initial hydroxylation forms 2-hydro-1,2-dihydroxyterephthalate (Choi et al. 2005), whereas isophthalate is acted upon by isophthalate 3,4-dioxygenase to form 4-hydro-3,4-dihydroxyisophthalate (Vamsee-Krishna et al. 2006). Generated intermediates leads to the formation of 3,4-dihydroxybenzoate and metabolized further *via* ring cleavage to central carbon pathway.

21.3.9 Phenylacetic Acid and Its Derivatives

Aerobic phenylacetic acid metabolizing bacteria was first reported in 1955 (Kunita 1955). Since then, several bacterial strains capable of utilizing phenylacetic acid were isolated however; the catabolic pathway for its degradation is revealed very recently (Teufel et al. 2010) (Fig. 21.4b). The catabolic pathway was similar to that reported for benzoate in *Azoarcus evansii* (Zaar et al. 2004; Gescher et al. 2005, 2006) (Fig. 21.4a). Like benzoate pathway, the catabolic route of phenylacetic acid also showed an unconventional strategy with involvement of a CoA-thioesters as intermediates. This was suggested with the induction of a phenylacetate-CoA ligase in *Pseudomonas putida* under aerobic conditions (Martinez-Blanco et al. 1990; Vitovski 1993). The phenylacetate-CoA ligase gene was found to be clustered together with 13 other phenylacetic acid (paa) genes in three transcriptional units, which were suspected to be involved in phenylacetic acid assimilation. Moreover, many of these genes were mandatory for growth on this substrate. A similar gene organization was reported in *E. coli* and in several *Pseudomonas* species (Teufel et al. 2010). It is the only known bacterial aerobic degradation pathway for phenylacetic acid. However, many attempts to identify pathway intermediates other than phenylacetyl-CoA led only to rough outlines. It has been reported that the metabolism of phenylacetic acid by certain species of *Pseudomonas* involves 4-hydroxyphenylacetic acid and 3,4-dihydoxyphenyl acetic acid as intermediates (Blakley et al. 1967) suggesting that 4-hydroxyphenyl acetic acid and phenylacetic acid could be catabolized by the same pathway. By contrast, other microorganism such as *Flavobacterium* (Van den Tweel et al. 1986) was proposed to catabolize phenylacetic acid by a different route which requires the formation of 3-hydroxyphenyl acetic acid and 2,5-dihydoxyphenyl acetic acid consecutively. A special catabolic variation is proposed to be performed by *Nocardia salmonicolor* which first oxidizes phenylacetic acid to 2-hydroxyphenyl acetic acid with the later formation of 2,5-dihydoxyphenyl acetic acid (Sariaslani et al. 1974).

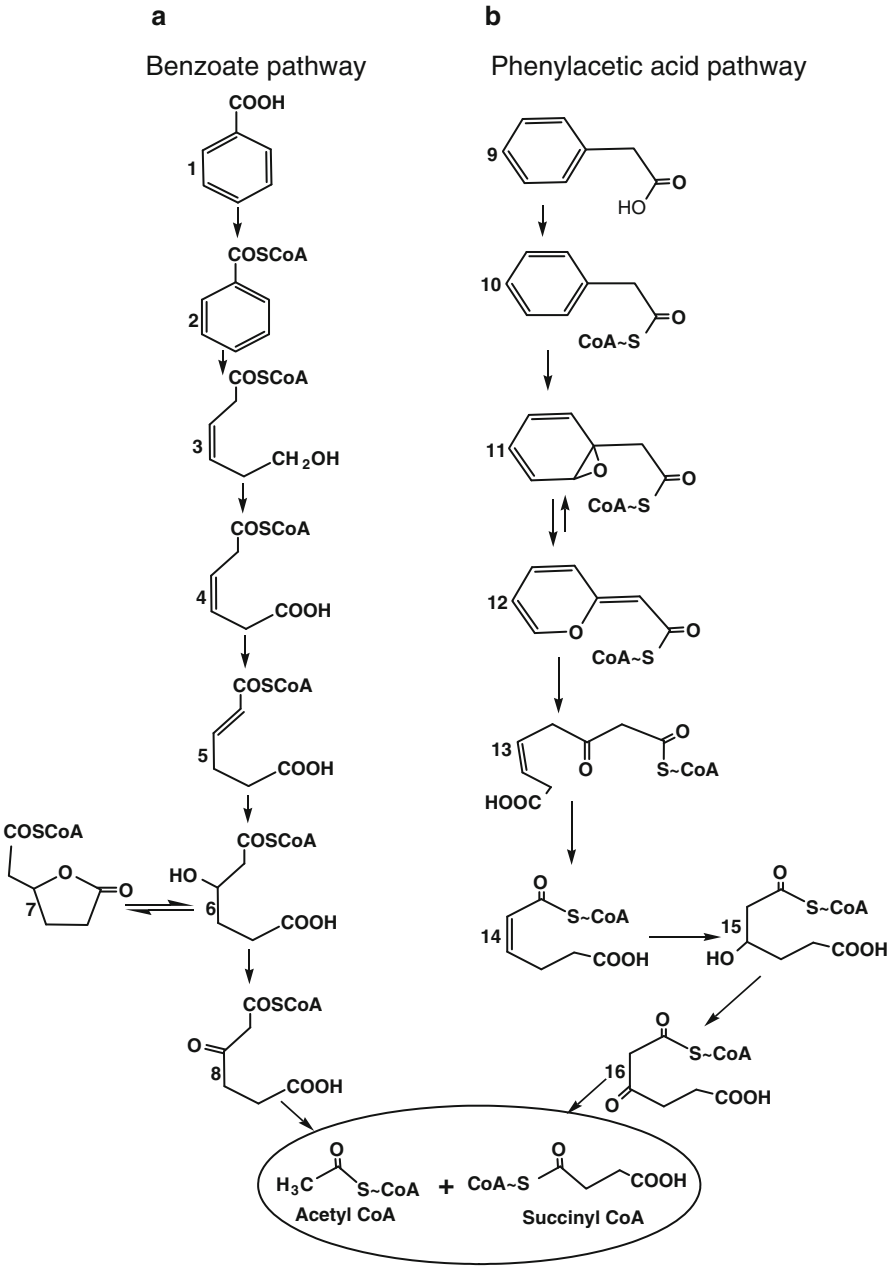

Fig. 21.4 Aerobic metabolic pathway for benzoate and phenylacetic acid degradation in bacteria. (**a**) Benzoate. 1, Benzoate; 2, benzoyl CoA; 3, 6-hydroxy-3-hexenoyl-CoA; 4, *cis*-3,4-dehydroadipyl-CoA; 5, *trans*−2,3-dehydroadipyl-CoA; 6, *β*-hydroxyadipyl-CoA; 7, *β*-hydroxyadipyl-CoA lactone; 8, *β*-ketoadipyl CoA. (**b**) Phenylacetic acid. 9, Phenylacetic acid; 10, Phenylacetyl CoA; 11, ring 1,2-epoxyphenylacetyl CoA; 12, 2-oxepin-2(3H)-ylideneacetyl CoA; 13, 3-oxo-5,6-dehydrosuberyl-CoA; 14, 2,3-dehydroadipyl CoA; 15, 3-hydroxyadipyl CoA; 16, 3-oxoadipyl CoA

The catabolic pathway of hydroxyl derivative of phenylacetic acid, i.e. 4-hydroxyphenylacetic acid in *Pseudomonas putida* and other unrelated microorganisms has been widely studied. This aromatic compound is first oxidized to 3,4-dihydroxyphenylacetic acid (homoprotocatechuic acid) and later, through a series of reactions involving the formation of 5-carboxymethyl-2-hydroxymuconic semialdehyde, pyruvic and succinic acids are produced (Sparnins et al. 1974).

21.4 Limitations to the Biodegradation

21.4.1 Bioavailability

Bioavailability may be defined as a limitation in the process of biodegradation due to the insufficient solubility of aromatic hydrocarbons. As a result, the rate of degradation decreases hindering the metabolism of such compounds. Significant amount of studies has been performed to elucidate methods for the effective solubilization of these hydrophobic molecules. This limitation during the process of biodegradation may be overcome by applying the so called biosurfactants. These are amphiphilic molecules consisting of both hydrophobic as well as hydrophilic domains and have the property to organize at interfaces such as oil/water or air/water and help lower the interfacial energy and tension. Incorporation of biosurfactants which can increase the solubility of aromatic hydrocarbons in water or isolating microorganisms which produce their own biosurfactants lead to efficient uptake and degradation of aromatic compounds. Hence, biosurfactants find a wide range of applications in various industries (Georgiou et al. 1992; Desai and Banat 1997; Banat et al. 2000; Mukherjee et al. 2006). In a microbial culture, there are three pools of biosurfactants: (1) the intracellular pool including membrane lipids, (2) the extracellular pool including excreted polysaccharide-protein-lipid complex, and (3) the interposed cell surface pool located on the cell wall and capsule, which includes the lipid and lipid-polymer complex. Some hydrocarbon-degrading microbes not only produces surface active compounds but also changes their cell surface hydrophobicity in response to the sparingly soluble compounds (Zhang and Miller 1994; Phale et al. 1995a, b; Makin and Beveridge 1996; Bouchez-Naitali et al. 1999; Al-Tahhan et al. 2000; Beal and Betts 2000; Tuleva et al. 2002; Noordman and Janssen 2002; Noordman et al. 2002; Prabhu and Phale 2003; Kumara et al. 2006). Production of biosurfactant by cells will help to pseudosolubilize hydrocarbons and facilitate their uptake (Zajic and Miller 1984; Rosenberg 1986; Haferberg et al. 1986; Rosenberg and Ron 1999).

Application of biosynthetic or chemically synthesized surfactants have led to the increased efficiency and rates of hydrocarbon degradation (Barkay et al. 1999; Schippers et al. 2000; Sandrin et al. 2000; Moran et al. 2000; Beal and Betts 2000; Gu and Chang 2001). Co-inoculation of rhamnolipid-producing *P. aeruginosa* with phenanthrene degrading *Pseudomonas* strains lead to increased levels of mineralization of phenanthrene (Dean et al. 2001). Similarly, external application

of rhamnolipid resulted in efficient utilization of hexadecane by *P. aeruginosa* (Shreve et al. 1995; Noordman and Janssen 2002; Noordman et al. 2002). Similarly, external addition of Triton-X-100 and Tween 80 has also been found to increase the rate of degradation of various aromatic compounds. In an study, enhanced degradation of diesel constituents was reported after addition of Triton-X-100 (Mohanty and Mukherji 2007). Rhamnolipids has been shown to have better biodegradability effect as compared to Triton-X-100 and rhamnolipids are also more biodegradable than Triton-X-100 under aerobic conditions (Mohan et al. 2006). Due to their low toxicity, effectiveness, and biodegradable nature, biosurfactants are ideal for various environmental applications like remediation of various aromatic and xenobiotic compounds from soils, oil contaminated water, and heavy metals from water (Desai and Banat 1997).

21.4.2 Carbon Catabolite Repression

Preferential utilization of structurally simple carbon sources like glucose over complex aromatic compounds is one of the major limitations in the process of biodegradation of aromatic compounds. The most extensively studied example is the repression of lactose utilization by glucose in *Escherichia coli* (Stulke and Hillen 1999, 2000). This is generally referred to as catabolite repression control (CRC) and is mediated through the elevated levels of cAMP (Notley-McRobb et al. 1997; Kimata et al. 1997). In the genus *Pseudomonas*, studies suggest that glucose uptake is inhibited and glucose-catabolizing enzymes are repressed by organic acids (Tiwari and Campbell 1969; Hylemon and Phibbs 1972; Roberts et al. 1973; Mukkada et al. 1973; Midgley and Dawes 1973; Phillips and Mulfinger 1981). c-AMP levels and adenylate cyclase activity in *P. aeruginosa* grown on repressing carbon source were similar to those cells grown in its absence. The external addition of c-AMP did not alter the repression (Siegel et al. 1977; Phillips and Mulfinger 1981). Organic acids have also been known to repress several enzymes (Smyth and Clarke 1975; Phillips and Mulfinger 1981; Zylstra et al. 1989; Duetz et al. 1994). On the other hand, glucose has been shown to repress xylene utilization in *P. putida* (Holtel et al. 1994) and phenyl acetic acid degradation operon in *P. putida* U (Schleissner et al. 1994). In *P. putida*, BADH and BZDH encoded by TOL plasmid, pWW0, were found to be repressed by acetate and glucose (Hugouvieux-Cotte-Pattat et al. 1990). Organic acids are known to suppress aromatic compound utilization (Schleissner et al. 1994; Duetz et al. 1994; Holtel et al. 1994; Muller et al. 1996; Collier et al. 1996; Ramos et al. 1997; McFall et al. 1997; Rentz et al. 2004; Choi et al. 2007). In a mixture of aromatic compounds, microbes utilize simple aromatic compounds or compounds that consume low energy to metabolize (i.e., compounds with higher oxidation level) over complex one. *Pseudomonas putida* and *Acinetobater* strains utilize benzoate over 4-HBA, while *Rhodococcus* prefers benzoate over phthalate when supplied as a mixture (Patrauchan et al. 2005; Choi et al. 2007).

Interestingly, *P. putida* CSV86 preferentially utilizes various aromatics like naphthalene, methylnaphthalenes, benzoate, and benzyl alcohol over glucose, thus showing a diauxic growth profile with utilization of aromatics in the first-growth phase. The strain cometabolizes aromatics with organic acids. However, like other pseudomonads, glucose utilization was suppressed by organic acids (Basu et al. 2006). Glucose uptake and metabolizing enzymes were found to be inducible in this strain. The preferential utilization of aromatic compounds over glucose in *P. putida* CSV86 could be due to the repression of the glucose uptake and glucose-metabolizing enzymes by aromatic and/or organic acids produced from the metabolism of aromatics (Basu and Phale 2006; Basu et al. 2006; Rojo 2010).

21.4.3 Heavy Metal Ion Pollution

Bioremediation and efficient utilization of aromatic hydrocarbons in sites contaminated with metal pollutants is a complex problem. According to the EPA, 40% of the hazardous waste sites are co-contaminated having both the organic and metal pollutants (Sandrin and Maier 2003). Metals like arsenic, barium, cadmium, chromium, lead, mercury, nickel, and zinc are the most frequently found at such sites and the common organic co-contaminants include petroleum, chlorinated solvents, pesticides, and herbicides. The process of biodegradation is generally considered to be an environmentally sound and cost-effective process for removing organic contaminants but the non biodegradable metal component must either be removed or stabilized within the site. Metal removal involves a combination of steps that may include mobilization, separation and collection, off-site transport, and disposal. Stabilization of metals requires that the site be permanently changed in some way. Most drastic is vitrification, wherein contaminated soil is heated to form a glasslike substance (Staley 1995). Alternatively, the site may be capped or paved to prevent water from entering the site and transporting metal contaminants, or site conditions may be imposed (e.g., anaerobiosis) that reduce the potential for metal mobilization and transport (Zoumis et al. 2001; Liu et al. 2001). Metal removal or metal stabilization, treatment of the organic component by biodegradation is likely to be the first step in remediation of co-contaminated sites (Roane et al. 1996).

Metal ions affect the growth of microorganisms through various mechanisms; however, microorganisms have also adapted and employ resistive measures using different strategies (Nies 1999). The metal ions exert toxicity on microorganisms by inactivating the metabolically essential enzymes or interfering into the cellular process. Metal toxicity is most commonly ascribed to the tight binding of metal ions to sulfhydryl (–SH) groups of enzymes essential for microbial metabolism. Besides this, metal ions may substitute for physiologically essential cations within an enzyme (e.g., Co^{2+} may substitute for Fe^{2+}), or metal oxyanions, like arsenate may replace structurally similar essential nonmetal oxyanions, such as phosphate. Such actions of metal ions will lead to the non-functionality of the enzyme. In addition, metals also impose oxidative stress on microorganisms (Kachur et al. 1998). It has

been well conceptualize that the effect of metal on biodegradation is calculated only by the amount of metal present in ionic form and not by the total amount of metal. This is generally called as bioavailable metal (Angle and Chaney 1989). Metals inhibit aerobic biodegradation of a variety of organic pollutants of concern including chlorinated phenols and benzoates (BENs), low molecular weight aromatics, and hydroxybenzoates. In a study, metal ion effect on the biodegradation by *Pseudomonas* sp. and *Micrococcus* sp. was studied (Benka-Coker and Ekundayo 1998). Individual impact of zinc, lead, copper and manganese was tested on crude oil biodegradation. Biodegradation was hindered maximally by zinc and minimally by manganese.

The mechanisms of metal ion toxicity differ in different environmental, physical and ecological conditions. Hence to remediate the co-contaminate sites, a more thorough bioremediational approach needs to be developed to eliminate the metal ion toxicity. Additions of metal-resistant microorganisms, pH adjustment or additives that reduce metal bioavailability are some of the major strategies which may form the basis of developing a single effective approach to combat metal ion toxicity.

21.5 Conclusions and Future Prospectives

Various mono-aromatic compounds are major pollutants in the environment. Bacterial degradation of these compounds has highlighted their enormous capability to be used during bioremediation. Several strains were isolated and shown to utilize a wide array of mono-aromatic compounds rapidly and completely to central metabolites. Even though, bacteria evolved to utilize these aromatics, there are environmental and biophysiochemical limitations that affect their ability to degrade pollutants. Hence, study of bacterial system that can utilize range of aromatic compounds in various environmental conditions as well as has non-limiting biochemical features would be ideal for the environmental clean up. The concept of a group of bacterial strains with all the possible features with minimum set of limitations can be designed and constructed using genetic engineering tools and can be used in the regulation of environmental sustainability.

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Chapter 22
Bioremediation of Arsenic from Contaminated Water

Anirban Pal and K.M. Paknikar

Abstract Presently, over 100 million people worldwide are exposed to arsenic contaminated groundwater making it one of the largest environmental catastrophes. Arsenic originates from the earth's crust which finds its way into groundwater as a result of various geological, biological and even anthropological processes. Toxicity effects of arsenic have been reported in plants, animals and most vividly in humans. WHO has recently brought down the permissible limit of arsenic in drinking water to 10 μg/L. Various physicochemical and biological arsenic remediation methods have been reported and applied to render groundwater potable for human use. Microbial remediation of arsenic from aquatic environments presents an interesting option because of its high efficiency, low cost and most importantly its ecofriendly nature. Microbial bioremediation of arsenic occurs through various processes. Microbially mediated arsenic redox reactions are one of the most important phenomena for arsenic remediation. We have reported *Microbacterium lacticum,* a unique bacterium that can tolerate up to 3,000 mg/L arsenite and oxidizes it rapidly to arsenate almost totally and applied this organism for arsenic bioremediation in field level operations in the arsenic affected areas. Besides microbial oxidation, biosorption and methylation are other options that have been tried with limited success. Thus many options are available for microbial bioremediation of arsenic from aqueous environments; these can be effectively used with or without other physicochemical methods. Our article will take an in-depth review of the fundamental processes involved as well as various laboratory scale, pilot scale and field scale technologies developed.

Keywords Arsenic • Arsenate • Bioremediation • *Microbacterium lacticum* • Biosorption • Aqueous environment

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22.1 Introduction

22.1.1 Arsenic Contamination of Water Resources

Arsenic contamination of groundwater is a huge problem affecting millions of people in several countries. A number of large aquifers in various parts of the world have been identified with arsenic occurring at concentrations above 10 μg/L, the maximum concentration limit (MCL) recommended for drinking water by the World Health Organization (WHO) (Wang and Zhao 2009). Most of these aquifers are in parts of Argentina, Bangladesh, Chile, northern China, Hungary, India (West Bengal), Mexico, Romania, Taiwan (China) and many parts of the USA. India (West Bengal) and Bangladesh is the region worst affected by groundwater arsenic problem (Harvey et al. 2002; Jahan et al. 2006). The maximum severity is in Bangladesh where groundwater is contaminated with naturally occurring arsenic from the alluvial and deltaic sediments that form the aquifers. It has been estimated that about 75 million people are at risk of developing health effects associated with the ingestion of arsenic (Karim 2000; Jahan et al. 2006). Arsenic problem was first identified in West Bengal in the 1990s when people started showing typical signs of arsenicosis, beginning with skin rashes and leading to cancers of major organs such as the lungs, kidneys, and bladder (Chowdhury et al. 2000). Similar problems were detected in Bangladesh, which is in close proximity to West Bengal and has a similar land pattern based on alluvial and deltaic sediments. It was found that more than half of the approximately four million wells that constitute Bangladesh's drinking water supply have levels of naturally occurring arsenic above the WHO standard of 0.01 mg/L, thus exposing as many as 50 million people to dangerous levels of arsenic through their drinking water (Jahan et al. 2006).

22.2 Sources of Arsenic Contamination

22.2.1 Geological Sources

Arsenic (As) is a toxic metalloid found in rocks, soil, water, sediments, and air. Despite its low crustal abundance (0.0001%), it is widely distributed in nature and is commonly associated with the ores of metals like copper, lead, and gold (Nriagu 2002; Oremland and Stolz 2003). The most important ores of arsenic are pyrites, realgar, and orpiment. Arsenic is introduced into soil and water during the weathering of rocks and minerals followed by subsequent leaching and runoff (Mahimairaja et al. 2005).

Based on arsenic geochemistry, three probable mechanisms have been proposed for arsenic mobility in groundwaters of West Bengal and Bangladesh by Bose and Sharma (2002) (Mahimairaja et al. 2005)

1. Mobilization of arsenic due to the oxidation of arsenic bearing pyrite minerals. Insoluble arsenic bearing minerals such as arsenopyrite (FeAsS) are rapidly oxidized (Eq. 22.1) when exposed to atmosphere, releasing soluble arsenite [As(III)], sulfate ($SO4^{2-}$), and ferrous iron [Fe(II)] (Mandal et al. 1996).

$$\mathrm{FeAsS} + 13\ \mathrm{Fe}^{+3} + 8\mathrm{H_2O} = 14\ \mathrm{Fe}^{+2} + \mathrm{SO4}^{-2} + 13^{\mathrm{H}+} + \mathrm{H_3AsO_4}\ (\mathrm{Aq.}) \qquad (22.1)$$

2. Dissolution of arsenic rich iron oxyhydroxides (FeOOH) due to onset of reducing conditions in the subsurface. Under oxidizing conditions, and in the presence of Fe, inorganic species of arsenic are predominantly retained in the solid phase through interaction with FeOOH coatings on soil particles. The onset of reducing conditions in such environments can lead to the dissolution of FeOOH coatings (McArthur et al. 2000; Nickson et al. 2000).
3. Release of arsenic sorbed to aquifer minerals by competitive exchange with phosphate ($H_2PO_4^-$) ions that migrate into aquifers from the application of fertilizers to surface soil (Acharyya et al. 1999).

The second mechanism involving dissolution of FeOOH under reducing conditions is considered to be the most probable reason for excessive arsenic accumulation in groundwater (Harvey et al. 2002; Smedley and Kinniburgh 2002; Mahimairaja et al. 2005a, b).

Volcanoes are also considered as a geological source of arsenic to the environment with the total atmospheric annual emissions from volcanoes being estimated at 31,000 mg (Smith et al. 1998; Walsh et al. 1979; Mahimairaja et al. 2005a, b).

22.2.2 Anthropological Sources

Arsenic is also being introduced into the environment through various anthropogenic activities. Major sources of arsenic discharged onto land originate from commercial wastes (~40%), coal ash (~22%), mining industry (~16%), and the atmospheric fallout from the steel industry (~13%) (Eisler 2004; Nriagu and Pacyna 1988; Mahimairaja et al. 2005a, b).

Industries manufacturing arsenic containing products are likely to contaminate soil and water bodies due to the arsenic laden liquid and solid wastes generated. For example, indiscriminate discharge of industrial effluents from the manufacturing of Paris Green (copper acetoarsenite, an arsenical pesticide) resulted in soil and groundwater contamination in residential area of Calcutta, India (Chatterjee and Mukherjee 1999; Mahimairaja et al. 2005a, b).

The use of compounds like sodium arsenite, lead arsenate, calcium arsenate, magnesium arsenate, zinc arsenate, zinc arsenite and Paris Green to control aquatic weeds in ponds and as herbicides in orchards has contributed to soil and groundwater arsenic contamination in many parts of the world (Adriano 2001; Merry et al. 1983; Peryea and Creger 1994; Mahimairaja et al. 2005a, b).

In New Zealand, the effluents from timber treatment plants are reported to be a major source of arsenic contamination in aquatic and terrestrial environments (Bolan and Thiyagarajan 2001; Mahimairaja et al. 2005a, b).

22.2.3 Biological Sources

Biological sources contribute only small amounts of arsenic into soil and water ecosystems. Arsenic accumulates readily in living tissues because of its strong affinity for proteins, lipids, and other cellular components (Ferguson and Gavis 1972; Mahimairaja et al. 2005). Aquatic organisms can accumulate arsenic easily thus accumulating considerably higher concentrations than their surroundings (i.e., biomagnification). These organisms contribute to environmental contamination upon consumption or disposal/degradation. Biomagnification of arsenic could lead to its hierarchical transfer from soil to plants followed by animals and humans, involving terrestrial and aquatic food chains. For example, poultry manure addition is considered to be one of the major sources of arsenic input to soils (Mahimairaja et al. 2005). In the Delaware-Maryland-Virginia peninsula along the eastern shore of the United States, 20–50 mg of arsenic is reportedly introduced annually to the environment through the use of arsenic compounds (e.g., Roxarsone, ROX) in poultry feed (Christen 2001; Mahimairaja et al. 2005a, b).

22.2.4 Microbial Transformations Leading to Arsenic Mobilization

The contribution made by microorganisms to the biogeochemistry of arsenic in the environment is extensive and detailed as it involves various oxidation, reduction, methylation, and demethylation reactions of its dominant chemical species (Oremland and Stolz 2003).

Several theories have been proposed to explain the subsurface mobilization of arsenic (Oremland and Stolz 2003), they are as follows:

1. The oxidation of arsenic containing pyrites (Chowdury et al. 1999)
2. The release of As (V) from reduction of iron oxides by autochthonous organic matter (e.g., peat) (McArthur et al. 2000),
3. The reduction of iron oxides by allochthonous organic matter (from dissolved organics in recharging waters) (Harvey et al. 2002),
4. The exchange of adsorbed As (V) with fertilizer phosphates (Nordstrom 2000).

Oremland and Stolz (2003) considered the microbial cycling of arsenic to be confined to microbial transformations between its +3 and +5 oxidation states.

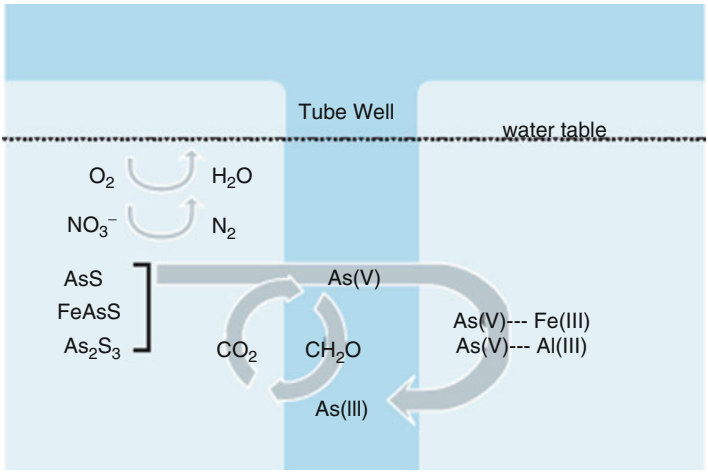


Fig. 22.1 A conceptual model of how arsenic-metabolizing prokaryotes may contribute to the mobilization of arsenic from the solid phase into the aqueous phase in a subsurface drinking water aquifer (Oremland and Stolz 2003)

They suggested that the above mentioned processes are not mutually exclusive processes. Over time microorganisms play an essential role in both the direct reduction and oxidation of the arsenic species, as well as the iron minerals contained in these aquifers. A conceptual model was formulated to explain the mobilization of arsenic in aquifers, which proposed that the initial process is the oxidation of the original As(III)-containing minerals (e.g., arsenopyrite) during transport and sedimentation by pioneering chemoautotrophic arsenite oxidizers (CAOs) and heterotrophic arsenite oxidizers (HAOs). This would result in the accumulation of As(V) onto surfaces of oxidized minerals like ferrihydrite. Subsequent human activity in the form of intensive irrigated agriculture, digging of wells, and lowering of groundwater tables provides oxidants (e.g., oxygen, nitrate) that further stimulate As(III) oxidation. This causes a buildup of microbial biomass (and its associated organic matter) and the creation of anoxic conditions. This organic matter, in conjunction with other sources either from decomposing buried peat deposits or from that dissolved in seasonal recharge from agricultural surface waters, would in turn promote the dissimilatory reduction of adsorbed As(V) by Dissimilatory arsenate reducing prokaryotes (DARPs) and the eventual dissolution of adsorbent minerals like ferrihydrite. The end result of these processes over time which is also accelerated by human activities would be the release of arsenic into the aqueous phase, as illustrated in Fig. 22.1.

22.3 Toxicity of Arsenic

22.3.1 Human

Arsenic is a metal that can generate multiple adverse health effects because of the many chemical forms it takes. Arsine gas (AsH_3) is the most toxic compound having a fatal dose of 250 mg/m^3 at an exposure time of 30 min (Bissen and Frimmel 2003a). The lethal dose (LD_{50}) for arsenic trioxide is 34.5 mg/kg (fatal dose for adults: 120–300 mg), sodium arsenite 4.5 mg/kg, sodium arsenate 14–18 mg/kg, monomethylarsonic acid 1,800 mg/kg, dimethylarsinic acid 1,200 mg/kg, and trimethylarsine 8,000 mg/kg (Yamauchi and Fowler 1994; Bissen and Frimmel 2003a).

As(III) is absorbed faster in biological systems than As(V), however both the oxidation states are reported to inhibit the energy-linked functions of the mitochondria. As(III) compounds have a high affinity to sulfhydryl groups in proteins and can cause deactivation of enzymes. As(V) competes with phosphate in cell reactions and can uncouple oxidative phosphorylation so that the high-energy – bonds of adenosine triphosphate are not conserved (Gorby 1994; Squibb and Fowler 1993; Pontius et al. 1994; Bissen and Frimmel 2003a).

Arsenic can cause both acute and chronic poisoning. Chronic arsenic poisoning involves non-specific symptoms such as weakness, loss of reflexes, weariness, gastritis, colitis, anorexia, weight loss, and hair loss. Long-term exposure through food or air results in hyperkeratosis, hyperpigmentation, cardiovascular diseases, disturbance in the peripheral vasculature and nervous systems, circulatory disorders, brittle loose nails with transverse white bands across the nails called Mees lines, eczema, suffering from liver and kidney disorder. Arsenic is deposited in hair, skin, nails, and bones (Vahter 1983; Hindmarsh and Mc Curdy 1986; Lu 1990; Hall 2002; Bissen and Frimmel 2003a).

Acute arsenic poisoning may cause vomiting, dryness of the mouth and throat, muscle cramps, colicky abdominal pain, tingling of the hands and feet, circulatory disorders, and nervous weakness, the skin may become cold and clammy, hallucinations, delirium, and diarrhoea may also appear, fatal shock can develop due to renal failure. Death may result within a short time due to hepatic failure, renal failure, or heart attack (Gorby 1994; Bissen and Frimmel 2003a). The human body can detoxify the inorganic arsenic compounds As(III) and As(V) by methylation to a certain amount to reduce the affinity of arsenic for tissue. The possibility of methylation of arsenic is limited to an arsenic uptake of 400–500 μg/day (Pontius et al. 1994; Bissen and Frimmel 2003a). Arsenic compounds are excreted in the urine after 3–4 days (Yamauchi and Fowler 1994; Pontius et al. 1994; Bissen and Frimmel 2003a). The individual sensitivity to arsenic differs. Humans who are not accustomed to the consumption of arsenic die at an arsenic uptake between 0.1 and 0.3 g/day. However, chronic arsenic consumption reportedly increased the tolerance to 1 g/day H_3AsO_3 without manifestations of acute poisoning (Morton and Dunnette 1994; Bissen and Frimmel 2003a).

Arsenic is found to inhibit the repair of DNA damage. It is also carcinogenic as uptake of arsenic causes lung cancer, bladder cancer, renal cancer, liver cancer, and skin cancer (Bates et al. 1992; Pontius 1994; Kessel et al. 2002; Roy and Saha 2002; Bissen and Frimmel 2003a).

22.3.2 Animals

Arsenic may be absorbed by animals through ingestion, inhalation, or through permeation of skin or mucous membranes; cells take up arsenic through an active transport system normally used in phosphate transport.

Episodes of arsenic poisoning are either acute or sub acute; chronic cases of arsenicosis are seldom encountered in any species except man. Single oral doses of arsenic fatal to 50% of sensitive species tested ranged from 17 to 48 mg/kg body weight (BW) in birds and from 2.5 to 33 mg/kg BW in mammals. Susceptible species of mammals were adversely affected at chronic doses of 1–10 mg arsenic/kg BW, or 50 mg arsenic/kg diet. Sensitive aquatic species were damaged at water concentrations of 19–48 μg arsenic/L, 120 mg arsenic/kg diet, or (in the case of freshwater fish) tissue residues >1.3 mg/kg fresh weight. Adverse effects to crops and vegetation were recorded at 3–28 mg of water soluble arsenic/L (equivalent to about 25–85 mg total arsenic/kg soil) and at atmospheric concentrations >3.9 ug arsenic/m^3 (Eisler 1988).

22.4 Conventional Technologies for Arsenic Remediation from Groundwater

22.4.1 Oxidation and Reduction

Oxidation is a pre required treatment step to convert the As(III) species in more easily removable As(V) species. Direct aeration is very slow (Bissen and Frimmel 2003b; Litter et al. 2010). However a number of chemicals, including gaseous chlorine, hypochlorite, ozone, permanganate, hydrogen peroxide, manganese oxides and Fenton's reagent (H_2O_2/Fe^{2+}) can be employed to accelerate oxidation (Feroze Ahmed 2002; Pirnie 2000; Litter et al. 2010). Chlorine is a rapid and effective oxidant, but it may react with organic matter, producing toxic and carcinogenic trihalomethanes as by-products (Litter et al. 2010).

22.4.2 Precipitation

Methods employing the insolubility of certain arsenical inorganic compounds such as As(III) sulfide, calcium arsenate and ferric arsenate may be proposed to remove

arsenic from water. Addition of calcium, magnesium, manganese (II) or iron (III) salts to As(V) solutions leads to the formation of arsenic containing solids that can be removed through sedimentation or filtration (Litter et al. 2010). However, the method has limitations such as the instability of the solids and inadequacy in direct disposal. However, it can be used to solve the problem in mining sites where those salts are present naturally (Ladeira et al. 2002; Litter et al. 2010).

22.4.3 Coagulation and Filtration

The most common technology for arsenic removal is coagulation and filtration. Arsenic is removed in the pentavalent form, which adsorbs onto coagulated flocs (formed by addition of flocculants) that can be then removed by filtration. As(III) has to be previously oxidized, generally with chlorine (Kartinen and Martin 1995; Litter et al. 2010). The most commonly used coagulants are aluminum sulfate [$Al_2(SO4)_3$], iron chloride ($FeCl_3$) and ferrous sulfate ($FeSO_4$), iron salts being generally better removal agents. $FeCl_3$ generates relatively large flocs, while smaller ones are formed with $FeSO_4$ (Edwards 1994; Hering et al. 1997; Newcombe and Moller 2008; Pirnie 2000; Ravenscroft et al. 2009; Litter et al. 2010). Filtration is a necessary step as it was reported that without filtration, arsenate removal is around 30%, but using a 0.1 or 1.0 mm filter, arsenate removal improves to more than 96% (Hering et al. 1996; Chwirka et al. 2000; Fields et al. 2000; Jekel and Seith 2000; Litter et al. 2010). The coagulation–filtration technology is simple, only common chemicals are used, installation costs are small and it can be easily applied to large water volumes. However, relatively large volumes of arsenic containing sludge are formed, typically disposed off in landfills which are a potential source of contamination (Litter et al. 2010).

22.4.4 Adsorption

Aluminum oxides (activated alumina), iron oxide/hydroxides, titanium dioxide, cerium oxide, or reduced metals can be used as adsorbents for arsenic (Ravenscroft et al. 2009; Litter et al. 2010). Granular activated alumina ($Al_2O_3/Al(OH)_3$) is a commercially available porous oxide, successfully applied at slightly acid pH (5–7), giving efficiencies higher than 95% for both As(V) and As(III) Efficiencies of alumina are found to be higher with As(V) than with As(III) (Pirnie 2000; Litter et al. 2010). Granular iron hydroxide, GFH, a synthetic alkaganeite proved to be a good material as it was able to retain As(V) and As(III) (Driehaus 2002; Hering et al. 1997; Wang et al. 2000; Litter et al. 2010). Microparticles with magnetic properties were developed to remove the adsorbent material after the treatment (Dahlke et al. 2003; Litter et al. 2010). Iron hydroxide nanoparticles were also used

as adsorbents. They were introduced into a polymeric network of ionic exchange resin. These materials gave good results in treating arsenic-contaminated groundwater of a village bordering Bangladesh and India (Cumbal and SenGupta 2009; DeMarco et al. 2003; Litter et al. 2010). However, despite their simplicity, the adsorption methods usually fail in lowering arsenic concentration to acceptable levels, and are recommended for treatment of water with low Fe/As content (Chaudhury et al. 2003; Driehaus et al. 1995; Litter et al. 2010). The alumina surface is saturated very rapidly at high arsenic concentrations which make regeneration necessary, usually with a caustic bath followed by an acid treatment (Kartinen and Martin 1995; Litter et al. 2010).

22.4.5 Membrane Processes

Microfiltration (MF) or ultrafiltration (UF) using low-pressure membranes (large nominal pore sizes, 10–30 psi) were found to be inadequate because the arsenical species are very small and can traverse the membranes, however, nanofiltration (NF) or reverse osmosis (RO), which use high-pressure membranes (75–250 psi) are found to be useful (Clifford 1999; Pirnie 2000; Litter et al. 2010). In case of reverse osmosis, the membrane allows water to pass through but rejects impurities especially polyvalent ions thus being suitable for arsenic oxyanions. The process is efficient over an extended pH range (3–11) (Litter et al. 2010). Small amount of the raw water (10–15%) passes through the membrane in NF and RO, hence these processes are suitable for household or applications which require only a small amount of treated water. In case of higher water volumes (e.g., municipal systems), multiple membrane units in series have to be used (Pirnie 2000; Litter et al. 2010). The main disadvantages, especially for RO, are low water recovery rates (typically 10–20%), high electrical consumption, relatively high capital and operating costs (expensive membranes), and the risk of membrane fouling (Litter et al. 2010).

22.4.6 Ion Exchange Resins

Synthetic ionic exchange resins, generally of polymeric matrix (polystyrene cross-linked with divinylbenzene), linked to charged functional groups like quaternary amine groups $N^{+}(CH_3)_3$ can be applied for arsenic removal (Litter et al. 2010). Arsenate removal by this technique is efficient, producing effluents with less than 1 mg/L of arsenic, while arsenite, being uncharged, is not removed, and a previous oxidation step is necessary (Pirnie 2000; Ravenscroft et al. 2009; Litter et al. 2010). Limitations of the technique include presence of competing anions, especially sulfate, TDS, selenium, fluoride, and nitrate, interfere strongly and can affect run length and suspended solids and precipitated iron which can cause clogging (Kartinen and Martin 1995; Pirnie 2000; Wang et al. 2000; Litter et al. 2010).

22.5 Emergent Technologies for Arsenic Remediation from Groundwater

New technologies are being developed for arsenic remediation to minimize costs of investment, operation and maintenance and technological development. These technologies focus more on small scale or household treatments for isolated populations. However, the social acceptance, waste production, treatment and the required handling needs to be assessed before considering the implementation of each remediation option (Litter et al. 2010).

22.5.1 In Situ Remediation

Different approaches for *in situ* remediation of arsenic contamination have been applied like permeable reactive barriers, air dispersion, chemical oxidation, multiphase extraction, supervised natural attenuation, etc. The use of permeable reactive barriers (PRB) and reactive zones are being predicted as one of the most efficient technologies for *in situ* removal of arsenic from groundwater. Fe (or Al) oxide-containing materials can be used as relatively cheap passive reactive barriers (Bhattacharya et al. 2002; Gavaskar et al. 1998; Gu et al. 1999; Lindberg et al. 1997; Litter et al. 2010).

22.5.2 Combined Coagulation/Flocculation and Adsorption Methods

Different technologies for single households were developed or adapted by scaling down and simplifying conventional methods used in water treatment plants, which use the oxidation, adsorption and coagulation sequence for arsenic removal from drinking water (Sastre et al. 1997; Litter et al. 2010).

22.5.3 Zerovalent Iron

Zerovalent iron (ZVI) is a material increasingly used for the treatment of several pollutants, particularly toxic metals. ZVI is one of the main components of permeable reactive barriers (PRBs). Use of ZVI for removal of arsenic has been object of different studies undertaken (Kanel et al. 2005; Leupin et al. 2005; Leupin and Hug 2005; Manning et al. 2002; Su and Puls 2001; Litter et al. 2010).

22.5.4 Low Cost Natural Adsorbent Materials

Litter et al. (2010) in their review have listed several natural adsorbent materials for removal of arsenic such as; materials based on iron/manganese oxides and other natural minerals for removal of arsenic (Deschamps et al. 2005; Mohan and Pittman 2007; Newcombe and Moller 2008; Pirnie 2000; Prasad 1994; Shevade and Ford 2004; Zeng 2003), simple sand filters for arsenic removal from groundwater having iron concentrations up to around 400 mg/L (Luzi et al. 2004), iron oxide coated sand (IOCS) to remove arsenic at small scale (Bhattacharya et al. 2002; Chen et al. 2004; Deschamps et al. 2005; Joshi and Chaudhury 1996; Thirunavukkarasu et al. 2004), manganese dioxide coated sand (Bajpai and Chaudhury 1999) and limestone particles covered by iron oxide (Banavali et al. 2008).

22.5.5 Photochemical Technologies

The use of solar or artificial light and dissolved iron has been object of several studies in the last decade, especially to facilitate oxidation of As(III) to As(V). Litter et al. (2010) proposed that to remove arsenic, a two-step process has to be designed: one for As(III) oxidation and the second one for elimination of the produced As(V), the two steps can be simultaneous or consecutive. The second mechanism is the photochemical reduction of As(V) or As(III) to elemental arsenic, a non mobile, relatively stable arsenic form, which can be removed from the aqueous phase.

22.5.6 Limitations of Conventional and Some Emergent Technologies

Conventional technologies though widely used have various limitations. The technologies like coagulation/filtration, adsorption, membrane processes and ion exchange need a pre-oxidation step for the As(III) in the contaminated water. Toxic by-products and sludge are produced in oxidation/reduction (carcinogenic trihalomethanes) processes, precipitation and coagulation/filtration. Limited efficiencies have been found in alumina adsorption and precipitation in case of high arsenic concentrations. Operation difficulties have been reported in processes like coagulation (requirement of pH adjustment), adsorption (requirement of regeneration), membrane processes (membrane fouling, high electrical consumption and low water recovery) and ion exchange (interference of TDS, F^-, NO_3^- and suspended solids) (Litter et al. 2010).

Some of the emergent technologies also have limitations as in case of *In situ* remediation problems are high impact of microbial and geochemical processes and

interference of sulfides, oxides, carbonates and hydroxides. The use of zero valent iron produces toxic solid wastes (Litter et al. 2010).

The limitations of conventional technologies and some emergent technologies calls for further search of better technologies for arsenic remediation which should be simple, efficient and having much lesser adverse impacts. Bioremediation processes for remediation of arsenic from groundwater may be considered as a possible alternative because of the variety of microbial processes involved in remediation of arsenic from natural environments which are discussed below.

22.5.7 Bioremediation of Arsenic from Contaminated Groundwater

22.5.7.1 Microbial Redox Reactions for Arsenic Remediation

Microbial arsenate metabolism was first identified by Green (1918). Green isolated an arsenate reducer, *Bacterium arenreducens*, and an arsenite oxidizer *Bacillus arsenoxydans*. The microbiological oxidation of As(III) to As(V) can impact the mobility and speciation of arsenic in the environment. The process has been known for many years and more than 30 strains representing at least nine genera have been reported to be involved, including α-,β-, and γ-Proteobacteria; Deinocci (i.e., *Thermus*); and Crenarchaeota (Stolz and Oremland 2003). Physiologically diverse, these include both heterotrophic arsenite oxidizers (HAOs) and the more recently described chemolithoautotrophic arsenite oxidizers (CAOs). Heterotrophic oxidation of As(III) is viewed as a detoxification reaction that converts As(III) encountered on the cell's outer membrane into the less toxic form i.e. As(V), perhaps making it less likely to enter the cell. CAOs couple the oxidation of arsenite (e.g., electron donor) to the reduction of either oxygen or nitrate and use the energy derived to fix CO_2 into organic cellular material and achieve growth (Stolz and Oremland 2003). The microbial reduction of As(V) to As(III) is another very important mechanism of arsenic detoxification. The most well studied mechanism of detoxification and resistance is the ArsC system (Rosen 2002; Mukhopadhyay et al. 2002), atleast three different but structurally related arsenate reductases have convergently evolved in bacteria and yeast. ArsC, a small–molecular mass protein (13–16 kDa), mediates the reduction of As(V) to As(III) in the cytoplasm. Although As(III) is more toxic, it can be excreted via an As(III)-specific transporter, ArsB (Stolz and Oremland 2003).

Weeger et al. (1999) isolated a heterotrophic arsenite-oxidizing bacterium (ULPAs1), belonging to the ß- Proteobacteria, (later named as *Cenibacterium arsenoxidans*) from arsenic contaminated waters. The growth characteristics of the bacterium were independent of the presence of arsenic [1.33 mM as As(III)] in minimum medium containing lactate as sole organic carbon source in which the doubling time was 1.5 h. However, there was no growth in the absence of an organic carbon source or in a rich medium like Luria–Bertani medium. The organism exhibited a

minimum inhibitory concentration of 6.65 mM for arsenic. The bacterium proved to be very efficient toward oxidation of high (1.33 mM) arsenic concentration in batch reactors. The oxidation rate of arsenite was 0.11 mM/h and maximum rate of 0.4 mM/h was observed with resting cells density of $5*10^8$ CFU/mL making this strain one of the fastest heterotrophic arsenite oxidizer reported.

Lievremont et al. (2003) combined the bio oxidation with the sorption of the arsenic on minerals like chabazite and kutnhorite which adsorb arsenic to different extents. Sorption processes were fast and depended on the arsenic speciation as well as on the type of solid phases. Results showed that As(V) was efficiently removed onto kutnahorite mineral (0.36 mmol/g). As(III) was shown to be oxidized by heterogeneous reactions with surface mineral components after contact with chabazite. Strain ULPAs1 (later named as *Cenibacterium arsenoxidans*) induced or not induced with arsenic performed fast As(III) oxidation at high initial arsenic concentration (1.33 mM) in batch reactors with chabazite. These experiments were useful for planning a two step detoxification process for arsenic contaminated effluents. The first step of the process could be As(III) biooxidation performed in a first column reactor filled with pure quartz sand or chabazite as supports for the biofilm. The second step could be the adsorption of As(V) in a second column filled with kutnahorite mineral adsorbent.

Simeonova (2004) took a step further and reported As(III) oxidation by immobilized cells of *C. arsenoxidans* in small calcium alginate beads, circumventing the drawbacks of handling a free suspension . High concentrations (100 mg/L) of As(III) were fully oxidized by *C. arsenoxidans*. The oxidation was shown to be faster than that of free cells. A rapid exploration of factors affecting the As(III) oxidation showed that temperature was the most important parameter. The bio-oxidation is most effective at a temperature of 25 ± 2°C. These results are promising and demonstrated the high potential of immobilized cells, i.e. improved biomass retention efficient and faster treatment of As(III) containing waste streams. Experiments to show that immobilized cells keep their As(III) oxidative ability during repeated batch and in continuous mode are under way.

Cenibacterium arsenoxidans has been renamed as *Herminiimonas arsenicoxydans* (Muller et al. 2007). Muller et al. (2007) carried out in depth exploration of *Herminiimonas arsenicoxydans* with regard to physiology, genetics, and proteomics. They reported that the bacterium possesses novels mechanisms for coping with arsenic. Besides multiple biochemical processes such as arsenic oxidation, reduction, and efflux, *H. arsenicoxydans* also exhibits positive chemotaxis and motility towards arsenic and metalloid scavenging by exopolysaccharides. These observations demonstrate the existence of a novel strategy to efficiently colonize arsenic-rich environments, which extends beyond oxidoreduction reactions. Such a microbial mechanism of detoxification is exploitable for bioremediation applications of arsenic contaminated sites.

Mokashi and Paknikar (2002) reported a culture of *Microbacterium lacticum* which could oxidize up to 50 mmol/L As (III) which was isolated from municipal sewage. Optimum pH and temperature for As (III) oxidation were found to be 7.5°C and 30°C respectively. Under optimal pH and temperature conditions the complete

oxidation of As(III) was observed in 5 h with $1*10^9$ CFU/mL. The bacterium could utilize different carbon sources such as citrate, acetate, sucrose, methanol and lactate, the oxidizing efficiency was found to be maximum with methanol as a carbon source. The bacterium could completely oxidize As(III) concentrations in the range of 0.5–3,500 mg/L with methanol as a carbon source. In the immobilized cell reactor operated in continuous mode, the microorganism could completely oxidize 0.5, 1, 5 and 10 mg/L of As(III) in 25, 60, 330 and 600 s under optimum conditions. *Microbacterium lacticum* described in this report not only has one of the highest MIC value for As(III) but it is able to retain As(III) oxidizing capacity at this concentration (50 mmol./L). As the optimal pH and temperature conditions of this bacterium are 7.5°C and 30°C, it can be easily used for remediation of arsenic in the field conditions in India having a tropical climate.

The oxidizing capacity of this microorganism of As(III) to As(V) was coupled with the removal of As(V) (contained in the effluent of the continuous reactor) on zero valent iron, activated charcoal and $FeCl_3$. Data showed that there was >99.9% removal of arsenic by all the three methods. None of the methods imparted any color, turbidity, odor or pH change (excepting zero valent iron) to the treated groundwater. Out of the three methods zero valent iron removed As(V) at a very slow rate. $FeCl_3$ method was faster than activated charcoal but ferric ions react with As (V) to form $FeAsO_4$. This secondary sludge containing As could pose disposal problems. Hence a system containing one column with immobilized cells of *Microbacterium lacticum* followed by a charcoal column was proposed as a probable arsenic removal system (Mokashi and Paknikar 2002).

Based on the above processes an integrated arsenic removal system was developed and operated. The process was demonstrated to treat 500 L of arsenic contaminated water per day for a period of >12 months, which was scaled up to treat 1,000 L/day. The integrated treatment system comprised of brick immobilized *M. lacticum* column (for arsenite oxidation), packed bed activated alumina column (for arsenate removal), packed bed charcoal column (filter for removing cells in the wash out) and UV irradiation assembly (for disinfection). Simulated groundwater with 1 mg/L arsenite and 0.03% sucrose (as a carbon source) was treated at a flow rate of 700 mL/min. in the scaled up system. The residual arsenic conc. was estimated to be 10–25 μg/L and the quality of treated water conformed to Indian standards of drinking water. The average life of each component *viz.* brick immobilized *M. lacticum* column, activated alumina column, charcoal column and UV irradiation assembly was >12, 6, 5 and >12 respectively. The operating cost of the integrated treatment system was calculated to be 0.18 Rs./L (US $ 0.0038/L) (Paknikar 2003).

Chemolithoautotrophic As (III) oxidizers couple the oxidation of As(III) (e.g., electron donor) to the reduction of either oxygen or nitrate and use the energy derived to fix CO_2 into organic cellular material and achieve growth (Wang and Zhao 2009). Santini et al. (2000) that *Pseudomonas arsenitoxidans* NT-26 grew chemolithoautrophically. *Pseudomonas arsenitoxidans* NT-26 is a gram-negative motile rod with two subterminal flagella. This organism grew chemolithoautrophically using As(III) as the electron donor, oxygen as the electron acceptor, and carbon dioxide or bicarbonate as the carbon sources. The growth

microorganism was greater in the presence of arsenite, which suggests that the organism is gaining energy from the oxidation of yeast extract. This discovery is significant because many arsenic oxidizing organisms have been discovered, such as *Bacillus arsenoxydans* and *Alcaligenes faecalis*, but none of these organisms have the ability to grow chemolithoautotrophically. Therefore, the discovery of this bacterial species is advancement in the understanding of microbial-arsenic interactions (Adeyemi 2009).

A new species of the genus *Thiomonas* (strain b6T) named *Thiomonas arsenivorans* reported by Battaglia-Brunet et al. (2006) which was isolated from Cheni gold mine site (Limousin, France) and was proved to use As(III) as sole energy source for chemolithoautotrophic growth. In another study, the As(III) oxidation capacity of *T. arsenivorans* was investigated in two up-flow fixed-bed reactors filled with sand, as biological support. Zerovalent iron (ZVI) was only added in a second fixed-bed reactor. The simultaneous biological oxidation of As(III) and chemical removal of As(III) and As(V) occurred in second fixed-bed reactor. Therefore, comparison of the results helped to evaluate the interaction between the chemical removal by ZVI and As(III) biological oxidation. The pilot unit was studied for 33 days, with HRT of 4 and 1 h. The maximal As(III) oxidation rate was 8.36 mg/h/L in first fixed bed reactor and about 45% of total As was removed in second fixed-bed reactor for an HRT of 1 h (Wan et al. 2010).

Michel et al. (2007) studied the formation and activity of an As(III)-oxidising biofilm in a bioreactor, using pozzolana (a sandy volcanic ash) as bacterial growth support for the purpose of optimizing fixed-bed bioreactors for bioremediation. After 60 days of continuous functioning with an As(III)-contaminated effluent containing 13 mg/L As(III), the active biofilm was found to be located mainly near the inflow rather than homogeneously distributed. Biofilm development by the CAsO1 (a bacterial consortium, obtained from Cheni gold mine site (Limousin, France)) and by *Thiomonas arsenivorans* was studied both on polystyrene microplates and pozzolana. Extra-cellular polymeric substances (EPS) and yeast extract were found to enhance bacterial attachment, and yeast extract also appears to increase the kinetics of biofilm formation. The specific As(III)-oxidase activity of *T. arsenivorans* was higher by ninefolds for planktonic cells (grown in a liquid medium containing As(III) in the absence of a growth support) than for sessile ones [bacteria attached to a support and grown in the presence of As(III)]and was induced by As(III). They suggested that the efficiency of fixed-bed reactors for the bioremediation of arsenic-contaminated waters can be thus optimised by controlling different factors such as temperature and EPS addition to increase biofilm density and activity.

Challan et al. (2009) continued the work and studied the colonization of pozzolana by an As(III)-oxidizing bacterial consortium from Cheni gold mine site (Limousin, France), which was monitored from the first hours of bacterial adhesion to 6 weeks of development under fed-batch conditions, using adapted ultrasonic dislodging and crystal-violet staining procedures to determine the biofilm adhering to the complex surfaces. The effect of temperature, arsenic concentration, and presence or absence of yeast extract (YE) on the amount of biofilm biomass and on the As(III)-oxidation ability was also studied. Fed-batch cultures allow twice as much

pozzolana colonization as that obtained under batch conditions. In addition, As(III) oxidation and the quantities of biomass under fed-batch culture conditions were the same at 14°C and 25°C. Preliminary tests for drinking-water bioremediation revealed the ability of Cheni Arsenic Oxidizing 1 (CAsO1) biofilm to remain and retain As(III) oxidation activity at low As(III) concentrations (50 μg/L).

Heterotrophic bacteria have not been shown to derive major energy from As(III) oxidation in growth. Heterotrophic oxidation of As(III) is viewed primarily as a detoxification reaction that converts As(III) encountered on the cellular outer membrane into less toxic form As(V), perhaps making it less likely to enter the cell (Wang and Zhao 2009).

Gihring et al. (2001) found that the organisms *Thermus aquaticus* and *Thermus thermophilus* were able to rapidly oxidize As(III) to As(V) but they were not able to grow with As(III) as the sole energy source, indicating that the ecological role of As(III) oxidation was detoxification of arsenic. Gihring and Banfield (2001) isolated a new *Thermus* strain (strain HR13) from an arsenic-rich terrestrial geothermal environment, which was capable of both As(III) oxidation for detoxification under aerobic conditions and As(V) dissimilatory reduction under anaerobic conditions (Wang and Zhao 2009).

Branco et al. (2009) investigated a highly arsenic resistant bacterium *Ochrobactrum tritici* SCII24 and found that this organism contains two different *ars* (arsenic resistance systems) operons and is able to oxidize arsenite to arsenate. The presence of arsenite oxidase genes in this organism was evaluated, and sequence analysis revealed structural genes for an As(III) oxidase (*aoxAB*), a *c*-type cytochrome (*cytC*), and molybdopterin biosynthesis (*moeA*). Experiments showed that the As(III) oxidation process in *O. tritici* requires not only the enzyme arsenite oxidase but also the cytochrome *c* encoded in the operon. The fundamental role of this cytochrome *c*, reduced in the presence of arsenite in strain SCII2 has not been found in other organisms. Such mechanisms may be exploited as bioremediation strategy in toxic environments.

Anaerobic As (III) oxidation was also observed in an arsenic contaminated industrial soil amended with inorganic C as the carbon source and nitrate as the electron acceptor by Azoarcus strain DAO1 and *Sinorhizobium* strain DAO10 (Rhine et al. 2006; Wang and Zhao 2009).

A bioremediation strategy based on injecting NO^{3-} to support the anoxic oxidation of ferrous iron (Fe(II)) and arsenite (As(III)) by chemolithotrophic As(III) oxidizing denitrifying bacteria in the subsurface, useful as a means to immobilize arsenic in the form of arsenate which would be adsorbed onto biogenic ferric [Fe(III)] (hydr)oxides was explored by Sun et al. (2009). During operation for 250 days, the average influent arsenic concentration of 567 μg/L was found to be reduced to 10.6 (9.6) μg/L in the effluent of the nitrate injected column. The cumulative removal of Fe(II) and As(III) in nitrate injected column was 6.5- to 10-fold higher than that in the column without nitrate. The dominant speciation of the immobilized iron and arsenic was Fe(III) and As(V) in nitrate injected column, compared with Fe(II) and As(III) without nitrate injection.

A very interesting finding has been reported by Osborne et al. (2010), where they reported a novel arsenite oxidizing bacteria designated GM1, isolated from a gold mine named Giant Mine in Canada. This mine is located in Northwest Territories, 62° north of the equator and 512 km south of the Arctic Circle. Gold was produced from 1948 to 1999 by roasting arsenopyrite (FeAsS)-bearing ore. The mine is reported to contain approximately 300,000 tonne of arsenic trioxide, stored in underground chambers (Clark and Raven 2004; Osborne et al. 2010). Temperatures in the underground areas range from 4°C to 10°C (Clark and Raven 2004; Osborne et al. 2010). GM1 is an aerobic psychrotolerant arsenite-oxidising bacterium from a subterranean biofilm in the Giant Mine. Unlike other characterized arsenite oxidisers, this organism is capable of growing below 10°C and is the first heterotrophic organism to oxidise arsenite in the early exponential phase of growth. GM1 is found to be a Gram-negative, rod-shaped, motile, heterotroph. Phylogenetic analysis of its full 16S rRNA gene sequence showed it to be a member of the β proteobacteria related to Polaromonas species (Osborne et al. 2010). Growth of GM1 was tested at 4°C, 10°C and 20°C in a minimal salts medium (MSM) with 0.04% (w/v) yeast extract in the presence and absence of 4 mM arsenite as studied. GM1 was unable to grow chemolithoautotrophically with arsenite (Osborne et al. 2010). They found that under all conditions arsenite was oxidised to arsenate and oxidation occurred in the early exponential phase of growth. The generation time of GM1 was shorter in the absence of arsenite, and decreased with increasing temperature (without arsenite at 4°C, 10°C and 20°C: 19, 16.5 and 7 h, respectively; with arsenite at 4°C, 10°C and 20°C: 21.5, 17.7 and 8.5 h, respectively). GM1 did not grow above 25°C. The arsenite-oxidising ability of GM1 was further confirmed by testing for arsenite oxidase (Aro) activity (Osborne et al. 2010). This discovery is very significant as this could pave the way for arsenic bioremediation in polar climates where the role of other mesophillic arsenite oxidizers is limited.

Rehman et al. (2010) reported a strain of *Pseudomonas lubricans*, isolated from heavy metal laden industrial wastewater which tolerated As(III) up to 3 mg/mL. *P. lubricans* showed optimum growth at pH 7 while optimum temperature for growth was 30°C and could oxidize As(III) 42%, 78% and 95% from the medium after 24, 48 and 72 h of incubation at optimal conditions, respectively. The arsenite oxidizing ability shown by *P. lubricans* indicates its potential application in biological treatment of wastewaters contaminated with arsenic.

Natarajan (2009) reported the role of *Acidithiobacillus* group of bacteria in acid generation and heavy metal dissolution which was studied with relevance to some Indian mines. Arsenite oxidizing *Thiomonas* and *Bacillus* group of bacteria were isolated from tailing dumps and their ability to oxidize As (III) to As (V) was established.

Katsoyiannis et al. (2002) reported the role of iron oxidizing bacteria i.e. *Gallionella* and *Leptothrix* in the co-removal of arsenic (III) with the oxidation of Fe (II) has been reported, using fixed bed up-flow bioreactors. The initial Fe (II) concentration was 1,500 mg/L and initial As (III) concentration was 100 mg/L. The removal efficiency was reported to be 80%. Further studies were conducted with iron oxidizing bacteria for removing both As (III) and As (V). The oxidation of

trivalent arsenic was found to be catalyzed by bacteria leading to an increased arsenic removal because trivalent arsenic cannot be efficiently adsorbed to iron oxides. The results indicated that both forms of arsenic could be efficiently removed to the permissible limits in drinking water. The removal efficiency went more than 90% and when the redox potential was around 320–340 mV, the removal of As(III) was increased up to 95% (Katsoyiannis and Zouboulis 2004). Zouboulis and Katsoyiannis (2005) conducted X-ray photoelectron spectroscopy (XPS) analyses to obtain information for the mechanism of As(III) removal by arsenic oxidizing iron bacteria. Results indicated that As(III) was partially oxidized to As(V) which enabled high arsenic removal efficiency (Jahan et al. 2006).

As(V) reduction can be achieved by various bacteria possessing cytoplasmic As(V) reductase (ArsC) (Silver and Phung 2005; Wang and Zhao 2009). ArsC is a small-molecular mass protein (13–16 kDa) that mediates the reduction of As(V) to As(III), which may be transported out of the cell by ArsAB arsenic chemiosmotic efflux system and by ATPase membrane system or sequestered in intracellular compartments, either as free As(III) or as conjugates with glutathione or other thiols (Rosen 2002; Oremland and Stolz 2003; Silver and Phung 2005; Wang and Zhao 2009). Macur et al. (2001) postulated that As(V) reduction under aerobic conditions is more likely a detoxification mechanism (Wang and Zhao 2009).

Another mechanism for As(V) reduction is dissimilatory anaerobic respiration, where microorganisms utilize As(V) as a terminal electron acceptor (Wang and Zhao 2009). Dissimilatory arsenate-reducing prokaryotes have been isolated from freshwater sediments, estuaries, soda lakes, hot springs and gold mines. These organisms can use a variety of electron donors including hydrogen, acetate, formate, pyruvate, butyrate, citrate, succinate, fumarate, malate and glucose (Oremland and Stolz 2003; Wang and Zhao 2009).

Zobrist et al. (2000) reported that the cell suspension of *Sulfurospirillium barnesii* was able to reduce As(V) to As(III) and Fe(III) in ferrihydrite to soluble Fe(II). The adsorption of As(III) onto the ferrihydrite or alumina phase controlled the extent of its release (Wang and Zhao 2009).

Table 22.1 summarizes the reports on role of different microorganisms in As(III) oxidation and As(V) reduction.

22.5.8 Role of Sulfate Reducing Bacteria in Arsenic Remediation

Groundwater extraction and treatment is the most widespread method for treatment of groundwater with elevated arsenic levels (US EPA report 2002). Although removal of dissolved arsenic from extracted groundwater in the treatment systems is usually effective, these systems generally cannot accomplish *in situ* decrease of groundwater arsenic on reasonable time scales (Voudrias 2001; Keimowitz et al. 2007). Therefore, alternate intervention strategies, such as *in situ* remediation, are of potential value, this remediation generally attempts to exploit a decreased arsenic

Table 22.1 Recent reports on microbial As(III) oxidation and As(V) reduction

Microorganisms	Mechanisms	Reference
Pseudomonas arsenitoxidans- NT26	Chemolithoautrophic As(III) oxidation under oxic conditions	Santini et al. (2000)
Thermus HR13	Heterotrophic As(III) oxidation under aerobic conditions and dissimilatory As(V) reduction under anaerobic conditions coupled with lactate oxidation	Gihring and Banfield (2001)
Thermus aquaticus *Thermus thermophilus*	Heterotrophic As(III) oxidation to As(V)	Gihring et al.(2001)
Azoarcus strain DAO1 *Sinorhizobium* strain DAO10	Anaerobic As(III) oxidation with inorganic C as the carbon source and nitrate as the electron acceptor	Rhine et al. (2006)
Microbacterium lacticum	Heterotrophic As(III) oxidation to As(V)	Mokashi and Paknikar (2002)
Escherichia coli *Staphylococcus aureus* *Staphylococcus xylosis*	As(V) reduction under anaerobic conditions	Tamaki and Frankenberger (1992)
Geospirillum barnseii (strain SES-3)	As(V) reduction using lactate as the electron donor	Laverman et al. (1995)
Anabaena oscillaroides	As(V) reduction to As(III)	McLaren and Kim (1995)
Chrysiogenes arsenatis	As(V) reduction using acetate as the electron donor	Macy et al. (1996)
Desulfotomaculum auripigmentum	Dissimilatory As(V) reduction using lactate as the electron donor	Newman et al. (1997)
Bacillus arsenicoselenatis (strain E1H) *Bacillus selenitireducens* (strain MLS10)	Dissimilatory reduction of As(V) to As(III) with the concomitant oxidation of lactate to acetate plus CO2	Blum et al. (1998)
Sulfurosirillum barnesii *Sulfurospirillum arsenophilum*	As(V) reduction using lactate, pyruvate or hydrogen and acetate as the electron donor	Stolz et al. (1999)
Pyrobaculum arsenaticum *Pyrobaculum aerophilum*	As(V) reduction using hydrogen as the electron donor	Huber et al. (2000)
Desulfomicrobium strain Ben-RB *Desulfovibrio* strain Ben-RA	As(V) reduction using lactate as the electron donor, oxidized lactate incompletely to acetate	Macy et al. (2000)
Sulforospirillum barnesii	Dissimilatory As(V) reduction under anaerobic conditions	Zobrist et al. (2000)
Sphingomonas *Caulobacter leidyi* *Rhizobium loti* *Pseudomonas*	As(V) reduction under aerobic conditions via a detoxification process	Macur et al. (2001)
Desulfitobacterium	Dissimilatory As(V) reduction using formate as the sole carbon source and electron donor	Niggemyer et al. (2001)

(continued)

Table 22.1 (continued)

Microorganisms	Mechanisms	Reference
Termite isolate (strain TSA-1)	Dissimilatory As(V) reduction using hydrogen as the electron donor	Herbel et al. (2002)
Rumen isolate (strain BRA-1)		
Hamster isolate (strain HT-1)		
Bacillus strain JMM-4	As(V) reduction while the lactate is oxidized to CO_2 via the intermediate, acetate	Santini et al. (2002)
Sphaerotilus	Iron oxidization and thus arsenic precipitation	Mouchet (1992)
Leptothrix ochracea		
Gallionella ferruginea		
Thiobacillus acidophilus, *Acidithiobacillus ferrooxidans*	Arsenic precipitation as ferric arsenate and arsenate-sulfate under acidic and high SO_4^{2-} conditions	Leblanc et al. (1996)
Acidithiobacillus ferrooxidans	Formation of schwertmannite under acidic and high SO_4^{2-} conditions concentrated arsenic up to several 10,000 mg/kg due to sorption and incorporation into the structure	Fukushi et al. (2003)
Gallionella ferruginea *Leptothrix ochracea*	Arsenic concentrations decreased from 50 to 200 mg/L to below 10 mg/L, As(III) partially oxidized to As(V)	Katsoyiannis and Zouboulis (2004)
Leptothrix ochracea	The first-order rate constant of As(III) oxidation was 0.23 min^{-1}. Residual arsenic concentration below 10 mg/L was achieved when the initial concentrations were 35 for As(III) and 42 mg/L for As(V).	Katsoyiannis et al. (2004)
Herminiimonas arsenicoxydans	A(III) oxidation to As(V)	Weeger et al. (1999) and Muller et al. (2007)
Herminiimonas arsenicoxydans	Bio oxidation combined with sorption of the arsenic on chabazite and kutnhorite.	Lievremont et al. (2003)
Herminiimonas arsenicoxydans	Cells immobilized in alginate beads	Simeonova (2004)
Thiomonas arsenivorans	Chemolithoautotrophic oxidation of As(III)	Battaglia-Brunet et al. (2006)
Thiomonas arsenivorans	As(III)-oxidising biofilm on pozzolana as support material	Michel et al. (2007) and Challan et al. (2009)
Ochrobactrum tritici	Role of arsenite oxidase and cytochrome C	Branco et al. (2009)
Acidithiobacillus group	Oxidation As (III) to As (V)	Natarajan (2009)
Pseudomonas lubricans	Oxidation As (III) to As (V)	Rehman et al. (2010)
GM1 (related to *Polaromonas* species)	Oxidation As (III) to As (V) under 4–25°C	Osborne et al. (2010)

Adapted and modified from Wang and Zhao (2009)

mobility under altered subsurface environments, such as more oxic conditions in which arsenic decreases due to sorption onto Fe (hydro) oxides (Dixit and Hering 2003; Pierce and Moore 1982; Keimowitz et al. 2007) or due to sorption onto zerovalent iron (Kober et al. 2005; Puls et al. 1999; Keimowitz et al. 2007) or more reducing, sulfidic conditions in which the arsenic mobility is relatively low due to sorption or coprecipitation with Fe sulfide minerals (Bostick and Fendorf 2003; Huerta-Diaz et al. 1998; Wolthers et al. 2005; Keimowitz et al. 2007) and/or formation of arsenic sulfides (Bostick et al. 2004; Keimowitz et al. 2007). Therefore, it is surmised that groundwater arsenic can be decreased *in situ* by enhancement of sulfate reduction and formation of sulfide minerals that are generally stable in suboxic groundwater (Morse et al. 1987; Keimowitz et al. 2007).

Arsenic-sulfide interactions are strongly pH-dependent and relatively complex (Wilkin et al. 2003). Arsenic solubility has been shown to decrease, then increase, then decrease again with progressively increasing sulfide concentrations; the maximum arsenic solubility occurs at about 0.1 mM sulfide, depending on the pH (Wilkin et al. 2003; Keimowitz et al. 2007).

Keimowitz et al. (2007) explored the feasibility of enhanced sulfate reduction by native microbial community having Sulfate Reducing Bacteria (SRBs) as an arsenic remediation strategy under neutral to mildly alkaline pH conditions at a landfill site in Winthrop, Maine, USA. At this site, groundwater is reducing and contains abundant sulfate. The native microbial community at this site reduced sulfate in the presence of added acetate. Acetate respiration and sulfate reduction were observed concurrent with dissolved iron concentrations initially increasing from 0.6 μM (0.03 mg/L) to a maximum of 111 μM (6.1 mg/L) and subsequently decreasing to 0.74 μM (0.04 mg/L). Dissolved arsenic concentrations initially varied with iron but subsequently increased again as sulfide accumulated, consistent with the formation of soluble thioarsenite complexes. Dissolved arsenic concentrations subsequently decreased again from a maximum of 2 μM (148 μg/L) to 0.3 μM (22 μg/L), consistent with formation of sulfide mineral phases or increased arsenic sorption at higher pH values. They postulate that these results indicate that *in situ* precipitation of sulfide mineral phases may be a viable remediation strategy at some arsenic contaminated landfill sites.

Natarajan (2008) reported the role of Sulphate Reducing Bacteria (SRB) isolated from some abandoned mines and acid mine drainage to precipitate dissolved metals such as copper, zinc, iron and arsenic. Arsenic bioremediation was demonstrated through the use of native microorganisms such *Thiomonas* spp. which could oxidize arsenite to arsenate. Bioremoval of arsenic through the use of jarosite precipitates generated by *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* was also found to be very effective. This processes could be used in the remediation of acid mine drainage and for efficient removal of toxic metal ions such as copper, zinc and arsenic.

Bioremediation of metal/metalloid-contaminated groundwater makes the use of sulphate-reducing bacteria (SRB) that reduce sulphate to sulphide while oxidising a carbon source (Malik 2004). Bioremediation of arsenic contaminated water by SRBs can be cost effective if a suitable carbon source and support matrix is available.

Teclu et al. (2008) used a mixed culture of sulphate-reducing bacteria to study the bioremediation of arsenic species [As(III) or As(V)] from groundwater. During growth of a mixed SRB culture which were isolated from anaerobic sediments from the Msunduzi river (Pietermaritzburg, South Africa) initial concentration of 1 mg/L of either As(III) or As(V) was reduced to 0.3 and 0.13 mg/L respectively. When 1 mg/L of arsenic either species was contacted for 24 h with the precipitate produced by batch cultured sulphate-reducing bacteria (SRB-PP) there was a removal of about 77% and 55% of As(V) and As(III) respectively. The adsorption data were fitted to Langmuir and Freundlich isotherms.

Teclu et al. (2009) also explored the role of molasses as a carbon source and polystyrene beads, sand and pine bark as support matrices for a consortium of SRBs which were isolated from anaerobic sediments from the Msunduzi river (Pietermaritzburg, South Africa). Polystyrene beads were found to be the best matrices with 49% removal of the original sulfate. Polystyrene was further examined for its durability as a long-term support material for the growing of SRB in the presence of As(III) and/or As(V) at concentrations of 1, 5 and 20 mg/L. An immobilized mixed culture of SRB with molasses as carbon source and polystyrene as support matrix was grown in laboratory-scale bioreactors to investigate the treatment of synthetic groundwater containing either As(III) or As(V) at initial concentration of 20, 10, 5 and 1 mg/L. Percentage removal of As(III) improved from about 10–47% when the concentration was reduced from 20 to 1 mg/L whereas the corresponding improvement for As(V) was from 39% to 92% during the 14-day experiment.

In an interesting study, Upadhyaya et al. (2010) developed a bioreactor system, consisting of two biologically active carbon (BAC) reactors in series for the simultaneous removal of nitrate and arsenic from a synthetic groundwater supplemented with acetic acid. A mixed biofilm microbial community that developed on the BAC was capable of utilizing dissolved oxygen, nitrate, arsenate, and sulfate as the electron acceptors. Nitrate was removed from a concentration of approximately 50 mg/L in the influent to below the detection limit of 0.2 mg/L. Biologically generated sulfides (by the SRBs) resulted in the precipitation of the iron sulfides mackinawite and greigite, which concomitantly removed arsenic from an influent concentration of approximately 200 μg/L to below 20 μg/L through arsenic sulfide precipitation and surface precipitation on iron sulfides. This study showed for the first time that arsenic and nitrate can be simultaneously removed from drinking water sources utilizing a bioreactor system.

It was found that under anaerobic conditions, arsenic can also be removed from groundwater by sequestering into insoluble sulfides indirectly by the metabolic activity of sulfate-reducing bacteria, utilizing a range of organic substrates with SO_4^{2-} as the terminal electron acceptor (Leblanc et al. 1996; Fukushi et al. 2003).

22.5.9 Microbial Biosorption for Arsenic Remediation

Biomass is biological materials derived from living or recently living organisms. Arsenic biosorption to biomass is a complicated process resulted from physicochemical

adsorption, ion exchange, coordination, complexation, chelation and precipitation. It may help to transfer soluble arsenic into solid phases, thus helping to remove it from groundwater or immobilize it in soils and sediments. It also can be used potentially for the drinking water treatment process (Wang and Zhao 2009).

22.5.10 Algae

Literature indicates that certain species of algae uptake heavy metals such as copper, cadmium, chromium, lead, and nickel (Qiming et al. 1999; Jahan et al. 2006). The uptake of metals by algae in aqueous solutions occurs when algae release a protein called metallothioneins. The alga releases metallothioneins which chemically binds to the metal as a defense mechanism to remove the metal from its regular cellular activity (Volesky 1999; Jahan et al. 2006). Algae respond to heavy metals by the synthesis of low molecular weight compounds such as carotenoids and glutathione, and the initiation of several antioxidants, as well as enzymes including superoxide dismutase, catalase, glutathione peroxidase and ascorbate peroxidase. *Chlorella sp.* and *Scenedesmus sp.* are the two most common algae species used for metal uptake. Suhendrayatna et al. (1999) exposed *Chlorella sp*. to concentrations of arsenite ranging from 0 to 100 μg/mL the maximum concentration being 100 μg As/mL. The cell growth of Chlorella sp. was not affected by the arsenite until it was exposed to concentrations higher than 50 μg/mL. At concentrations greater than 50 μg As/mL, the cell growth of the species was suppressed (Jahan et al. 2006). Beceiro-Gonzalez et al. (2000) concluded that *Chlorella sp.* retained approximately 50% of arsenite from a solution. Taboada-de la Calzada et al. (1999) observed that most of the biosorption was rapid and occurred in the first 15 min by the *Chlorella sp*. (Jahan et al. 2006). Harris and Ramelow (1990) studied the use of *Chlorella sp.* and *Scenedesmus sp.* for biosorption of gold, copper, cadmium, and zinc. It was found that approximately 90% of sorption took place within 15 min and that the remnants adsorbed at a slower rate. Experiments also indicated that the metal binding ability of Scenedesmus sp. was much greater than that of Chlorella sp. (Jahan et al. 2006). Terry and Stone (2002) reported on the biosorption of cadmium and copper contaminated water by *Scenedesmus abundans*. It was shown that both living and non living *S. abundans* removed cadmium and copper from water, with better removal rates with the living algae (Jahan et al. 2006).

Jahan et al. (2006) investigated As(III) biosorption by the alga *Scenedesmus abundans* as a possible cost effective method of bioremediation of arsenic from water. Algal biomass (40 mg/L) was exposed to varying concentrations of As(III) i.e. 1, 5, 10, 20, 50 and 100 mg/L and samples were obtained at certain time intervals to analyze for residual arsenic concentrations. The removal of As(III) was found to be around 70%. Algal morphology changed in presence of arsenic. Sorption of arsenic with algae could be modeled by the conventional Langmuir isotherm. The isotherm constants indicate a high adsorptive capacity of the select alga for arsenic.

Hansen et al. (2004) studied As(V) biosorption using dried algae (*Lessonia nigrescens*) collected in Valparaiso bay, Chile. The experiments were performed using laboratory solutions (200 mg/L, pH 2.5, 4.5 and 6.5). *Lessonia nigrescens* showed very good adsorption capacities and its use may be interesting for small-scale drinking water treatment, deserving further investigation (Litter et al. 2010).

Bundschuh et al. (2007) investigated the effectiveness and suitability of dried macro-algae (*Spyrogira spp*.) in removing arsenic from acid mine drainage (AMD) and other waters from the Poopo lake basin (Bolivia, Andean highlands) finding higher efficiency i.e. 80–90% of As removal was attained within 4 days (Litter et al. 2010).

22.5.11 Bacteria

Corynebacterium glutamicum, which is used for the industrial production of amino acids and nucleotides, is one of the most arsenic-resistant microorganisms described to date (up to 12 mM arsenite and >400 mM arsenate). Analysis of the *C. glutamicum* genome revealed the presence of two complete *ars* operons (*ars1* and *ars2*) comprising the typical three-gene structure *arsRBC*, with an extra *arsC1´* located downstream from *arsC1* (*ars1* operon), and two orphan genes (*arsB3* and *arsC4*) (Mateos et al. 2006). Mateos et al. (2006) confirmed the involvement of both *ars* operons in arsenic resistance in *C. glutamicum* by disruption and amplification of those genes. The strains obtained by them were resistant to up to 60 mM arsenite, one of the highest levels of bacterial resistance to arsenite so far described. They are attempting to obtain *C. glutamicum* mutant strains able to remove arsenic from contaminated water.

Mondal et al. (2008) reported that arsenic removal by three bacterial strains namely, *Ralstonia eutropha* MTCC 2487, *Pseudomonas putida* MTCC 1194 and *Bacillus indicus* MTCC 4374, from wastewater (pH 7.1, 29 _C) containing 15 mg/L arsenic were 67%, 60% and 61%, respectively. It was also observed that arsenic concentration of 15 mg/L prolonged the stationary phase of these strains (Wang and Zhao 2009).

Genetically engineering methods could be used to enhance intracellular accumulation of both As(III) and As(V). Kostal et al. (2004) found that *Escherichia coli* overexpressing *ArsR* accumulated 5- and 60-fold-higher levels of As(III) and As(V) than cells without *ArsR* overexpression. The level of arsenic accumulation was 1.5–2.2 nmol/mg dry weight (110–173 μgAs/g dw). The engineered cells removed 98% of 0.05 mg/L As(III) from contaminated water after 1 h (Wang and Zhao 2009).

Singh et al. (2008a) expressed an arsenic chelating metallothionein (fMT) from the arsenic-tolerant marine alga *Fucus vesiculosus* in *Escherichia coli* and also studied the coexpression of the As(III)-specific transporter GlpF with fMT which further improved arsenic accumulation and offered high selectivity toward As. At a cell concentration of 5 g (Dry Cell Weight)/L, cells expressing fMT lowered the As(III) concentration from 35 to 10 ppb within 20 min while cells expressing fMT and

GlpF achieved the same reduction within 1 min, with all the added As(III) removed within 20 min. In comparison, control cells reduced the As(III) concentration only to 25 ppb, primarily due to nonspecific cell surface adsorption. This substantial improvement in As removal can be attributed to the enhanced uptake provided by the additional As(III) transporter GlpF.

Takeuchi et al. (2007) studied arsenic resistance and removal was evaluated in nine bacterial strains of marine and non-marine origins. Among the strains tested, *Marinomonas communis* exhibited the second-highest arsenic resistance with median effective concentration (EC50) value of 510 mg As/L, and was capable of removing arsenic from culture medium amended with arsenate. Arsenic accumulation in cells amounted to 2,290 μg As/g (dry weight) when incubated on medium containing 5mgAs/L of arsenate. This is the highest value ever reported in bacteria. Given the efficiency with which *M. communis* can accumulate arsenic, it is likely that the bacterium would be a strong candidate for bioremediation of arsenic contaminated water.

Bag et al. (2010) performed kinetic studies on removal of trivalent arsenic from a simulated aqueous solution of arsenic oxide (As_2O_3) using an arsenic resistant bacterial strain, *Rhodococcus equi* (JUBTAs02). Batch studies were conducted to determine the arsenic-intoxicated growth kinetics of the bacteria. The kinetic parameters like saturation constant K_S and maximum specific growth rate, the inhibition constant, K_i, has been determined. From the studies with ground water a first order kinetics with respect to arsenic concentration has been determined for arsenic uptake rate. The same microorganism has been used in immobilized form to treat simulated water as well as naturally occurring arsenic laden ground water in a continuous packed bed reactor using initial arsenite concentration and inlet flow rate as parameters. A maximum value of arsenite removal efficiency of 95% has been achieved in this process. Deterministic mathematical models capable of explaining the trend of removal of arsenic from simulated and ground water have been developed using the kinetic parameters of intrinsic growth of the microorganism.

22.5.12 Fungi

Fungi play fundamental roles in the natural environment especially regarding decomposition, transformation and nutrient cycling. Accumulation of heavy metals by fungal biomass may be particularly relevant because of its potential low cost application in bioremediation and recovery of metals, in this case, to the detoxification of arsenic polluted habitats.

Adeyemi (2009) investigated the bioaccumulation of arsenic in three filamentous fungi, *Aspergillus niger, Serpula himantioides* and *Trametes versicolor* and their possible application in remediation of arsenic. They were exposed to arsenopyrite (FeAsS) in concentrations 0.2%, 0.4%, 0.6% and 0.8% (W/V). *T. versicolor* was the most efficient in accumulation with all amounts, accumulating up to 15 times the amounts accumulated by *A. niger* which was the least effective in accumulation.

Sathishkumar et al. (2004) studied biosorption of arsenic by modified mycelial pellets of *Aspergillus fumigatus*. Groundwater sample was brought from Kolkata having 1.3 mg/L As(III) and 0.9 mg/L As(V), the pellets showed 85% removal of As (III) with equilibriation time of 135 min and 83% removal of As (V) with equilibrium time of 150 min .

Say et al. (2003) reported that the maximum adsorption capacities of As(III) onto *Penicillium purpurogenum* fungal biomass reached 35.6 mg/g under noncompetitive conditions and 3.4 mg/g under competitive conditions by other ions [e.g., Cd(II), Pb(II), Hg(II)] after 4 h (pH 5, 20°C). The fungus could be used for ten cycles for biosorption (Wang and Zhao 2009).

Loukidou et al. (2003) reported that *Penicillium chrysogenum* (a waste byproduct from antibiotic production) pretreated with surfactants (hexadecyl-trimethylammonium bromide and dodecylamine) and a cationic polyelectrolyte was able to remove significant amounts of As(V) from waters. At pH 3, the removal capacities of the modified biomass ranged from 33.3 to 56.1 mg arsenic/g biomass (Wang and Zhao 2009).

Murugesan et al. (2006) reported that a tea fungus, a waste produced during black tea fermentation, was able to sequester arsenic in groundwater samples collected from Kolkata, West Bengal. $FeCl_3$ pretreated and autoclaved fungal mats removed 100% of As(III) and Fe(II) after 30 min and 77% of As(V) after 90 min. The optimum adsorbent dosage was 20 g/L.

Iron oxide-coated fungal (*Aspergillus niger*) biomass was able to remove 95% of As(V), 75% of the As(III), and 50% of DMA from contaminated water at pH 6 (Pokhrel and Viraraghavan 2006, 2008; Wang and Zhao 2009).

Ceransky et al. (2007) studied the biosorption of arsenic by a fungi *Neosartorya fischeri,* where the fungal biomass, one as pellet and the other as compact biomass were exposed to 2.5 mg of arsenate for 1 h and then compact and pelletized biomasses were analyzed for the total content of arsenic by Hydride Generation Atomic Absorption Spectrometry (HG AAS). Because of the higher wet weight of pellets, biosorption of arsenic by this form of biomass is also higher and represents 31.5% (0.783 mg) of the original amount of arsenic (2.5 mg) in the system. Biosorption of arsenic by the compact form of mycelium is lower and represents 15.2% (0.388 mg) of the original arsenic content.

Singh et al. (2008b) introduced phytochelatins (PCs) (which are naturally occurring peptides with high-binding capabilities for a wide range of heavy metals including arsenic) in the yeast *Saccharomyces cerevisiae* by expressing *Arabidopsis thaliana* phytochelatin synthase (AtPCS) in it for enhanced arsenic accumulation and removal. PCs production in yeast resulted in six times higher arsenic accumulation as compared to the control strain under a wide range of arsenic concentrations. These results open up the possibility of using cells expressing AtPCS as an inexpensive sorbent for the removal of toxic arsenic.

Vala and Upadhay (2008) investigated the arsenic tolerance and accumulation capacity of a facultative marine *Aspergillus sp*. The fungus exhibited different tolerance pattern towards different forms of arsenic when exposed to 100 mg/l As(III) and As(V)when incubated for 9–10 days. The fungus exhibited luxuriant growth in

presence of As(III) similar to Control, while in case of As(V) growth was observed after day 6. There was a rise in biomass accumulation in case of both test arsenic forms though biomass accumulation was less than Control in both the cases. The highest accumulation of As(III) and As(V) was found to be 13.8663 and 16.5725 mg/g. Biomass accumulation and arsenic content data are indicative of high tolerance and shows promising metal removal potential of the test isolate, hence its application potentiality for bioremediation purpose can be envisaged.

Mukherjee et al. (2010) determined the arsenate tolerance limit in wild-type *Aspergillus niger*. Because of its high tolerance, toxic effects of arsenate concentrations ranging from 25 to 100 mg/L were investigated in regard to various physiological parameters and cellular arsenate uptake was also analyzed. Growth of *A. niger* increased at 25 mg/L arsenate, and it survived up to 100 mg/L. Results indicated that *A. niger* had high arsenate uptake potential and could tolerate oxidative stress by manipulating its anti-oxidative defense mechanism, a property that may be exploited for removal of arsenate from contaminated aqua-environment.

Table 22.2 summarizes reports on arsenic removal through microbial biosorption.

22.5.13 Microbial Methylation and Demethylation for Arsenic Remediation

Methylation is the process in which certain species of fungi and bacteria methylate inorganic arsenic species to form methylarsenicals (Rodriguez 1999). The methylation of arsenic occurs via alternating reduction of pentavalent arsenic to trivalent arsenic and subsequent addition of a methyl group. After their formation, methylarsenicals are released from the microbe as a gaseous product (Fig. 22.2), this is called as biovolatization. From a bioremediation perspective, several species of soil dwelling microorganisms may be significant as they have shown arsenic volatizing potential (Frankenberger and Arshad 2002). This includes species of *Penicillium* and *Aspergillus,* which can volatilize both organic and inorganic arsenic compounds, and *Pseudomonas*, which are capable of volatilizing inorganicarsenic (Verdell 2008). Huysmans and Frankenberger (1991) reported a strain of *Penicillium* sp. isolated from evaporation pond water which was found to be capable of methylating and subsequently volatilizing organic arsenic. They investigated the conditions for optimum production of trimethylarsine which could be useful in developing a bioremediation approach in trapping the arsenic gas evolved from soil or water as a mitigation alternative in the cleanup of arsenic contamination.

Maki et al. (2006) investigated the microbial population of organoarsenic contaminated soils of Ohkunoshima Island. Forty-eight isolates were screened for mineralization of monomethylarsonic acid MMAA(V). Only nine isolates reduced 140 μg/L of MMAA(V), giving decreasing percentages ranging from 5% to 100% within 14 days. Among the nine isolates, two remarkably converted 140 μg/L of MMAA to more than 71 μg/L of inorganic arsenic. Phylogenetic analysis using

Table 22.2 Arsenic removal through microbial biosorption

Microorganisms/biomass	Mechanisms and observations	References
Penicillium chrysogenum	At pH 3, the removal capacities of As(V) by a modified biomass with common surfactants and a cationic polyelectrolyte ranged from 33.3 to 56.1 mg arsenic/g	Loukidou et al. (2003)
Penicillium purpurogenum	The maximum adsorption capacities of As(III) were 35.6 mg/g under noncompetitive conditions and 3.4 mg/g under competitive conditions	Say et al. (2003)
Genetically engineered *Escherichia coli*	Cells overexpressing ArsR accumulated 5 and 60 fold higher levels of As(III) and As(V) and removed 98% of 0.05 mg/L As(III) from contaminated water after 1 h.	Kostal et al. (2004)
Scenedesmus abundans Wastewater bacteria	Microbes could remove up to 70% of the As(III) from contaminated water (0.05–1.5 mg/L for bacteria and 1–100 mg/L for algae)	Jahan et al. (2006)
Tea fungus (a symbiont of two yeasts *viz*., *Pichia sp*. and *Zygosaccharomyces sp.* and a bacterium *Acetobacter sp*.)	FeCl3 pretreated and autoclaved fungal mats removed 100% of As(III) after 30 min and 77% of As(V) after 90 min	Murugesan et al. (2006)
Aspergillus niger	95% of As(V) and 75% of As(III) removed at pH 6	Pokhrel and Viraraghavan (2006)
Scytonema	The sorbent exhibited a 100% affinity for As(III) at pH 6.9 and demonstrated a recycleability of up to 59 cycles of sorption–elution	Prasad et al. (2006)
Ralstonia eutropha *Pseudomonas putida* *Bacillus indicus*	Arsenic removal capacities were 67%, 60% and 61%, respectively. Arsenic concentration of 15 mg/L prolongs the stationary phase of these strains.	Mondal et al. (2008)
Aspergillus niger	50% of DMA removed at pH 6 after 7 h	Pokhrel and Viraraghavan (2008)
Chlorella species	Cell growth was not affected up to 50 µg/mL of arsenic	Suhendrayatna et al. (1999)
Chlorella species.	Retains approx. 50% arsenic from water	Beceiro-Gonzalez et al. (2000)
Chlorella species	Biosorption of arsenic was rapid and occurred in first 15 min	Taboada-de la Calzada et al. (1999)
Chlorella species Scenedesmus abundans	90% absorption of heavy metals took place in 15 min. Scenedesmus showed better metal binding ability	Harris and Ramelow (1990)

(continued)

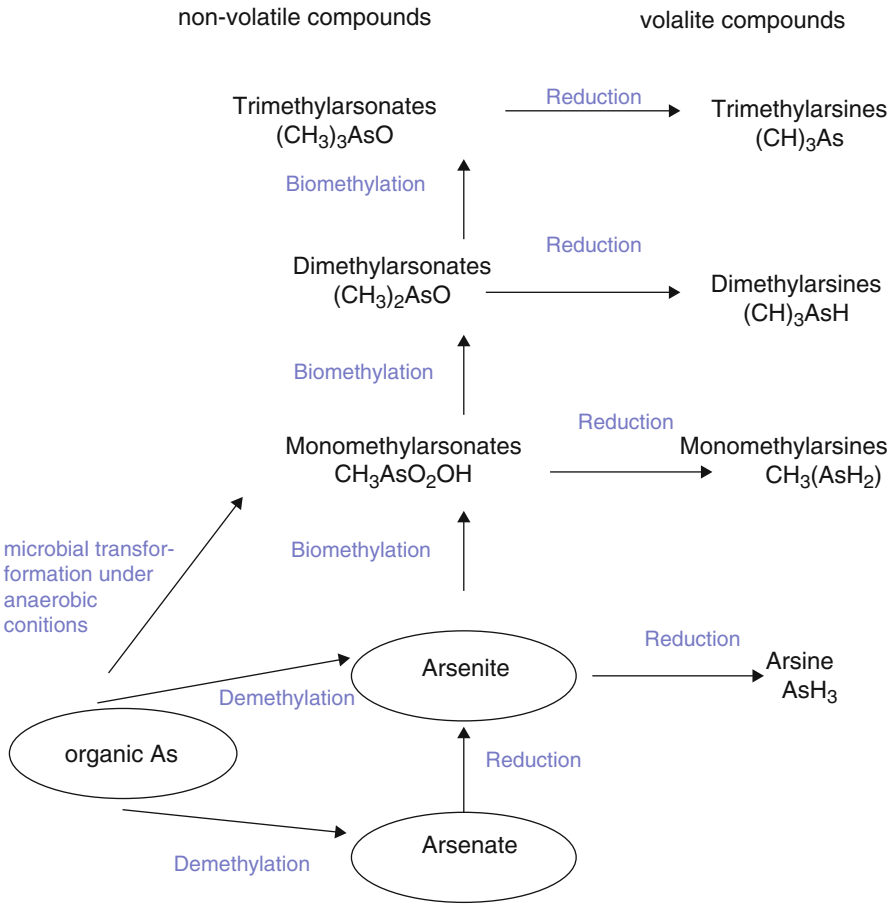
Table 22.2 (continued)

Microorganisms/biomass	Mechanisms and observations	References
Lessonia nigrescens	Good absorption capacity of As(V) at 200 mg/L	Hansen et al. (2004)
Spyrogira spp	80–90% removal of arsenic in 4 days	Bundschuh et al. (2007)
Corynebacterium glutamicum	Strains obtained by them tolerated upto 60 mM As(III)	Mateos et al. (2006)
Aspergillus niger, Serpula himantioides and *Trametes versicolor*	*Trametes versicolor* showed maximum uptake of FeAsS, 15 more than *A. niger,* which showed least uptake	Adeyemi (2009)
Aspergillus fumigates	855 As(III) removal and 83% As(V) removal	Sathishkumar et al. (2004)
Genetically engineered *Escherichia coli*	cells expressing fMT lowered the As(III) concentration from 35 to 10 ppb while cells expressing fMT and GlpF removed all the added As(III) removed within 20 min.	Singh et al. (2008a)
Marinomonas communis	Arsenic accumulation in cells amounted to 2,290 µg As/g (dry weight) when incubated on medium containing 5mgAs/L of arsenate	Takeuchi et al. (2007)
Rhodococcus equi (JUBTAs02)	arsenite removal efficiency of 95%	Bag et al. (2010)
Saccharomyces cerevisiae	Saccharomyces cerevisiae by expressing Phytochelatin Synthase (AtPCS), showed six times higher As accumulation	Singh et al. (2008b)
Aspergillus sp	accumulation of As(III) and As(V) was found to be 13.8663 and 16.5725 mg/g respectively from initial 100 mg/L	Vala et al. (2008)
Aspergillus niger	it survived up to 100 mg/L by manipulating its anti-oxidative defense mechanism	Mukherjee et al. (2010)

Adapted from Wang and Zhao (2009)

16S rDNA sequences showed that the two isolates belonged to the *Pseudomonas putida* strains**.**

The conversion of As(V) to small amounts of volatile methylarsines was first described in a pure culture of a methanogen, *Methanobacterium bryantii* (McBride and Wolfe1971; Wang and Mulligan 2006). Recently, several pure cultures of anaerobes, including a methanogen (*Methanobacterium formicicum*), a fermentative bacterium (*Clostridium collagenovorans*) and sulfate-reducing bacteria (*Desulfovibrio vulgaris* and *D. gigas*), were also implicated in the formation of methylarsines (Michalke et al. 2000; Wang and Mulligan 2006). As(V) can be converted to monomethylarsine and dimethylarsine by *Achromobacter* sp. and *Enterobacter* sp., and to monomethylarsine,



Source: Kenneth J. Verdell (2008), senior honors thesis.

Fig. 22.2 Arsenic volatilization pathways (all processes are reversible) Frankenberger and Arshad (2002) (Source: Kenneth J. Verdell (2008), senior honors thesis)

dimethylarsine and trimethylarsine by *Aeromonas* sp. and *Nocardia* sp. (Cullen and Reimer 1989; Wang and Mulligan 2006). Anaerobic microcosms established from the sediments of a gold mine impacted lake were found to be able to methylate As(V) forming MMAA(V) and DMAA(V) (Bright et al. 1994; Wang and Mulligan 2006). On the other hand, the trivalent methylated intermediates such as MMAA(III) and DMAA(III) are found to be readily oxidized chemically and biologically (Sanders et al. 1979; Shariatpanahi et al. 1983; Wang and Mulligan 2006). Shariatpanahi et al. reported that the rates of methylation and demethylation of monosodium methylarsonate at 10 and 100 mg/L of normal culture media by *Aeromonas*, *Nocardia*, *Enterobacter*, *Flavobacterium*, *Achromobacter*, *Pseudomonas*, and *Alcaligenes* species followed first order composite kinetics (Wang and Mulligan 2006).

Sanders (1979) measured an average demethylation and oxidation rate of DMA(III) to As(V) which was found to be approximately 1 ng DMA(III)/L/day by mixed bacterial cultures isolated from an estuarine system (Wang and Zhao 2009).

Biovolatilization (Methylation) might be developed as an *ex-situ* method for arsenic removal under controlled conditions. Visoottiviseth and Panviroj (2001) reported that the fungal species *Penicillium* sp. were capable of volatilizing 25.8–43.9 mg of arsenic during a 5-day cultivation period (Wang and Zhao 2009). Edvantoro et al. (2004) showed that augmenting contaminated soils (1,390 mg arsenic/kg soil) with methylating fungi (*Penicillium* sp. and *Ulpcladium* sp.) significantly increased the arsenic volatilization rates (up to eightfold increase) (Wang and Zhao 2009).

Urik et al. (2007) quantified the arsenic biovolatilization by microscopic filamentous fungi *Aspergillus clavatus*, *Aspergillus niger*, *Trichoderma viride* and *Penicillium glabrum*. The fungi were cultivated on a liquid medium enriched with inorganic As(V). Filamentous fungi volatilized 0.010–0.067 mg and 0.093–0.262 mg of arsenic from cultivation systems enriched with 0.25 and 1.00 mg of arsenic respectively. These results represent the loss of arsenic after a 30-day cultivation from cultivation systems. The *Aspergillus niger* and *Aspergillus clavatus* strains were then used for determination of the production of volatile arsenicals in a cultivation system on 29th day enriched with 1.00 and 2.5 mg of inorganic arsenic, respectively. The amount of volatile arsenicals was 40.7 and 39.98 ng of arsenic for the *Aspergillus clavatus* and *Aspergillus niger* strains, respectively.

Cernansky et al. (2009) studied the production of volatile derivatives of arsenic using pure cultures of different fungal strains *viz. Neosartorya fischeri, Aspergillus clavatus* and *A. niger* under laboratory conditions. The average amount of volatilized arsenic for all fungal strains ranged from 0.026 to 0.257 mg of As(III) and 0.024–0.191 mg of As(V), respectively. Approximately 23% of arsenic was volatilized from all culture media originally enriched with approximately 4 and 17 mg/L of As(III). The average amount of biovolatilized arsenic from culture media originally enriched with 4 and 17 mg/L of As(V) was 24% and 16%, respectively. The order of ability of arsenic biovolatilization is *Neosartorya fischeri* > *A. clavatus* > *A. niger* (Wang and Zhao 2009).

Methylation and demethylation may play a significant role in influencing the toxicity and mobility of arsenic in soils and groundwater. As(III) and As(V) methylation may form volatile species leading to the escape of arsenic from water and soil surfaces by volatilization. Though the methylated arsenic species are generally considered less toxic than the inorganic species, the methylation processes do not necessarily contribute to the detoxification mechanism. Recent research has demonstrated that trivalent methylated arsenic species are more effective in destroying DNA. The potency of the DNA damage decreases in the order DMAA (III) > MMAA (III) > [As(III), As(V)] > MMAA(V) > DMAA(V) > trimethylarsine oxide [TMAO(V)] (Dopp et al. 2004; Wang and Zhao 2009).

Table 22.3 summarizes reports on microbial methylation and demethylation.

Table 22.3 Reports on arsenic biomethylation

Microorganisms	Mechanisms and observations	Reference
Penicillium sp.	Methylarsonic and dimethylarsinic acid methylation to rimethylarsine	Huysmans and Frankenberger (1991)
Polyohysa peniculus	As(V) methylation into dimethylarsine	Cullen et al. (1994)
Chlorella vulgaris	Biosorption and accumulation of arsenic and converted into (CH3)AsO(OH)	Kaise et al. (1997)
Scopulariopsis brevicalue	As(V) methylation to (CH3)3As species	Andrews et al. (2000)
Methanobacterium formicicum *Clostridium collagenovorans* *Desulfovibrio vulgaris* *Desulfovibrio gigas*	As methylation and demethylation under favorable conditions	Michalke et al. (2000)
Closterium aciculare	As(V) methylation into methylarsenic(III) species	Hasegawa et al. (2001)
Fusarium oxysporum/meloni	As(V) accumulation and methylation into dimethylarsine	Granchinho et al. (2002)
Fucus gardneri	As(V) methylation into dimethylarsine	Granchinho et al. (2002)
Penicillium sp. *Ulocladium sp.*	Eight fold increase in As(V) methylation to arsine	Edvantoro et al. (2004)
Achromobacter sp. *Enterobacter sp*	As(V) methylation to monomethylarsine and dimethylarsine	Cullen et al. (1989)
Aeromonas sp. *Nocardia sp*	As(V) methylation to monomethylarsine, dimethylarsine and trimethylarsine	
Aeromonas, *Nocardia,* *Enterobacter,* *Flavobacterium,* *Achromobacter,* *Pseudomonas,* *Alcaligenes*	methylation and demethylation of monosodium methylarsonate follows first order kinetics at 10 and 100 mg/L	Shariatpanahi et al. (1983)
Penicillium sp.	Volatilization of 25.8–43.9 mg of arsenic during a 5-day cultivation period	Visoottiviseth and Panviroj (2001)
Neosartorya fischeri *Aspergillus clavatus* *Aspergillus niger*	production of volatile derivatives of arsenic. The order of ability of arsenic biovolatilization is *Neosartorya fischeri* > *Aspergillus clavatus* > *A. niger.*	Cernansky et al. (2009)
Aspergillus clavatus, *A. niger,* *Trichoderma viride* *Penicillium glabrum*	volatilized 0.010–0.067 mg and 0.093–0.262 mg of arsenic from initial 0.25 and 1.00 mg of arsenic respectively	Urik et al. (2007)
Pseudomonas putida	Converted 140 μg/L of MMAA to more than 71 μg/L of inorganic arsenic	Maki et al. (2006)

Adapted from Wang and Zhao (2009)

22.5.14 Microbial Assimilation of Arsenic Instead of Phosphorus

A very interesting discovery was made by Wolfe-Simon et al. (2010) who isolated a bacterium GFAJ-1 of Halomonadaceae from Mono Lake, California which can incorporate arsenate instead of phosphate in its macromolecules, most notably nucleic acids and proteins. Mass spectrometry reports show that arsenic was inside the cell. When radio labeled arsenate was supplied to the culture it was detected in protein, lipid, nucleic acid, and metabolite fractions of the cells, suggesting that arsenic had been incorporated in molecules forming each fraction. The bacterial DNA isolated and DNA was analyzed that revealed the presence of arsenic. Further analysis indicated that at least some of the arsenic in the bacteria was in the form of arsenate that replaced the phosphates with molecular bonds to carbon and oxygen atoms to replace the phosphates in DNA and other molecules (Pennisi 2010).

Thus it can be seen that microorganisms can employ a large variety of arsenic transforming mechanisms in order to sustain themselves in arsenic contaminated environments.

22.6 Arsenic Removal Technologies Operated in India

22.6.1 Community Level Arsenic Treatment Technologies

A number of community level Arsenic removal technologies were developed by various agencies, both national and international. These were based on different scientific principles mentioned earlier and Arsenic treatment plants were installed based on these technologies in various parts of India (Planning commission's task force report, GOI 2007).

Details are given below:

22.6.1.1 Apyron Technology

Apyron's Arsenic treatment units remove Arsenic by adsorption using enhanced activated alumina media called *Aqua-Bind™-Arsenic*. The adsorption media is a composite of manganese oxide and activated alumina (Planning commission's task force report, GOI 2007).

22.6.1.2 Water Systems International (WSI) Technology

WSI uses an ion exchange process for the removal of Arsenic. The unit called "bucket of resin" is attached to a tube well hand pump. WSI has designed the unit to a tolerance level of reducing arsenic from 1,500 μg/L to <50 μg/L. The targeted

resin life at the level of 1,000 μg/L of arsenic is 300,000 L before resin regeneration (Planning commission's task force report, GOI 2007).

22.6.1.3 RPM Technology

RPM technology is based on activated alumina (adsorption) enhanced by a proprietary additive engineered to accomplish excellent Arsenic removal. The system is easy to install and works with gravity force (Planning commission's task force report, GOI 2007).

22.6.1.4 Pal Trockner Technology

Pal Trockner uses Granular Ferric Hydroxide (GFH) as adsorbent, which can eliminate arsenic of any concentration from drinking water bringing down its level to below the permissible limit for potable use. The Arsenic removal capacity of GFH is 5–10 times higher than that of other similar adsorbent-based systems (Planning commission's task force report, GOI 2007).

22.6.1.5 Anir Engineering

Anir Engineering uses the proven technology of adsorption with slurry/granular ferric hydroxide. The technology offers high efficiency, cost-effective Arsenic removal, is simple to operate/maintain, with easy disposal of non-toxic sludge (Planning commission's task force report, GOI 2007).

22.6.1.6 School of Fundamental Research (SFR) Technology

SFR device is designed to be fitted to 'force & lift' pump which is connected to a vertical PVC cylinder filled with silicate matrix (ceramic material) coated with an additional oxidizing element for removal of the major portion of iron before the water enters into arsenic removal system. The Arsenic removal system consists of PVC cylinders filled with goethite compound specially deposited on activated alumina (Planning commission's task force report, GOI 2007).

22.6.1.7 Public Health Engineering Department (PHED) Technology

The removal of Arsenic in this technology is accomplished in four chambers. Groundwater is abstracted and lifted by force and lift hand pump to spray into droplets over a bed containing packed hematite lumps before it is then led to sedimentation

chamber at bottom. The settled water is conveyed through three chambers placed in series containing red hematite lumps, quartz and dual media (Sand-Activated Alumina), respectively (Planning commission's task force report, GOI 2007).

22.6.1.8 All India Hygiene and Public Health (AIIH&PH) Technology

This technology follows oxidation followed by coagulation/co-precipitation route for the removal of Arsenic from groundwater. Bleaching powder is used as the oxidizing agent and alum is used as the coagulant (Planning commission's task force report, GOI 2007).

22.6.1.9 Oxide India (Catalysts) Pvt. Ltd. (BE College Model)

This technology uses adsorption technique in which activated alumina is used as the adsorption medium. Activated alumina can adsorb As(V) better than As(III). Therefore, a preoxidation of As(III) to As(V) is done to increase the efficiency. Activated alumina plant is relatively more expensive. The adsorption medium is also expensive and requires regular backwash to remove deposition of iron onto it and thus making alumina site unavailable to arsenic (Planning commission's task force report, GOI 2007).

22.6.1.10 Central Glass and Ceramic Research Institute (CGCRI) Technology Using Ceramic Filter

CGCRI, Kolkata has developed a technology in which a fine iron based adsorbent is mixed with the contaminated water. The water along with the adsorbent is pushed through a series of ceramic filters. Clean Arsenic free water comes out through the sidewalls of the filter and the remaining water along with the adsorbent goes back to the reactor. This cycle is continued with fresh input of contaminated water. A demonstration plant using this technology was first installed by CGCRI in the year 2001 which had a capacity of 60 L/h.

Subsequently, four Arsenic removal plants each with a capacity of 2,500 L/h have been installed in the year 2003. These plants are being evaluated. CGCRI technology is cost intensive at smaller scale of operation (Planning commission's task force report, GOI 2007).

22.6.1.11 Agharkar Research Institute (ARI), Pune Technology

An ideal example of use of bioremediation technology has been set by Agharkar Research Institute, Pune by integrating microbial arsenite oxidation and alumina

adsorption for arsenic removal. Five integrated arsenic removal systems with 1,000 L/day filtration capacities have been designed and installed by Paknikar and his group in two arsenic affected villages namely Kaurikasa and Muraithitola in Rajnandgaon block in Chhattisgarh, India. These plants are fully operational since 2006. The integrated plants are being operated on the bio-oxidation alumina adsorption technology proposed (Mokashi and Paknikar 2002; Paknikar 2003). This involves pumping of groundwater and addition of carbon source, microbial oxidation of arsenite, arsenate removal, filtration of cells and disinfection in a step wise manner. The systems are run on a daily basis with periodic analysis of various parameters like Arsenic concentration, pH, color, odor etc. of both the inlet groundwater and the treated water. The results are very encouraging and deployment of the technology to other affected areas is being considered. Figure 22.3 shows the schematic diagram of the operation of field scale system and Fig. 22.4 shows the photograph of the field scale system (Planning commission's task force report, GOI 2007).

Though a number of technologies developed for arsenic removal from groundwater have been developed as reported above, many of them failed during actual field application. The systems of WSI, AIIHPH, Apyron, RPM, School of Fundamental Research, PHED, Anir Engineering were not working or missing from field application sites. The reasons for failure of these technologies may be due to lack of awareness among affected people, lack of sense of belonging, lack of willingness to take responsibility, lack of safe methods for sanitary disposal of Arsenic-bearing sludge, no provision of salary for operating staff and irregular supply of chemicals needed to run the plants (Planning commission's task force report, GOI 2007).

22.6.2 Domestic Arsenic Treatment Technologies

22.6.2.1 School of Environmental Studies (SOES), Jadavpore – Filter Tablet System

SOES developed a tablet and filter system for the removal of Arsenic at household level. The removal is based on the principle of oxidation-coagulation-precipitation-filtration route. The unit consists of two containers, the upper container is fitted with a filter candle and the lower container is collector for Arsenic free water. The tablet consists of an oxidizing agent, Fe (III) salt and activated carbon. The tablet is added to the contaminated water. The oxidizing agent converts entire Arsenic into As (V). Fe (III) salt gets hydrolyzed into hydroxide which attaches As (V) with it and eventually gets adsorbed onto activated carbon. Water is then transferred into the top container containing the filter candle and the clean water is collected into the bottom container through filtration. Scientists from SOES have reported field testing of their filter-tablet system during 1993–1995 with Arsenic removal of 93–100% (Planning commission's task force report, GOI 2007).

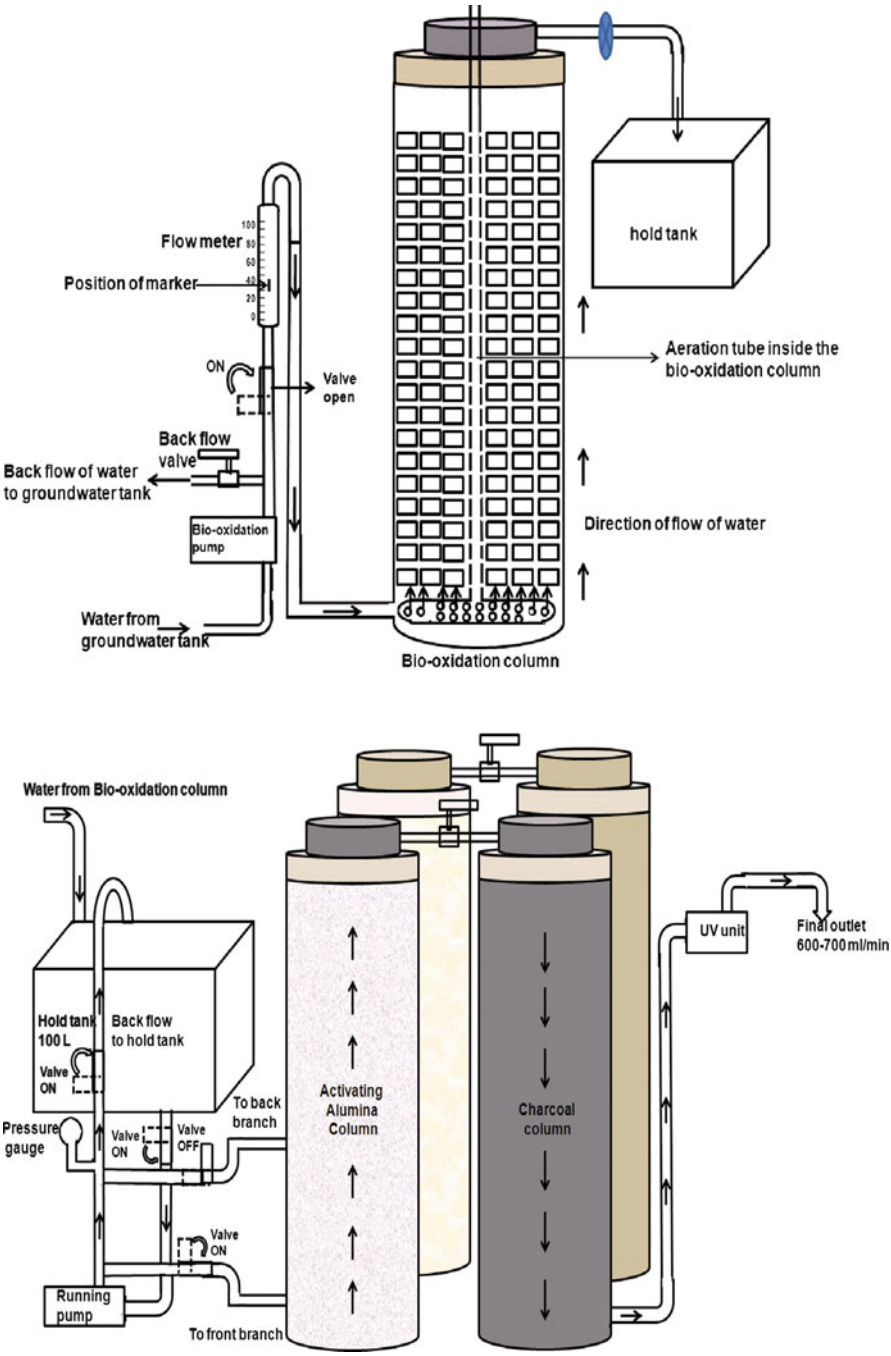


Fig. 22.3 Schematic diagram of scaled up field arsenic removal system developed by Paknikar et al.

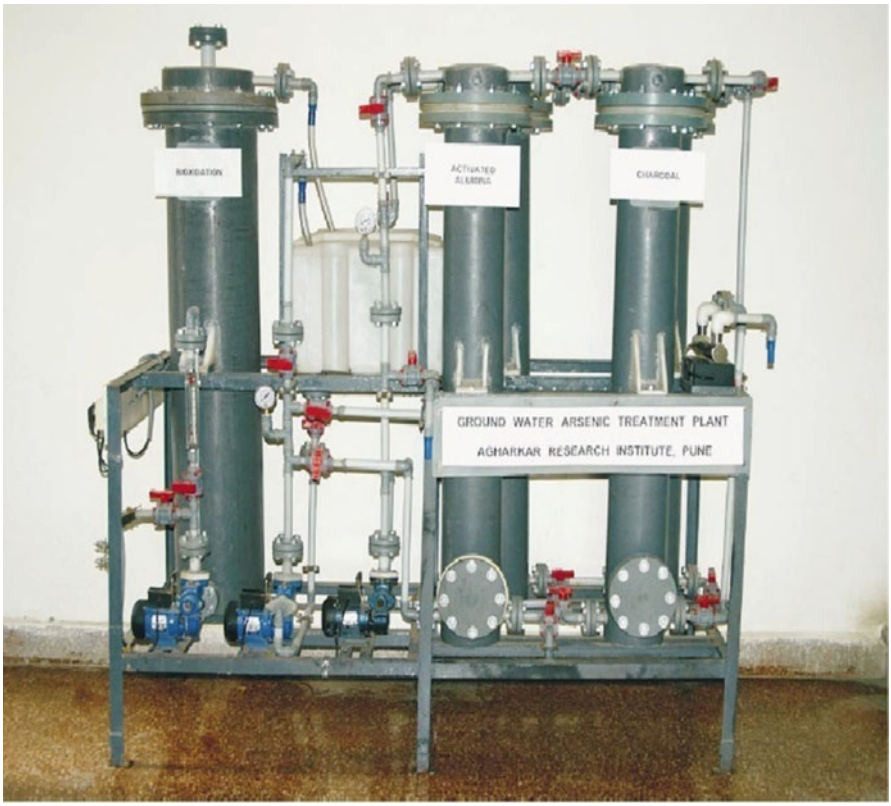


Fig. 22.4 Integrated field scale system working on bio-oxidation alumina adsorption technology installed in arsenic affected areas of Rajnandgaon District, Chhattisgarh

22.6.2.2 AIIH&PH Domestic Arsenic Removal System

AIIH&PH domestic Arsenic removal units use the same route as SOES, i.e., oxidation coagulation-precipitation-filtration. They use bleaching powder solution as the oxidizing agent, and ferric alum as the coagulator. Both SOES and AIIH&PH domestic Arsenic removal techniques adopt a robust principle for Arsenic removal, which perhaps, is also the oldest Arsenic removal technique (Planning commission's task force report, GOI 2007).

22.6.2.3 NML's Domestic Arsenic Removal Unit

National Metallurgical Laboratory, Jamshedpur's domestic Arsenic removal unit was designed keeping in mind the general shortcomings of oxidizing-coagulation-

precipitation filtration technique. NML technique uses adsorption route for Arsenic removal. It is essentially a three container system. A naturally occurring mineral is used as the adsorption medium. Finely divided powders of the mineral is mixed with the water in the top most containers and slowly agitated for about 5 min. The water is allowed to settle for about an hour. The mineral being heavy in nature has a natural tendency to settle. The settling may be expedited by adding a little flocculent, which is normally used in lowering the turbidity in surface water. The supernatant water is allowed to flow into the middle container attached with a common ceramic filter candle. Arsenic free water is slowly collected into lower container through filtration. Fifty units of NML's Arsenic removal system is being field tested in Sahebgunj district of Jharkhand (Planning commission's task force report, GOI 2007).

The applicability and efficiency of the above mentioned domestic arsenic treatment technologies in arsenic contaminated regions needs to be studied.

22.7 Future Perspectives

It is evident from the reports that though there are number of conventional technologies being employed for remediation of arsenic from water. The conventional methods of arsenic remediation have many limitations like requirement of arsenite pre-oxidation, disposal of toxic sludge, limited efficiencies, operational difficulties and costs. The solution to these limitations may lie in the interaction of microorganisms with arsenic.

Interaction of micro-organisms with different forms of arsenic and their biologically mediated conversions in the contaminated areas are a very plausible means of bioremediation of arsenic. The microorganisms being naturally present in the contaminated regions are freely available and well suited with those particular environments. Thus, the vast repertoire of mechanisms employed by microorganisms for biotransformation of arsenic in contaminated environments can be harnessed along with conventional and emerging technologies to provide affordable, on site and efficient arsenic remediation technologies.

A number of biologically mediated transformations of arsenic have been mentioned in the present chapter which may be useful to be employed for bioremediation of arsenic. Most attractive among them are the microbial arsenic oxidation-reduction reactions. Several bacteria have been reported which mediate the oxidation of arsenite to arsenate which is a less toxic form and also easily removable from water as compared to arsenite. A number of organisms including *Cenibacterium arsenoxidans* (renamed as *Herminiimonas arsenicoxydans*) (Lievremont et al. 2003; Muller et al. 2007), *Microbacterium lacticum* (Mokashi and Paknikar 2002), *Pseudomonas arsenitoxidans* NT-26 (Santini et al. 2000), *Thiomonas arsenivorans* (Battaglia-Brunet et al. 2006), *Gallionella* and *Leptothrix*

(Katsoyiannis et al. 2002) have been shown to oxidize arsenite to arsenate and these microbial oxidation have been applied in bioremedial techniques by the investigators. The results have been encouraging and the microbial oxidation can be further joined with physico- chemical techniques to present very efficient technologies for arsenic remediation in the target areas.

The role of sulfate reducing bacteria in the bioremediation of arsenic contamination has been explored and applied by a number of investigators. Consortia of Sulfate Reducing Bacteria (SRBs) has been used by Keimowitz et al. (2007) in Maine, USA, Teclu et al. (2008) in Pietermaritzburg, South Africa, Upadhyaya et al. (2010) for remediation of arsenic contamination.

Arsenic remediation by biosorption on microbial biomass has been investigated for algae, bacteria and fungi and has been reportedly used for arsenic remediation. Algae like *Chlorella sp.* and *Scenedesmus sp.* (Jahan et al. 2006), *Lessonia nigrescens* (Hansen et al. 2004) and *Spyrogira sp.* (Bundschuh et al. 2007) has been used in has been used for arsenic sorption in contaminated regions. Bacteria like *Corynebacterium glutamicum* (Mateos et al. 2006), *Marinomonas communis* (Takeuchi et al. 2007), *Rhodococcus equi* (Bag et al. 2010) etc. have shown very high resistance to arsenic and can be used as a biosorbent for arsenic from contaminated waters. A large number of algae have been investigated and shown to be useful for arsenic biosorption. These include species like *Aspergillus fumigates* (Sathishkumar et al. 2004), *Trametes versicolor* (Adeyemi 2009), *Penicillium chrysogenum* (Loukidou et al. 2003), *Penicillium purpurogenum* (Say et al. 2003), *Neosartorya fischeri* (Cernansky et al. 2007) etc. have shown good arsenic uptake capacity and thus can be an effective method of arsenic remediation from contaminated waters.

The methylation and demethylation reactions of arsenic carried out by microorganisms can be used effectively for detoxification of arsenic from contaminated water bodies. Reports on *Pseudomonas putida* (Maki et al. 2006), *Penicillium* sp. (Visoottiviseth and Panviroj 2001), *Aspergillus clavatus* and *Aspergillus niger* strains (Urik et al. 2007), *Neosartorya fischeri* (Cernansky et al. 2009) showed encouraging results for arsenic detoxification and thus provide a possible means of arsenic contamination bioremediation.

The work of Wolfe-Simon et al. (2010), who isolated a bacterium GFAJ-1 of Halomonadaceae from Mono Lake, California which can incorporate arsenate instead of phosphate in its macromolecules, most notably nucleic acids and proteins brings to the fore a revolutionary new concept of bacterial interaction with arsenic which can open totally new avenues of arsenic bioremediation methods.

The microorganisms provide a number of bioremediation methods of arsenic from contaminated water, which are found to be effective and well suited for the local conditions in which they are present. These microbial bioremedial interactions with arsenic can be used as such or in combination with conventional and emergent technologies to provide case specific as well as robust technologies for arsenic remediation in different areas (having different conditions) at affordable costs and with minimum operational and disposal problems.

22.8 Conclusions

Arsenic bioremediation mechanisms present a very realistic and attractive option for cleansing of arsenic contaminated groundwater that can be used for drinking and domestic purposes. Ironically the people who are most affected by arsenic contamination of groundwater are from poor regions of the world. Their total dependence on groundwater as a cheap source of drinking and domestic water along with their inability to afford costly conventional and emergent technologies for safe water compounds the problem. Bioremediation technologies with their simplicity, economy and *in situ* feasibility will go to a long way in solving the arsenic contaminated groundwater problem thus benefitting millions of people.

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Chapter 23
Metagenomic Approaches in Microbial Bioremediation of Metals and Radionuclides

Pinaki Sar and Ekramul Islam

Abstract Microbial bioremediation has been emerged as a potential alternative to clean up toxic contaminants from our environment effectively yet inexpensively. In contrast to microbial transformation of toxic organics, bioremediation of metals and radionuclides does not deal with degradation and elimination of these contaminants. Nevertheless, remediation goals may be achieved by microbial interaction with toxic metals leading towards immobilization, compartmentalization or concentration of contaminated wastes within the environment. In order to realize the promise of bioremediation appropriately, it is essential to understand how microbes affect *in situ* partitioning of metals into different phases (gaseous, solid and liquid) and what control(s) microbial growth and activity within contaminated environment. For the successful development of bioremediation strategies, knowledge on indigenous microbial communities, their metabolic capabilities, and how beneficial reactions are controlled *in situ* are considered critical. Rapid advancement in molecular techniques including high throughput DNA sequencing, amplification and cloning technology, coupled with microarray and other "-omic" tools have shown promise to provide global insight in the metabolic potential and activity of microbes living in contaminated environments. This "-omics era" of bioremediation, has opened up new perspectives and pointed towards new opportunities in pollution abatement and environmental management. Compared to traditional pure culture based studies, metagenomic approaches address specific environmental issues by exploring and exploiting unexplored resources of uncultivable and uncharacterized microorganisms. The present chapter will describe the concept and application of metagenomics in exploring microbial diversity, dynamics, and function within the metal/radionuclide contaminated environment.

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Keywords Metagenomics • Bioremediation • Metals • Radionuclides

23.1 Introduction

Environmental contamination with toxic metals and radionuclides released from diverse anthropogenic and natural activities pose a severe threat to both structure and function of our biosphere affecting human and other organism's health and global sustainability. Multidisciplinary studies have been undertaken to develop appropriate technologies to mitigate all environment related issues including removal or reduction of hazards due to contamination with heavy metals and radionuclides. Although, there are several traditional physicochemical methods developed and used to treat metal contaminated wastes of various nature and dimensions, microbe based bioremediation has increasingly being considered as the most effective strategy to deal with all such contaminants. Bioremediation technology exploits microbial catabolic abilities to reduce, eliminate, contain, or transform various environmental contaminants to benign products (Tabak et al. 2005; Palmisano and Hazen 2003). Though the word "bioremediation" was first used in peer-reviewed scientific literature in 1987 (Hazen 1997); the concept had its link to early days of human civilization (about 6000 BC). Relatively "modern" use of bioremediation was apparent only in 1891, with the opening of first biological sewage treatment plant in Sussex, UK (Tabak et al. 2005). Compared to other non biological methods, microbially mediated bioremediation is a more promising, uses low-cost and low-technology techniques and have high public acceptance, which generally and can often be carried out on site. In last 25 years the concept of bioremediation has emerged as one of the most applicable and some cases only appropriate technology to clean up diverse types of pollutants (including solvents, explosives, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and radioactive and metallic contaminants) from contaminated soil, water and even air. Microorganisms with their astonishing metabolic versatility, ubiquity and adaptability can aid in remediation by oxidizing, binding, immobilizing, volatilizing or otherwise transforming contaminants (Lovley 2003). However, in contrast to microbial transformation and degradation of toxic organic contaminants to innocuous forms, heavy metals and radionuclides can not be degraded. Remediation of metallic contaminants therefore can be achieved only by microbial interaction that leads to enzymatic and/or redox transformation (to their less toxic forms), immobilization, compartmentalization and complexation, overall reducing their environmental mobility and toxicity. In last three decades extensive research has been conducted on basic and applied aspects of microbial interaction with metals with a bioremediation perspective. These studies mainly include isolation of superior metal resistant and/or accumulating or metal transforming microorganisms, their identification, biochemical and genetic characterization, elucidation of microbe-metal interactions

mechanisms and all related studies relevant for bioremediation. Molecular approaches including '-omics' tools were used relatively recently in several studies to get better insight in to the bacterial interaction with toxic metals. Such extensive studies have shown beyond doubt that microbial interaction with metals and minerals have potential for treatment of environmental pollution (Lloyd and Lovley 2001; Lloyd and Ranshaw 2005; Gadd 2010). In spite of considerable research interest and including few commercial applications, the success of bioremediation remains limited and yet to be appropriately realized. Reasons for such failures includes, but not limited to (i) lack of information on the type of bacteria occupying contaminated sites (since most of the natural microbial flora are not readily culturable in the laboratory) (ii) poor understanding of their metabolic capabilities, particularly of mechanisms controlling growth and activity of microorganisms in contaminated environment (iii) lack of knowledge on how the indigenous communities respond to changes in environmental conditions within such habitats (Lovely 2003). It has been suggested that to develop successful bioremediation strategies, be it natural attenuation, bioaugmentation or biostimulation, understanding the geochemistry of the contaminated site as well as detail description of the microbial communities involved in key physiological processes are extremely essential. More precisely, microbial communities must be characterized in terms of structure (to know who is there?), phenotypic potential (to know what metabolic capabilities they have?), function (to know the realized metabolic potential) and interaction with the environments (Rittmann et al. 2006). Effective bioremediation strategies should encompass an understanding of fundamental physiological and molecular processes extant in the remediating populations. Ideally, one would like to perform such studies in the most ecologically relevant context, but the trade-off is that *in situ* it is difficult to control enough variables to make the results interpretable (Thompson et al. 2010). Although developments in molecular approaches, particularly 16S rRNA gene based identification of most appropriate bacteria and analysis of genes involved in bioremediation helped substantially to improve our understanding of bioremediation, the subject still remains more empirical than knowledge based (Lovely 2003). Lack of detail insight to develop adequate models that predict indigenous microbial activity during bioremediation is considered to be a major lack over physicochemical treatments that use more mature geochemical and hydrological models to provide better understanding of physical and chemical processes occurring in contaminated environments. In recent years, however, the emergence of 'genomics era' with application of high throughput DNA sequencing and methods to analyze expression of genes and their functions together have shown potential to revolutionize the field as it provides a global insight in to the microbial metabolic potential and activity within contaminated environment, irrespective of their culturability. The present chapter reviews the concept of metagenomics and metagenomics based strategies for bioremediation of metals and radionuclides, microbial interactions and processes relevant for bioremediation, and a brief summary of case studies.

23.2 Critical Review and Analysis

23.2.1 Cultivation Independent Approaches, Metagenomics and Bioremediation

Microbial world, the "foundation of the biosphere" and the engine of Earth's biogeochemical cycle constitutes the most extraordinary reservoir of life in the biosphere and is extremely rich in resources that we have begun to explore and understand (Staley et al.1997; Torsvik et al. 2002). Despite having enormous metabolic potential, it remains highly challenging to microbiologist to access the microbial world, as most of the environmental microorganisms are not readily culturable in laboratory conditions (Handelsman 2004). Only a small minority (1% or less, rarely up to 5% or slightly more of the total populations) of the microorganisms from environmental samples are readily culturable on standard media and therefore not accessible for basic research or biotechnological applications. As per the 16S rRNA gene sequence information available in Ribosomal Database Project (till January 2011), out of the 44 bacterial phyla, 6 Eubacterial and 2 Archeal phyla do not have any entry for culturable representative (Fig. 23.1). Noticeably, within the bacterial groups, *Firmicutes*, *Proteobacteria*, *Actinobacteria* have maximum entries and among these the percent of culturable bacteria are 13, 30 and 18, respectively.

Recent development in culture-independent molecular tools have revolutionized the field of microbial ecology by providing new path to assess the unculturable world (Rape and Giovanoni 2003; Handelsman 2004; Gilbert and Dupont 2011). In the days of pre genomics era, isolation and characterization (non molecular) of organisms responsible for remediation was more emphasized (Treatability studies). Such studies not only provided great opportunities to investigate their role in cleaning up the contaminants but other aspects of their physiology that control their growth and activity in contaminated environment were elucidated too. However, without the intervention of molecular tools, it was difficult to ascertain whether the isolated organisms were among the primary organisms responsible for bioremediation *in situ* or not (Lovely 2003). Advent of culture independent molecular approaches for the assessment of diversity, structure, dynamics and functions of the microbial communities within contaminated environment have subsequently revolutionized the concept of bioremediation. Analysis of 16S rRNA gene sequences derived from contaminated habitat gave more insight in to the indigenous microbial communities. Over the years, a number of molecular tools have been successfully developed for qualitative and quantitative analyses of microbial community structure and function. Among the qualitative techniques, fingerprinting such as ARDRA (amplified ribosomal DNA restriction analysis), AFDRA (amplified functional DNA restriction analysis) and DGGE (denaturing gradient gel electrophoresis) along with DNA sequencing are widely used. While the quantitative technologies include dot blot and fluorescence *in situ* hybridization (FISH) and real time PCR. All these techniques emphasize the enormous genetic and

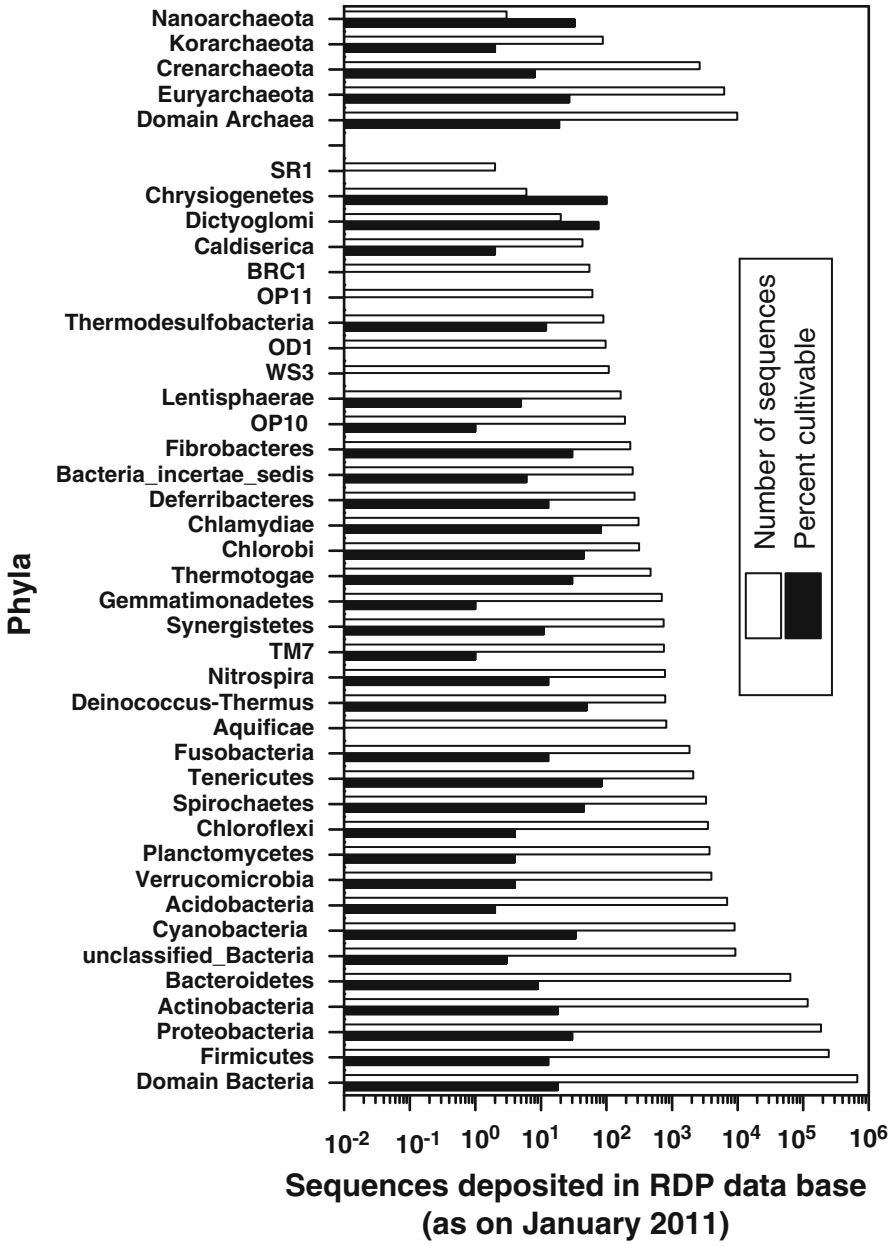

Fig. 23.1 Total number of 16S rRNA gene sequences deposited in each phyla and percent of cultivable members (Data obtained from RDP data base)

metabolic diversity of microbial communities and repeatedly knocked our vision that how little we know about the microbial world. Presently molecular techniques are getting popularity to access the whole microbial communities present in a particular habitat, bypassing the hurdle of laboratory based cultivation. As rightly pointed by Lovely (2003), one of the surprises from the application of 16S rRNA approach was the findings that in some instances, microorganisms that predominate during bioremediation, are closely related to organisms that can be cultured or already cultured. The example of close phylogenetic relatedness of organisms active in a contaminated aquifer to culturable *Geobacter* spp. implicated that simultaneous hydrocarbon degradation by oxidation and reduction of Fe^{3+} and U^{6+} in the aquifer could be possible *in situ*. However, one of the major limitations of 16S rRNA technique is that prediction of physiology from phylogeny may not be always correct, and in cases where there is no closely related organism available as pure culture it becomes more difficult (Lovely 2003). Along with identifying the kind of microorganisms, analysis of functional genes involved in bioremediation revealed what the microorganisms can do. Molecular approaches in detection, analysis and expression of genes revealed the catabolic potential of microorganisms relevant for bioremediation (George et al. 2010). Compared to DNA based methods, extraction and analysis of RNA was found to be more useful in this regard, as it could reflect the actual expression levels of genes of interest. Recent application of genome sequencing applied to both pure culture isolates and environmental genomes (metagenomics) have further strengthened our understanding on microbial physiology and catabolic activity within contaminated niches. Coupled with genome sequencing, developments in whole genome microarrays and proteomic tools facilitated the analysis of expression of all the genes in each genome under various environmental conditions. The availability of genetic systems of environmentally relevant organisms and genome wide expression analysis provides a number of advantages: (i) identification of regulatory circuits, (ii) elucidation of function of many genes previously unknown for their function and (iii) deciphering the pathways for bioremediation (Lovely 2003). The availability of the genome sequences for *P. putida* strains F1 [DOE Joint Genome Institute (JGI); http://genome.jgi-psf.org/finished_microbes/psepu/psepu.home.html] and KT2440 (Nelson et al. 2002) provides an opportunity to access its metabolic versatility and explore the molecular basis for its enhanced ability to adapt to the various environmental conditions present in pristine and metal-contaminated soils. Thompson et al. (2010) have recently investigated core molecular response of *Pseudomonas putida* F1 to acute chromate challenge by using proteomics tools. Comparative analysis of the proteomes revealed that an adaptive strategy employed by *P. putida* F1 in response to acute chromate exposure by up-regulating proteins involved in sulfate transport, cysteine biosynthesis, and the uptake and utilization of alternative sulfur sources such as aliphatic sulfonates. He et al. (2010a) have studied molecular mechanism of chromate resistance and reduction by *Bacillus cereus* SJ1, by using the obtained genome sequence. By genome sequencing analysis, these investigators identified a putative chromate transport operon chrIA1 and two additional *chrA* genes encoding putative chromate transporters that confer

chromate resistance. An azoreductase gene *azoR* and four *nitR* genes that encode nitroreductases which may catalyze reduction of chromate were also found. The expression of the chromate transporter gene *chrA1* was inducible by Cr(VI) and most likely regulated by *chrI*. Even though the physiological function of ChrI has not been verified due to the absence of a genetic system for this Gram positive strain, ChrI is most likely the first identified chromate responsive regulator. In addition, genome analysis identified a number of putative genes encoding gene products with possible functions in chromate resistance and reduction that may be the basis for the observed high chromate resistance and reduction ability of this strain. Furthermore, possible horizontal gene transfer events indicated in this study may have enabled *B. cereus* SJ1 to survive in metal (loid) contaminated environments. Recently, emergence of new tool 'metagenomics' enable us to study microbes in the complex communities where they live and to begin to understand how these communities work (Box 23.1) simply bypassing isolation of pure culture and their characterization. Traditional microbiological approaches have already shown the usefulness of microbes; the new approach of metagenomics will greatly extend scientists' ability to discover and benefit from microbial capabilities (Gilbert and Dupont 2011).

23.2.2 Microbial Interactions with Metals

Microorganisms have coevolved along with metals and minerals that constitute the earth crust (Hazen 2008). This long term coexistence (from 3.6 billion years ago) have allowed natural microbial flora to developed strategies to interact and modify these metals and metal bearing minerals sometimes to their benefit (in various metabolic reactions or just for detoxification) or as part of other secondary consequences. Bioremediation of metal pollution strongly relies on complex interplay of biological, chemical and physical processes, particularly, the mechanistic understanding of interrelated microbial processes, chemical reactions and contaminants mobility. Microorganisms exist in complex biogeochemical matrices within soil and subsurface environments. Their interaction with metals is subjected to several environmental factors like solution chemistry, sorptive reactive surface, availability of organic ligands and reductants (Tabak et al. 2005). Microorganisms interact with toxic metals and in various ways depending on the metal species, organism and environments.

Structural components and metabolic activity influence metal speciation and their solubility, mobility, bioavailability and toxicity; some of which may be harnessed as the basis for potential bioremediation strategies (Tabak et al. 2005; Gadd 2010). It is well known that many metals are essential for life which plays vital roles such as enzyme cofactors, catalysts, activity in redox process, but at excess concentration these metal ions can become detrimental to living organisms. Other metals such as Hg, Cs, Al, Cd, Pb have no known biological function but all can be accumulated and found to be toxic to organisms. Many microorganisms

Box 23.1

What Is Metagenomics?

Metagenomics describes the functional and sequence based analysis of the collective microbial genomes contained in an environmental sample (Riesenfeld et al. 2004). The term meta refers to meta-analysis, i.e. process of statistically combining separate analysis, while genomics is the comprehensive analysis of an organisms genetic material (Vo et al. 2007).

By definition metagenomics excludes studies that use PCR (with specific, degenerate or random primers) to amplify genes or gene cassettes, since these methods do not provide genomic information beyond the genes that are amplified.

Why Metagenomics?

One gram of soil or sediment may contain more than 10^{10} bacteria which may constituted by 10^4–10^5 different species (Torsvik et al. 2002). For technical reasons, the diversity and population density of microorganisms inhabiting the biosphere (6.3×10^{30} kg total biomass) is too high to be studied using traditional cultivation strategies (Raes et al. 2007; Ferrer et al. 2009; Gilbert and Dupont 2011). So far, we have only tapped into the "tip of the iceberg" of microbial resources as only 1% or less of the microorganism can be cultured by standard techniques in laboratory conditions (Amann et al. 1995). This cultivation bottleneck has skewed our view of microbial diversity and limited our appreciation of the microbial world. Meta genomics provides a relatively unbiased view not only of the community structure (species richness and distribution) but also of the functional (metabolic) potential of a community. Metagenomics facilitates the following aspect:

- Organisms can be studied directly in their environment bypassing the need to isolate each species.
- There are significant advantages for viral metagenomics, because of difficulties cultivating the appropriate host.
- Genomic information has advanced research in diverse arrays of fields, including forensic science and biomedical research.
- A main promise of metagenomics is that it will accelerate drug discovery and biotechnology by providing new genes with novel functions.

could grow and flourish in the metal rich or polluted environments with the help of variety of active and incidental mechanisms which contribute to resistance. Widespread resistance to toxic metal in microbe has been observed ranging from few percent (pristine environments) to nearly 100% (heavy metal rich or polluted environments) (Silver and Phung 2009). Variety of survival mechanisms developed

among the microbes for propagate their life in metal rich or polluted environments. All these mechanisms rely on reduction of toxicity by decrease or increase in mobility of metal ions. These include (i) transformation of redox state, (ii) active efflux, (iii) chelation by metal binding ligands (including proteins, peptides, etc.), (iv) active transport, and intracellular compartmentalization and (v) metal binding by cell wall and other structural components. Microbiological responses and geochemical cycles of metals are evident from such metal transformation which play central role in metal biogeochemistry (Tabak et al. 2005; Gilmour and Riedel 2009; Gadd 2010). Microbial metal resistance mechanisms generally conferred by metal resistance genes encoded on plasmids or chromosomes. Many metals and metalloids such as Ag^{+}, AsO_2^{-}, AsO_4^{-}, Cd^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Zn^{2+}, CrO_4^{2-}, TeO_3^{2-}, Sb^{3+} and $T1^{+}$ resistant genes located on plasmids. Related systems are also encoded frequently on bacterial chromosomes e.g. Hg^{2+} and Cd^{2+} resistance in *Bacillus* and arsenic efflux in *E. coli* (Silver and Phung 1996; Rosen 2002). The panoply of bacterial responses to toxic metals and radionuclides showed promise towards metal bioremediation specifically (i) by harnessing the metal precipitation or mobilization, thus affecting their environmental availability-toxicity, (ii) by designing or favoring (in case of in situ applications) metal resistant strains to better adopt and function within toxic environments and (iii) by combination of natural (indigenous communities for in situ applications) and engineered/superior strains (Valls and de Lorenzo 2002).

23.2.3 Bioremediation Options for Metal Contaminated Sites

Implementation of bioremediation strategy is often faced with two fundamental questions. First, where will the bioremediation process be carried out? Second, how aggressively will site remediation be approached? Regarding the first question, remediation process can be carried out *in situ* or *ex situ*. Aggressiveness depends on intrinsic or engineered bioremediation strategies (Madsen 2002). The major bioremediation options are shown in the following flow diagram (Fig. 23.2).

***In situ* bioremediation**: This option generally utilizes microbial processes that may mobilize or immobilize contaminants on site, where they are found in the landscape (Vrionis et al. 2005; Hwang et al. 2009). This option can be divided in two more options; intrinsic bioremediation which relies on the innate capacity of microorganisms present in field sites and is considered as passive and *in situ* engineered which relies on variety of engineering processes (control of water flow, aeration, chemical amendments, physical mixing of potent microorganisms) to accelerate intrinsic bioremediation. The latter option can be divided into several categories such as biostimulation, bioaugmentation, bioventing etc. depending on the engineering process utilization.

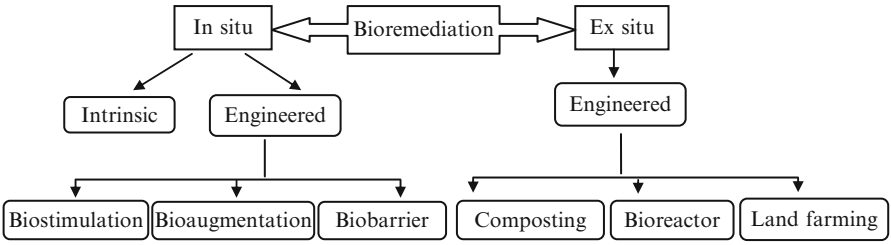

Fig. 23.2 Flow diagram of bioremediation strategies

Intrinsic Bioremediation: Intrinsic bioremediation is carried out *in situ* utilizing the inherent metabolic processes of indigenous microorganisms and is a component of natural attenuation, which includes physical and chemical processes. Cleanup activities that rely on natural attenuation to reduce contaminant levels and monitoring to determine the remedial effectiveness are referred to as "monitored natural attenuation". The intrinsic bioremediation should be monitored over a period of time to know whether the process actually occurring at a sufficient rate in the subsurface, contaminant plume size and associated microbial activity (biodegradation and/or biotransformation). This strategy is found to be very successful in case of organically polluted environments. However, promising results have been obtained with intrinsic bioremediation of metal (Selenium) polluted agricultural drainage water in marshlands (NABIR).

Biostimulation: Generally metal and radionuclide contaminated environments are lack of proper electron donor or accepter to support potent bioremediating microorganisms. Therefore, stimulation of growth of these microbes by addition of nutrients (usually sources of carbon, nitrogen, and/or phosphorus), oxygen, or other electron donors or acceptors is necessary and the process is known as biostimulation. These amendments serve to increase the number or activity of naturally occurring microorganisms available for bioremediation. Amendments can be added in either liquid or gaseous form, via injection. Liquids can be injected into shallow or deep aquifers to stimulate the growth of microorganisms involved in bioremediation. This strategies is undertaken as part of uranium bioremediation in Oak Ridge Field Research Center in Tennessee and Old Rifle UMTRA field site in western Colorado (Hwang et al. 2009; Yabusaki et al. 2007)

Bioaugmentation: Bioaugmentation is the introduction of microorganisms that can biotransform or biodegrade a particular contaminant in a particular environment. Until recently, bioaugmentation had not been consistently effective in a subsurface environment as it was not clear whether the introduced species could be effectively distributed through the complex geologic structures of most subsurface environments or compete over the long term with the indigenous microbiota.

Biobarrier: Biobarriers are effective bioremediation strategy if the intention is to contain the contaminant plume. Another successful biobarrier strategy is the

injection of ultramicrobacteria (<0.2 μm), formed by stressing bacteria so that they are more easily injected. This is followed by injection of nutrients that cause the ultramicrobacteria to return to their normal size and plug the pore structure so that ground water flow will be inhibited in that area.

***Ex situ* bioremediation**: *Ex situ* is defined as "in a position or location other than the natural or original one." Ex situ bioremediation usually refers to aboveground treatment in which soils have been excavated and washed, or water or sediments have been extracted from the subsurface and then decontaminated.

Composting: Composting is a technique that involves combining contaminated soil with non hazardous organic amendments such as manure or agricultural wastes. The presence of these organic materials supports the development of a rich microbial population and elevated temperature characteristic of composting. Composting in prepared beds holds a number of possibilities for bioremediation of metals degrading organic chelating agents, altering pH, redox potential and production of surfactants.

Bioreactor: Slurry reactors or aqueous reactors are used for *ex situ* treatment of contaminated soil and water pumped up from a contaminated plume. Bioremediation in reactors involves the processing of contaminated solid material (soil, sediment, sludge) or water through an engineered containment system.

Land farming: Land farming is a simple technique in which contaminated soil is excavated and spread over a prepared bed and periodically tilled until pollutants are degraded. The goal is to stimulate indigenous biodegradative microorganisms and facilitate their aerobic degradation of contaminants.

23.2.4 Key Steps in Metagenomic Techniques

Selection of study site: Metagenomic analysis of microbial communities should be carried out by addressing the scientific questions underlying for a particular site. Prior information of chemical, physical and ecological characteristic of the selected habitat are helpful to derive more insight from metagenomic data. Specific hypothesis could be proposed based on well characterized sites. In respect to this NABIR (Natural accelerated bioremediation) field research center is the good example (http://www.esd.ornl.gov/orifrc/). To develop uranium bioremediation strategies scientists vigorously characterized the sites before embark on metagenomic analysis. Interdisciplinary collaborations with scientists those who have been engage in studying the non-microbial aspects of selected sites. Different habitats require different depths of sequencing depending on their complexity and degree of completeness needed to address the questions being posed. Pilot studies to determine the required depth of sequencing may be also be necessary (He et al. 2007; Cardenas et al. 2010).

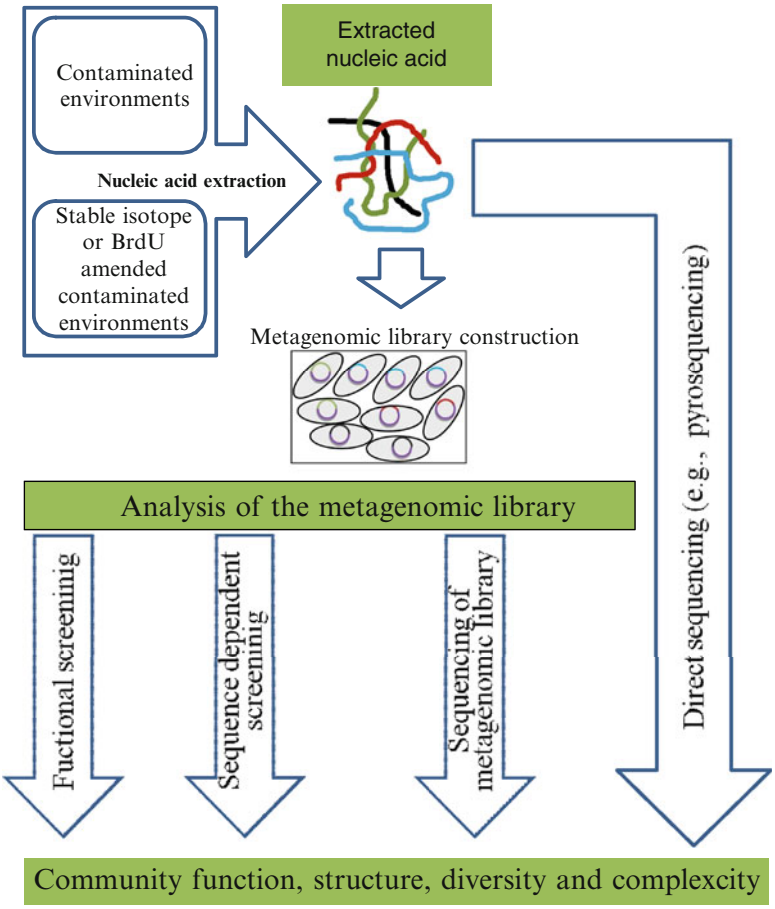

Fig. 23.3 Flow digramme of key steps involve in metagenomic analysis

Sample Collection and Nucleic Acid Extraction: Every metagenomic study started with the extraction of nucleic acid from the sample (key steps of metagenomic analysis is shown in Fig. 23.3). When a community is selected for metagenomic analysis, its species composition should be assessed with respect to the amount of allocated sequences. A complex microbial community usually includes bacterial, archaeal, microbial eukaryotes and viruses. Therefore the extracted pool of nucleic acid comes from all organisms present in the sample. Most of the metagenomic studies carried out so far has considered microbial communities in central part. However, metagenomic sequencing of communities containing eukaryotes, in particular protists, is mostly cost-prohibitive because of their enormous genome sizes and low gene-coding densities. Therefore selecting a community that does not contain eukaryotes, or from which eukaryotes or their DNA can be excluded, is an important consideration.

However, simply excluding eukaryotes from a metagenomic analysis is not ideal from an ecological point of view as it compromises our ability to assess a microbial

community in its entirety. An alternative or complementary strategy could be adopted to obtain molecular data at the RNA (metatranscriptomics) or protein (metaproteomics) level, thus bypassing the problem of large amounts of non-coding eukaryotic sequence data (Cowan et al. 2005). In recent literatures nucleic acid extraction methods have been reviewed (Cowan et al. 2005; Siddhapura et al. 2010). The two principal strategies for the recovery of metagenomic DNA are cell recovery and direct lysis. Usually physical and chemical methods are applied to recover total community genome. Bead beating has been shown to recover more diversity compared to chemical treatment. However, chemical lysis is a more gentle method, recovering higher molecular weight DNA and can also select for certain taxa by exploiting their unique biochemical characteristics. The technologies for recovering RNA from environmental samples are largely similar to those used for DNA isolation. Protocols are optimized for better yield of intact mRNA and often co-extraction methodologies have also been preferred (Griffiths et al. 2000; Popova et al. 2010; Mettel et al. 2010).

Genome and Gene Enrichment: Active microbial populations within contaminated environments can be assessed by genome enrichment followed by metagenomic analysis (Chen and Murrell 2010). Stable Isotope Probing (SIP) labeling technology can be used to selectively enrich the DNA, RNA or phospholipids derive fatty acids of active microbial populations. For SIP analysis, stable isotope (^{13}C, ^{15}N or ^{18}O) labeled substrates are added to the environment subjected to study for active microbial communities. As these substrates are consumed by active organisms, labeled atoms are incorporated in their DNA or RNA. Total nucleic acid is then extracted from the samples and labeled and non-labeled DNA or RNA is separated by density gradient centrifugation and 'heavier' labeled fraction can be used for construction of metagenomic libraries. Bromodeoxyuridine (BrdU, an analogue of thymidine) can also be used as alternative labeling substrates. Microorganisms actively growing in polluted environment containing BrDu will incorporate this labeled molecule to their DNA. The labeled DNA can be separated from the extracted DNA pool by density gradient centrifugation or immunocapture (Cowan et al. 2005).

Construction of Metagenomic Libraries: Analysis of environmental DNA or RNA by constructing metagenomic libraries is considered to be powerful tools for exploring microbial diversity. The basic steps which have been used over the years for construction of metagenomic libraries are generation of suitably sized DNA fragments and cloning the sized DNA into suitable vector. Cloning vectors such as cosmid, fosmid or BACs which could carry large sized DNA fragment are generally used to construct the metagenomic libraries (Gilbert and Dupont 2011).

23.2.4.1 Screening the Metagenomic Libraries

Function based screening: Screening strategies for metagenomic libraries can be designed to explore functional and/or genetic diversity. Functional screening of metagenomic libraries has enormous potential for discovery of new metabolic

activities and enzymes relevant to bioremediation. In the function-driven analysis, clones of interest are selected based on the detection of heterologous expression of desired traits like (i) growth on selective medium containing pollutant as sole electron accepter or C and energy source, (ii) metabolic activity (clearing zone, or coloration around colonies on specific solid media), (iii) Growth inhibition of indicator bacteria (Handelsman 2005). Library screening can be aided by high throughput automated colony picking, robotic pipetting, use of microtitere plates, informatics-assisted data management and sensitive assay targeting a broad range of biomolecules. Generally, screening "hits" in the metagenomic library are very low. However, rate of gaining positive hits can be improved by (i) the use of diverse strategies to enrich/select for community genomes with desired traits before construction of metagenomic libraries, (ii) the use of different expression hosts, (iii) the development of novel high throughput strategies for screening compatible with the fluorescence activated cell shorter (Stenuit et al. 2009). Recently, Mirete et al. (2007) discovered novel nickel resistance determinants from rhizosphere metagenome of plants adapted to acid mine drainage by metagenomic library construction and screening.

Sequence Based Screening: The sequence driven approach involves complete sequencing of clones containing the phylogenetic anchors such as the 16S rRNA genes or functional genes coding for key process. Suitable primers or target specific probes which are designed with information from database are utilized in commonly used screening methods; PCR and hybridization. Today, massive parallel screening of metagenomic libraries can be done by using microarrays. For example, Zhou and co-workers developed a comprehensive microarray termed "Geochip" which has been used to obtained direct lineages between biochemical processes and functional activities of microbial communities in a variety of environments (He et al. 2007, 2010b; Liu et al. 2010). In many cases whole community sequences were assessed by shotgun sequencing all inserts in the metagenomic library. In this case, the purpose is to get global picture of the microbial ecosystem and not to focus on particular genes, pathways or organisms. Sequence data are usually assembled into sequence contigs and scaffolds *in silico*.

Metagenomic analysis by direct sequencing: Preparation and maintenance of metagenomic library is a big hurdle. Today, development of new sequencing technology allows much greater depth of sequencing that revolutionizes the field of metagenomics and eliminates needs of cloning and library construction. For example, the pyrosequencing technology developed for the 454 Life Sciences Genome sequencer FLX (Roche) achieves an approximately 100 fold increase in throughput over current Sanger sequencing technology for fraction of cost (Droege and Hill 2008). In present day with advent of such powerful sequencers, generation of sequence data is not the limitation, however, storage, assembly and analysis of huge amount of data facing limitation (Cardenas and Tiedje 2008). On the other hand, pyrosequencing generates short read length (250 bases) which is considered as chronic drawback of the method and requires development of new algorithms to predict protein coding genes in the metagenome (Krause et al. 2006).

23.2.5 Genome Sequencing of Bacteria Relevant to Uranium Bioremediation

So far the primary uranium remediation strategies rely on reducing the soluble oxidized form of U(VI) to the insoluble form U(IV). Precipitation of U from contaminated groundwater/subsurface sediments prevents its further mobility. *Geobacter* species in the subsurface environment is the primary organism able to use U as an electron accepter for their respiration and reduced it to insoluble form (Lovely et al. 1991; Palmisano and Hazen 2003). Therefore, one promising uranium bioremediation technique consists of injection of appropriate substrate(s) into the contaminated aquifer in order to boost the natural *Geobacter* community performing uranium reduction (Anderson et al. 2003). Additionally, *Geobacter* spp. can oxidize organic pollutants in the subsurface environment using Fe(III) as an electron accepter (Lovley et al. 1989; Zhang et al. 2010). In order to get insight into enormous metabolic capability of *Geobacter* species several whole genome sequencing projects for selected isolates have been undertaken and some are already completed (Table 23.1) (Holmes et al. 2007). Sequencing of the genomes of *G. metallidurans* GS-15, *G. sulfurreducens* PCA (Caccavo et al. 1994; Methé et al. 2003), *G. bemidjiensis Bem* (Nevin et al. 2005), *G. uranireducens* Rf4 are now completed and whole genome sequencing of more isolates are under process (http://www.jgi.doe.gov/genome-projects/). These significant sequencing approaches not only enriched the catalogue of *Geobacter* biology but also paved the way for the construction of whole genome DNA microarrays which are very useful to analyze gene expression patterns in *Geobacter* genomes under various environmental conditions (Holmes et al. 2009).

Regulatory circuits in these organisms can be identified by genome wide-expression and proteome analysis and these circuits can be used for construction of genome-scale metabolic models that can be utilized to predict the behavior of *Geobacter* in changing environmental conditions (Mahadevan et al. 2006; Sun et al. 2009; Ueki and Lovley 2010). Such models have been recently coupled to transport models of contaminants in an attempt to model *in situ* uranium bioremediation (Scheibe et al. 2009). This represent a significant step ahead in the exploitation of genome based technologies for improving uranium bioremediation strategies. Although *Geobacter* species represent an abundant bacterial group with uranium sequestrating abilities in U-contaminated environments, the global picture of microbial community dynamics in such environments is much more complex and involves syntrophic association of other bacteria (Brodie et al. 2006; Chandler et al. 2006; Cardenas et al. 2010). For instance, Geobacteriaceae population maintains high cell densities with low U(VI) concentration and declined U(VI) reduction ability. This observation suggests the existence of a syntrophic association with organisms acting as biological electron accepter (Brodie et al. 2006). Therefore, the dynamics of growth and activity of *Geobacter* species and other microorganisms *in situ* and their interaction with other environmental factors such as groundwater flow and geochemistry are yet to be understood.

Table 23.1 Whole genome/metagenome sequencing project available at Joint Genome Institute (JGI)

Project name	Phase status	Program	JGI project ID
Cupriavidus metallidurans CH34	Finished	DOEM 2001	16314
Ralstonia eutropha JMP-134	Finished	DOEM 2002	16313
Geobacter bemidjiensis Bem	Finished	GTL 2007	16055
Geobacter metallireducens GS-15	Finished	DOEM 2002	16056
Geobacter sp. FRC-32	Finished	DOEM 2005	16057
Geobacter sp. M18	Awaiting external Release	GTL 2007	100065
Geobacter sp. M21	Finished	GTL 2007	97123
Geobacter sp. Ply4	Abandoned	GTL 2005	96881
Geobacter sp. Strain SZ	Finished	DOEM 2006	16058
Geobacter uraniireducens Rf4	Finished	GTL 2005	16059
Geobacter Uranium Bioremediation Rifle Site DLES2[a]	Abandoned	GTL 2007	52046
Geobacter Uranium Bioremediation Rifle Site DLES3[a]	Awaiting UA	GTL 2007	52047
Shewanella amazonesis SB2B	Finished	DOEM 2004	16362
Shewanella baltica OS155	Finished	DOEM 2004	16363
Shewanella baltica OS185	Finished	CSP 2005	16364
Shewanella baltica OS195	Finished	DOEM 2005	16365
Shewanella baltica OS223	Finished	DOEM 2005	16366
Shewanella denitrificans OS217	Finished	DOEM 2004	16368
Shewanella frigidimarina NCMIB400	Finished	DOEM 2004	16369
Shewanella halifaxensis HAW-EB4	Finished	DOEM 2006	16370
Shewanella loihica PV-4	Finished	DOEM 2004	16877
Shewanella pealeana ATCC 700345	Finished	DOEM 2006	16371
Shewanella putrefaciens 200	Finished	DOEM 2004	16372
Shewanella putrefaciens CN-32	Finished	DOEM 2004	16373
Shewanella sediminis HAW-EB3	Finished	DOEM 2006	16375
Shewanella sp. ANA-3	Finished	DOEM 2005	16876
Shewanella sp. MR-4	Finished	DOEM 2005	16376
Shewanella sp. MR-7	Finished	DOEM 2005	16377
Shewanella sp. W3-18-1	Finished	DOEM 2005	16378
Shewanella woodyi ATCC 51908	Finished	DOEM 2006	16379
Diaphorobacter sp. TPSY	Finished	CSP 2008	202766
Desulfovibrio desulfuricans G20	Finished	DOEM 2002	16714
Desulfovibrio desulfuricans ATCC 27774	Finished	GTL 2007	98774
Desulfovibrio fructosovorans JJ	Finishing	GTL 2007	400127
Sphingomonas wittichii RW1	Finished	DOEM 2006	16890

[a]Metagenomic project

For better insight into the microbial communities inhabiting U-impacted sites several metagenomic project have been undertaken which could help in the bioremediation strategies.

23.2.6 Metagenome Projects Related to Uranium Bioremediation

In order to provide better understanding of inhabitant microbial communities from metal and uranium contaminated sites for development of bioremediation strategies several metagenome projects are completed and some are in progress. Among these Heme et al. (2010) recently completed and published one metagenome project on heavy metal contaminated groundwater microbial community. This project was undertaken to answer several highly relevant questions that include: How does anthropogenic contamination affect groundwater microbial community structure and diversity? How does a microbial community adapt to severer environmental change such as heavy metal contamination? And can molecular mechanisms responsible for such environmental change be predict from the metagenomic sequences? Shotgun sequencing approach was adopted to complete this project. Initially, the extracted DNA was fragmented and three metagenomic libraries of 20.04 Mb small insert (3 kb) in pUC, 23.13 Mb medium insert (8 kb) in pMCL and 9.27 Mb large insert (40 kb) pCCiFos. Major findings from this project are: (i) Heavy metals, nitric acid organic solvents contamination resulted in massive decrease in species, allelic diversity as well as metabolic diversity, (ii) The microbial community primarily composed of denitrifying bacteria γ- and β-*Proteobacteria,* (iii) Over abundant of key genes conferring resistance to various metals and (iv) Lateral gene transfer mechanism function rapidly to response environmental changes.

The second project was on microbial community of uranium contaminated site at Hanford, Richland, Washington (CSP 2009) (http://ifchanford.pnl.gov). This project was started with the aim to understand the factors such as geochemical, hydrophysical and microbiological which control the migration of uranium through vados zone and groundwater of a former plutonium production site (from 1943 to 1989) adjacent to a river. This work is one component of integrated, multidisciplinary research efforts supported by DOE-BER's Environmental Remediation Sciences Program (ERSP) at Pacific Northwest National Laboratory to examine how geochemical and biogeochemical processes that operate at different spatial scales affect the fate and transport of radionuclides and other contaminants in the subsurface at DOE's Hanford Site. Presently it is in production state. Being the part of an integrated multidisciplinary field research program this project utilized the massive sequencing of the ribosomal gene only.

The third project dealt with *Geobacter* uranium bioremediation communities D05, D07-1 and D07-2 (GTL program 2007): Genome sequencing in D05 is in assembly state while D07-1 and D07-2 are completed. Samples from these projects

were collected from Rifle Site, Rifle, Colorado. In these project 454 based sequencing was adopted. Knowledge of whole genome sequence of *Geobacter* was found to very helpful for assembly and analysis of rather short read and data, respectively, generated by sequencing approaches. Surprisingly, the data analysis revealed that nearly one third of the open reading frames were not closely related to sequenced *Geobacter*, although the community was thought to be composed of mainly *Geobacter* spp. The result from this project suggested that a greater diversity of *Geobacter* is present in the contaminated site than that currently captured in pure culture and whose whole genome sequence has been done.

The fourth important project was sequencing the mixed waste (nitrate, heavy metals, radionuclides and organic contaminants) groundwater community [community sequencing program (CSP) 2005] (http://www.jgi.doe.gov/sequencing). The samples for this project were obtained from the Natural and Accelerated Bioremediation Research (NABIR) Field Research Center (FRC), well no. FW-010. The overall objective of this project was to provide (i) fundamental and comprehensive understanding of microbial community diversity in response to mixed waste (ii) insights into how the microbial community adapts to extremely toxic mixed-waste environments and (iii) how the sequence information could be integrated with the studies of biogeochemistry, hydrology, microbiology, and engineering to understand the impacts of contaminants and remediation treatments on microbial community dynamics. The manageable diversity and complexity (~20 phylotypes) of the ground water community was assessed by shotgun sequencing approach. Metagenomic data from the contaminated site provided sequence information related to novel uncultured bacteria which have been utilized to developed whole-community microarrays. These microarrays were used to address the ecological questions and help to understanding of how microorganisms are affected and adapt in contaminated sites as well as help scientist in designing efficient bioremediation strategies (He et al. 2007; Hwang et al. 2009). The whole-community sequence information provides baseline information for understanding how contaminants affect microbial communities, helped scientists to design strategies for remediating mixed contaminants and creates an unprecedented opportunity to test ecological and evolutionary theories about the relationship between phylogenetic diversity and the functional properties of ecosystems. These issues are central to current concerns about biodiversity and are also directly relevant to understanding and managing microbial communities for bioremediation and other purposes.

23.2.7 Community Microarrays

In current years, on the basis of metagenomic data community-microarrays (phylochip and geochip) have been developed to assess microbial community structure, function and dynamics in contaminated sites (Andersen 2010). Several groups have now developed microarrays for the analysis of functional genes (Waldron et al. 2009) and mRNA

(Gao et al. 2007) in the environment, and the direct detection of 16S rRNA (Brodie et al. 2006). There is also an Affymetrix array containing probes targeting the entire Ribosomal Database Project (Wilson et al. 2002), a system that has been applied to the analysis of microbial communities undergoing uranium reduction and reoxidation (Brodie et al. 2006). Comprehensive functional gene array (GeoChip) developed based on metagenomic technologies have been used to assess functional structure of microbial communities during pilot scale uranium *in situ* bioremediation (Xu et al. 2010). In this study, the authors observed that at inner loop (designated as bioremediation site) amount of uranium reduced to drinking water standard (<30 μg L^{-1}) along with abundance of metal reducing populations including *Desulfovibrio*, *Geobacter*, *Anaeromyxobacter*, *Shewanella* and other bacteria such as *Rhodopseudomonas* and *Pseudomonas*. This study able to correlate the data obtained from geochemical analysis and geochip analysis of stimulated indigenous microbial communities for bioremediation of uranium Chandler et al. (2010) developed field-portable microarray analysis system. They have used this system in monitoring the microbial community structure and dynamics during *in situ* U(VI) bioremediation.

23.3 Conclusions

There is no doubt that microorganisms play key role(s) in biological treatment of various environmental contaminants. Comprehensive understanding of both structure and function of relevant microbial community is required to achieve effective and reliable cleaning up of contaminants. However, this is particularly challenging to microbiologist as most of the environmental microorganisms are not readily culturable and therefore their biology is not fully characterized. Undoubtedly, the emergence of metagenomics has revolutionized this field. Metagenomics provides direct access to indigenous microbial communities from the contaminated environments independently of their culturability. Metagenomic approaches are expected to boost the discovery of new catabolic activities and provide valuable information for the management and cleanup of polluted sites. Though we have just begun to appreciate the usefulness of enormous prospect of metagenomics in bioremediation, form the technical point of view its application is not yet properly realized. So far only few metagenomic projects have given concrete results on true understanding of microbial activity necessary for cleaning up the contaminants. Paucity of knowledge of functional screening of metagenomic libraries; lack of suitable host organisms to express metagenome derived genes in anaerobic condition; lengthy sequence analysis processes are among the few tailbacks. Considering the developments in the filed of next generation sequencing, data analysis etc. the decade ahead seems to be crucial to bring real scientific breakthroughs in the field of bioremediation. Metagenomics is further facing with two challenges. One lies on the dynamical behavior of microbial communities in response to changing environmental conditions of the contaminated sites undergoing bioremediation. From the metagenomics data it remains difficult to reconstruct a faithful picture of the functioning of complex

microbial communities and the contribution of particular, low-abundant microbial taxa to specific process. As bioremediation involves complex interaction of microbial communities and therefore it is envisioned that coupling of other '-omics' technologies (metatranscriptomics, metabolomics, metaproteomics etc.) will be necessary to get comprehensive understanding of community attributes associated with successful bioremediation.

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Chapter 24
Microbial Concrete, a Wonder Metabolic Product That Remediates the Defects in Building Structures

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Abstract Microbes play key geoactive roles in the biosphere, particularly in the areas of element biotransformations, biogeochemical cycling and mineral transformations and formations. Recently, the ability of bacteria to produce minerals such as carbonates has been studied in detail with various uses in civil engineering. Natural processes, such as weathering, faults, land subsidence, earthquakes, and human activities create fractures and fissures in concrete structures, which can reduce the service life of the structures. 'Microbial Concrete' a novel metabolic byproduct of microbially induced calcite precipitation by way of urease (urea hydrolyzing enzyme) presents a promising novel biotechnology for the enhancement of durability of building materials and structures. The ubiquity and importance of microbes in inducing calcite precipitation make "microbial concrete" a most important metabolic product of biomineralization that can remediate and restore such structures. Urease producing bacteria when added to cement, helps in the improvement of compressive strength, reduction of permeability and reduced corrosion rate of reinforced structure. The promising results using this metabolic product encouraged different research groups world wide and in-depth studies of these different approaches have been discussed in this chapter. The methodologies to such approach have also been discussed. The present chapter suggests a potential use of the microbially induced calcite precipitation process with environmental significance as a safe consolidation tool in remediation of defects in building structures and towards enhancement in its service life.

Keywords Microbial calcite • *Bacillus* sp. • Compressive strength • Building materials • Sand columns • Crack remediation • Corrosion • Permeability

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24.1 Introduction

In recent years, it has been pointed out that not only structural safety but also durability is important when designing building or concrete structures. However, in traditional structural design the degradation of structures over long periods of time is not regarded as a serious problem; the destruction of structures has a much greater impact on people or society. Taking these social factors into consideration, it is obvious that we should not consider the problem of durability of building materials as a problem of the past. It is necessary to develop some new eco-friendly self-remediating techniques to support already designed and future constructing buildings to meet various demands to enhance their durability.

Selection of materials and technologies for the building construction should satisfy the felt needs of the user as well as the development needs of the society, without causing any adverse impact on environment. Due to high construction costs and the social importance, the durability demands for large building structures are becoming more and more important. Building material is any material which is used for construction purpose. Many naturally occurring substances, such as clay, sand, wood and rocks, even twigs and leaves have been used to construct buildings. Apart from naturally occurring materials, many man-made products are in use, some more and some less synthetic. Concrete is the most widely used building material throughout the world. Current concern about the degradation of concrete and the economic impact of the maintenance and repair of concrete structures, have drawn the attention to processes of concrete deterioration, and to the methods to slow down or even to eliminate concrete degradation. Durability of concrete is the ability of a concrete to resist deterioration, particularly deterioration due to weather exposure, chemical exposure or surface abrasion. A durable concrete must maintain its form, quality and serviceability for its prescribed service life.

Natural processes, such as weathering, faults, land subsidence, earthquakes, and human activities create fractures and fissures in concrete structures or monuments. These fractures and fissures are detrimental since they can reduce the service life of the structure (Achal et al. 2011). Weathering induces an increased porosity, structural weakening of surface layers and unattractive appearance. Synthetic agents such as epoxies and surface treatments with water repellents such as silanes or siloxanes, or with pore blockers are applied for remediation of these structures. However, these and other treatments with organic and inorganic products involve some disadvantages, such as different thermal expansion, degradation with age and the need for constant maintenance. Appearance of cracks and fissures is an inevitable phenomenon during the aging process of concrete structures when exposed to weather changes. Such cracking leads to easy passage for aggressive environment to reach the reinforcement and initiate corrosion. Moreover, sometimes repair is carried out in the areas where it is not possible to shut down the plant or it is hazardous for human beings. Hence, in such situations a way should be found out to self healing materials that seal the cracks automatically. Recently, a novel technique has been reported that utilizes microorganisms in remediation of cracks and fissures in natural and man-made structures by precipitation of calcium carbonate.

Using bacteria for remediation of building structures? It sounds like a strange idea, but it has become a reality and this new method should be ready to be used in practice in the near future (Achal et al. 2011). Using the latest microbial biotechnology, a new type of construction material, microbial concrete, can be developed to enhance the durability of building structures. Microbial concrete is made of naturally occurring microorganisms at ambient temperature and thus requires much less energy to produce. It is sustainable as microorganisms are abundant in nature and can be reproduced easily at low cost. The microorganisms that are suitable for making microbial concrete are non-pathogenic and environmentally friendly. Furthermore, unlike the use of cement, soils or contaminated land can even be treated or improved without disturbing the ground or environment as microorganisms can penetrate and reproduce themselves in soil or any such environments. Harnessing this natural, unexhausted resource may result in an entirely new approach to geotechnical or environmental engineering problems and bring in enormous economical benefit to construction industries (Jian and Ivanov 2009). The application of microbial biotechnology to construction may also simplify some of the existing construction processes and revolutionize the ways of new construction processes.

24.2 Critical Review

Microbial metabolic activities often contribute to selective cementation by producing relatively insoluble organic and inorganic compounds intra and extracellularly. Microorganisms and microbiologically mediated mineralization processes are active in almost every environment on earth. The application of bacteria for bioremediation purposes in building materials and structures is becoming increasingly popular as is reflected by recent studies. The applicability of specifically mineral-producing bacteria for sand consolidation and limestone monument repair (Gollapudi et al. 1995; Dick et al. 2006; Achal et al. 2009a) and filling of pores and cracks in concrete have been recently investigated (Bang et al. 2001; De Muynck et al. 2008; Achal et al. 2010, 2011). In most of these studies ureolytic bacteria of the genus *Bacillus* were used as agent for the biological production of calcium carbonate based minerals. The mechanism of calcium carbonate formation by these bacteria is based on the enzymatic hydrolysis of urea to ammonia and carbon dioxide.

24.2.1 Microbially Induced Calcium Carbonate Precipitation (MICCP)

Microbially induced calcium carbonate precipitation generally results from a series of complex biochemical reactions (Stocks-Fischer et al. 1999) involving

urease (urea amidohydrolase; EC 3.5.1.5). The basic reaction of the calcocarbonic system is:

$$CO_3^{2-} + Ca^{2+} \leftrightarrow CaCO_3 \left(Ksp = 3.8 \times 10^9\right) \tag{24.1}$$

Where, Ksp the solubility product.

The driving force for precipitation of calcium carbonate ($CaCO_3$) is the supersaturation level S, defined by the ratio of the ionic product:

$$S = \left(Ca^{2+}\right) \times \left(CO_3^{2-}\right) / Ksp \tag{24.2}$$

During microbial urease activity, 1 mole of urea is hydrolyzed intracellularly to 1 mole of ammonia and 1 mole of carbamate (Eq. 24.3), which spontaneously hydrolyses to form an additional 1 mole of ammonia and carbonic acid (Eq. 24.4) (Burne and Chen 2000).

$$CO\left(NH_2\right)_2 + H_2O \rightarrow NH_2COOH + NH_3 \tag{24.3}$$

$$NH_2COOH + H_2O \rightarrow NH_3 + H_2CO_3 \tag{24.4}$$

These products subsequently equilibrate in water to form bicarbonate and 2 moles of ammonium and hydroxide ions (Eqs. 24.5 and 24.6).

$$H_2CO^3 \leftrightarrow HCO_3^- + H^+ \tag{24.5}$$

$$2NH_3 + 2H_2O \leftrightarrow 2NH_4^+ + 2OH^- \tag{24.6}$$

The latter two reactions give rise to a pH increase, which in turn shifts the bicarbonate equilibrium, resulting in the formation of carbonate ions (Eq. 24.7). This pH increase takes place initially in the local micro-environment around the bacterial cell, and propagates in the bulk solution of the bacterial cell suspension.

$$HCO_3^- + H^+ + 2NH_4^+ + 2OH^- \leftrightarrow CO_3^{2-} + 2NH_4^+ + 2H_2O \tag{24.7}$$

Thus, the carbonate concentration will increase, inducing an increase in S (according to Eq. 24.2) and resulting in $CaCO_3$ precipitation around the cell, in the presence of soluble calcium ions as shown in Eqs. 24.8 and 24.9.

$$Ca^{2+} + Cell \rightarrow Cell - Ca^{2+} \tag{24.8}$$

$$Cell\text{ - }Ca^{2+} + Co_3^{2-} \rightarrow Cell\text{ - }CaCO3^- \tag{24.9}$$

Calcium carbonate precipitation is a rather straightforward chemical process governed mainly by four key factors: (1) the calcium concentration, (2) the concentration of dissolved inorganic carbon (DIC), (3) the pH and (4) the availability of nucleation sites (Hammes and Verstraete 2002).

Calcite precipitation in solution occurs via the overall equilibrium reaction of $Ca^{+2} + CO_3^{-2} \leftrightarrow CaCO_3$ as described in Eq. 24.1. Because the production of CO_3^{-2} from bicarbonate (HCO_3^-) in water is strongly pH dependent, an increase in CO_3^{-2} concentration occurs under alkaline conditions. Therefore, calcium carbonate precipitation readily occurs in alkaline environments abundant of the calcium (Ca^{+2}) and carbonate (CO_3^{-2}) ions (Stocks-Fischer et al. 1999; Ramachandran et al. 2001; Qian et al. 2010a). Urease activity promotes this precipitation outside the cells. Briefly, calcium ions in the solution are attracted to the bacterial cell wall due to the negative charge of the latter. Upon addition of urea to the bacteria, dissolved inorganic carbon and ammonium are released in the microenvironment of the bacteria. In the presence of calcium ions, this can result in a local supersaturation and hence heterogeneous precipitation of calcium carbonate occurs on the bacterial cell wall. Furthermore, it has been demonstrated that specific bacterial outer structures (glycocalyx and parietal polymers), a variety of organic polymers outside the cell wall (Lappin-Scott et al. 1988; MacLeod et al. 1988) or exopolymeric substances consisting of exopolysaccharides, proteins and amino acids play an essential role in the morphology and mineralogy of microbially induced carbonate precipitation (Braissant et al. 2003; Ercole et al. 2007). The actual role of the bacterial precipitation remains, however, a matter of debate (De Muynck et al. 2010). Some authors believe this precipitation to be an unwanted and accidental by-product of the metabolism (Knorre and Krumbein 2000) while others think that it is a specific process with ecological benefits for the precipitating organisms (Ehrlich 1996; McConnaughey and Whelan 1997). The evidence of microbial involvement in carbonate precipitation has subsequently led to the exploration of this process in the field of bioremediation. The use of MICCP has also been proposed for the removal of heavy metals (Warren et al. 2001) and biodegradation of pollutants (Simon et al. 2004; Chaturvedi et al. 2006).

As discussed before, urease is the key enzyme involved in the process of calcite precipitation induced by bacteria. Bacteria are known to hydrolyze urea by urease for the purposes of: (1) increasing the ambient pH (Burne and Marquis 2000), (2) utilizing it as a nitrogen source (Burne and Chen 2000), and (3) using it as a source of energy. There are many species of *Bacillus* reported that produce large amount of urease, further helps in calcite precipitation and biocementation (De Muynck et al. 2008; Achal et al. 2009a). Urease occurs in many bacteria, several species of yeast and a number of higher plants including jack beans (*Canvalia ensiformis*) (Dixon et al. 1980), soybean leaf and seed (*Glycine max*) (Kerr et al. 1983), pigweed (*Chenopodium album*) (El-Shora 2001) and mulberry leaf (*Morus alba*) (Hirayama et al. 2000). Most organisms with ureolytic ability use urea as a source of nitrogen by actively transporting or passively diffusing urea into the cell cytoplasm, where urease hydrolyses urea releasing two ammonium molecules, which can then be directly assimilated into biomass via the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway or by the action of glutamate dehydrogenase (GDH) (Tyler 1978). To ensure this process is energy efficient, the production of urease in organisms such as *Pseudomonas aeruginosa*,

Alcaligenes eutrophus, *Bacillus megaterium* (Kaltwasser et al. 1972) and *Klebsiella aerogenes* (Friedrich and Magasanik 1977), is repressed by the presence of ammonium. There are however some exceptions to this regulation, such as *Proteus vulgaris*, which can produce urease even in the presence of high concentrations of ammonium (Mörsdorf and Kaltwasser 1989). Some specialist organisms exist that have additional uses for urease, beyond nitrogen assimilation. *Helicobacter pylori,* an inhabitant of the low pH gastric juices in the stomach, not only has intracellular but also extracellular urease that is located on the cell surface. The extracellular urease plays a protective role from the low pH environment of the stomach, by providing a microenvironment of more neutral pH, generated from production of ammonium near the cell surface (Marshall et al. 1990; Dunn and Grütter 2001; Ha et al. 2001). *Sporosarcina pasteurii* is another specialist organism that has a different use for urease, other than nitrogen assimilation. *S. pasteurii* is a moderately alkaliphilic organism with a growth optimum at pH 9.25. Alkaliphiles present a special problem for the generation of ATP due to a reversed chemiosmotic proton gradient. In neutrophilic organisms, ATP is produced from the proton motive force that is generated by pumping protons out of the cell from the electron transport chain. This generates a proton concentration gradient (high outside/low inside) and causes protons to be driven back into the cell through the ATP-synthase, resulting in ATP generation (Prescott et al. 1993). When the external environment is highly alkaline, the proton concentration gradient is reversed and the gradient favours protons to be fluxed out of the cell, but not back into it. Alkaliphilic organisms must create a high membrane potential (charge difference across the membrane) by pumping out cations, to drive protons back into the cell against the concentration gradient (Ivey et al. 1998). For *S. pasteurii*, the effluxed cation used to create a high membrane potential to drive ATP synthesis is ammonium, which can be supplied directly as ammonium or indirectly as urea (Jahns 1996). As the biocementation reaction results in the generation of high concentrations of ammonium, only those bacterial sources where urease is not down regulated by the presence of ammonium are useful. These organisms include *Sporosarcina pasteurii* and *Proteus vulgaris*. For biocementation purposes, an ideal microbial source of urease has the following properties:

i. High urease production capacity
ii. Ability to produce urease in the presence of ammonium
iii. High stability (robust)
iv. Consistent production (reliable)
v. Does not require further down-stream processing prior to use in biocementation

Urea hydrolysis is the most easily controlled of the carbonate generating reactions, with the potential to produce high concentrations of carbonate within a short time. Beside conventional bioremediation measurement, a number of applications involving MICCP have been attempted in the area of construction industry. Following section deals with detailed analysis regarding parameters affecting the durability of building structures and how role of bacteria has been reported to enhance durable aspects.

24.3 Analysis

Concrete is a strong and relatively cheap construction material widely used worldwide. The major drawback, however, is that its massive production exerts negative effects on the environment, increasing energy consumption, greenhouse gas emissions and landscape mutilation. It is estimated that cement (Portland clinker) production alone contributes 7% to global anthropogenic CO_2 emissions, what is particularly due to the sintering of limestone and clay at a temperature of 1,500°C, as during this process calcium carbonate ($CaCO_3$) is converted to calcium oxide (CaO) while releasing CO_2 (Worrell et al. 2001). Therefore, from an environmental viewpoint, concrete does not appear to be a sustainable material (Gerilla et al. 2007). Another aspect of concrete is its liability to cracking, a phenomenon that hampers the material's structural integrity and durability. The impact of durability-related problems on national economies can be substantial and is reflected by the sums of money spent on maintenance and repair of building structures.

24.3.1 Durability of Building Materials and Structures

The main aim of any specification is to ensure the quality of the final product. Strength and durability are considered to be the requirements for concrete or any other building materials. Nowadays, performance based durability criteria are becoming more prevalent in specifications for building materials. A major goal of any performance based specification is to ensure the durability of building structures. Durability is defined as the ability of the structure to perform satisfactorily with minimum maintenance over the anticipated service life of the structure. The quality of any building material depends on three major parameters, viz. (i) compressive strength, (ii) permeability and (iii) corrosion. For an efficient microbial concrete it should produce more compressive strength, less permeability and should not affect corrosion of any reinforcement.

To improve the durability of structures constructed in concrete, engineers started using mineral additives in the form of waste materials from industries such as fly-ash, ground granulated blast furnace slag. Many additives and admixtures were developed for concrete as it is one of the most widely used construction material not only for small elements like concrete sleepers but also in many giant structures which are constructed to satisfy the needs of infrastructure of growing population. In spite of so much development in the ingredients of concrete final product, concrete was not made to react with environment and have the ability of self healing as seen in naturally built structures described above. Most of these additives were passive in nature. More research and development is required to tackle the problems of permeability, ingress of chloride and carbonation to protect the reinforcing material added in concrete from deterioration. There is a need to develop an additive which will react with environment, grow as per needs and will have property of self healing in case of distress.

In existing structures, it is not possible to change the nature of the concrete easily. As deterioration starts to occur, the emotively termed 'concrete cancer' steps have to be taken to arrest the decline and repair the damage. Several tests are available to the engineer for diagnosing the condition of the concrete. The alkalinity of the concrete at the surface and at various depths down to the position of the reinforcement will give an indication of how much and how soon the reinforcement may be at risk. This is known as the depth of the carbonation front. The presence of chlorides can be determined, again at varying depths. The permeability of the concrete will give an indication of its susceptibility to absorption of air and water. Particularly cracking of the surface layer of concrete reduces material durability as ingress water and detrimental chemicals cause a range of matrix degradation processes as well as corrosion of the embedded steel reinforcement (Neville 1996).

Durability problems such as crack formation are typically tackled by manual inspection and repair, *i.e.* by impregnation of cracks with cement or epoxy-based or other synthetic fillers (Neville 1996). There are so many synthetic agents or latex binding agents (such as acrylic, polyvinyl acetate, butadiene styrene) which are used to avoid any kind of fractures and fissures in the concrete structures or used in repair applications such as the bonding of fresh concrete, sprayed concrete or sand/cement repair mortar to hardened concrete. There is reluctance by some repair product manufacturers to adequately address the problem, with a very limited range of appearances available from their materials in different countries. There is a propensity for specifiers to recommend overcoating of the whole building, such as with a high-build acrylic paint in order to achieve a uniform appearance, which is also easily matched in future if further repairs become necessary. This may well be a blessing in disguise for some buildings, but for others the original appearance is their single most important feature, and it should not be discarded lightly. It also needs to be said that a building which is painted may mean a lifetime of higher maintenance costs. It will probably need repainting every 10 years, perhaps less, to retain its smartness. Despite the advice to use an appearance-changing coating only as a last resort, these products have their place. Indeed, they may be essential as a means of preventing the absorption of moisture and air, in the latter case being classed as anti-carbonation coatings. In selecting a coating, one should have in mind what is intended to be achieved by that coating, if it is not simply a change of appearance. In particular it must be noted that some are waterproof but permeable to air. These will not necessarily arrest the advancement of the carbonation front. Others are not vapour-permeable, which can lead to blistering of the coating if there is moisture trapped behind it.

In brief, these traditional repair systems have a number of disadvantageous aspects such as different thermal expansion coefficient compared to concrete, weak bonding, disappearance, environmental and health hazards and even costly. Therefore, researchers from various group proposed microbially induced calcium carbonate precipitation as an alternative and environmental friendly technique to enhance the durability of building materials and structures. To date, most of the published work has focused on MICCP for purposes of strength development and materials remediation (Table 24.1).

Table 24.1 The reaction conditions reported in the literature for the production of $CaCO_3$ via microbial production of carbonate from urea hydrolysis

Target	Urea concentration (mM/L)	Calcium concentration (mM/L)	Urease activity/IU	References
Sand column	33	25	412	Achal et al. (2009a)
Sand column	3,000	3,000	n/s	Qian et al. (2010b)
Crack remediation	332	50	n/s	Ramachandran et al. (2001)
Stone remediation	332	12–50	190	Stocks-Fischer et al. (1999)
Stone remediation	65	25	40	Bachmeir et al. (2002)
Rock pros plugging	330	0.025	n/s	Gollapudi et al. (1995)

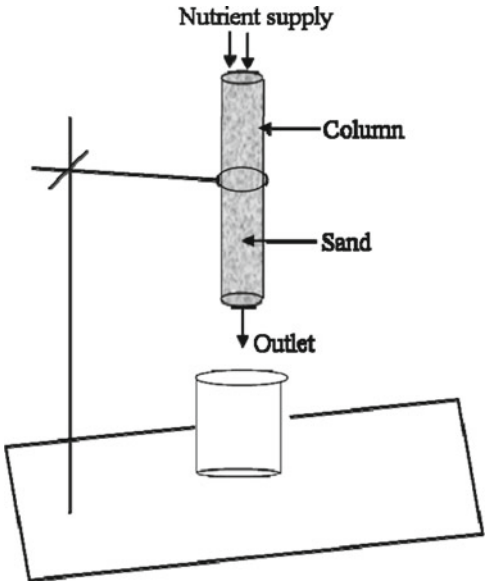

Fig. 24.1 A typical set-up for the sand consolidation experiment

24.3.2 Sand Consolidation and MICCP

Sand is the common material used to make most of the building materials and structures. As such there is no carbonate mineral found in sand, so it is easy to understand the mechanism of calcite precipitation induced by bacteria in it. There are many reports where various researchers have successfully defined MICCP in sand consolidation (Achal et al. 2009a; Qian et al. 2010b). They prepared a sand column by introducing bacterial solution into sands and treated the column with nutrient media. A typical set-up of this experiment has been shown in Fig. 24.1 to get brief idea about sand consolidation by bacteria.

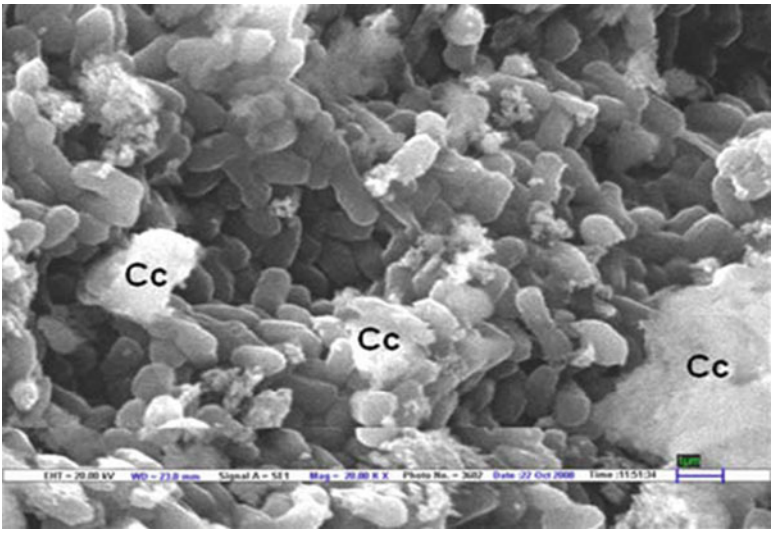

Fig. 24.2 Scanning electron micrograph of microbiologically induced calcite precipitation by Bp M-3 mutant in sand consolidation (*Cc* calcite crystals) (Source: Achal et al. (2009b))

The nutrient media supplied from the top provides nutrition for the bacteria, subsequently urease starts acting on substrate and as a result, the pH increases. Later the concentration of CO_3^{2-} increases and, at the same time, anionic group of urease producing bacteria attracts Ca^{2+} in the surrounding environment and increases the concentration of CO_3^{2-} in a certain part of the crystal. The more amount of Ca^{2+} get attracted until the concentration of the crystal precursors is high enough to nucleate, and the granular $CaCO_3$ finally gets precipitated (Dick et al. 2006; Qian et al. 2010b).

Jenneman et al. (1984) have shown that nutrients necessary for growth and metabolism can be transported through sandstone cores. Shaw et al. (1985) found that bacteria produce an exopolysaccharide film, which contributes to pore plugging by suspending clay particles, cells, and other suspended solids. The decrease in permeability in sand column by MICCP is believed to be the result of the *in situ* growth and metabolism of microorganisms, rather than abiotic factors (Raiders et al. 1989). Kantzas et al. (1992) reported that sand consolidation by *B. pasteurii* reduced porosity by up to 50% and permeability by up to 90% in the areas where the cementation took place. Qian et al. (2010b) reported compressive strength up to 2 MPa (Mega Pascal) in sand column of size 32.10 mm × 18.40 mm, while Achal et al. (2009b) reported a maximum of 40% calcite deposition in the upper layer of sand column consolidated by a mutant of *Sporosarcina pasteurii*.

The results of sand consolidation based on MICCP attracted many researchers to think beyond this building material. Microbiological sand plugging suggests that calcite precipitation induced by bacteria can be successfully employed on building materials to improve the durability of such structures (Fig. 24.2).

Table 24.2 An overview of the applications based on MICCP in which microbial concrete has been used to improve durability of building materials

Target	Microorganism used	Advantage	References
Mortar	*Sporosarcina pasteurii*, *Bacillus* sp., *B. halodurans*, *B. cohnii*, *B. pseudofirmus*, *Shewanella* sp.	Improvement in the compressive strength	Ghosh et al. (2005), Jonkers and Schlangen (2008), and Achal et al. (2009a, 2010)
Concrete	*Bacillus* sp., *B. sphaericus*, *B. pseudofirmus*	Reduction in permeation properties	De Belie and De Muynck (2009) and Jonkers et al. (2010)
	B. sphaericus, *S. pasteurii*	Remediation of cracks in concrete	Ramachandran et al. (2001) and De Belie and De Muynck (2009)
	B. sphaericus, *S. pasteurii*	Surface protection of concrete	De Muynck et al. (2008) and Qian et al. (2009)
Stone and granite	*S. pasteurii*, *Micrococcus* sp., *Bacillus subtilis*, *Myxococcus xanthus*	Remediation of cracks in granites, protection of stones and monument repair	Gollapudi et al. (1995), Zhong and Islam (1995), Tiano et al. (1999), Rodriguez-Navarro et al. (2003), and Dick et al. (2006)

The durability of any building structure depends on its permeation properties and strength. Moreover, microbially induced calcite precipitation has been investigated for its potential to improve the durability of construction materials by many research groups (Achal et al. 2010, 2011; De Muynck et al. 2010 and references therein).

The first step in selection of repair techniques and products must always be a thorough investigation to understand the nature of the problem; otherwise a repair exercise may prove to be completely fruitless. We need to look into some other repair techniques to improve the durability of building materials that also should be environmentally friendly. However, a promising sustainable repair methodology is currently being investigated and developed in several laboratories, *i.e.* a technique based on the application of mineral-producing bacteria. An overview of different applications of microbial concrete of building materials or structures has been presented in Table 24.2. In next section, the role of MICCP towards enhancement in compressive strength and reduction in permeation properties has been discussed.

24.3.3 Compressive Strength Based on MICCP

The compressive strength of the concrete is one of the most important technical properties. Compressive strength is considered as an index to assess the overall quality of concrete and it is generally assumed that an improvement in the compressive strength results in improvement of all other properties. Hence, strength investigations are generally centered on compressive strengths. Concrete mixes are proportioned on the basis of achieving the desired compressive strength at the specified age. The compressive strength of concrete or mortar is usually determined by submitting a specimen of constant cross section to a uniformly distributed increasing axial compression load in a suitable testing machine until failure occurs. The strength is expressed as the ultimate compression load per cross sectional area, usually in psi, Pa or Kg/cm^2. Compressive strength test results are used to determine that the concrete mixture as delivered meets the requirements of the specified strength in the job specification. This result is used for quality control and acceptance of concrete. By keeping these points in mind, it is very important to study the effect of microbes on the compressive strength of mortar and concrete. In general, a mortar refers to a workable paste consisting of cement, sand and water to bind building materials together and to fill the gaps between them. In particular, a biological mortar refers to a mixture of bacteria along with a nutritional medium containing a calcium salt in mortar. The term biological refers to the microbial origin of the binder, *i.e.* microbiologically produced calcium carbonate. In contrast to mortar, concrete contains coarse aggregate as an extra material.

Several studies have shown that MICCP can be used to improve the compressive strength of mortar (Bang et al. 2001; Ghosh et al. 2005; Achal et al. 2009a, b, 2011; Park et al. 2010) where microorganisms have also been applied in the concrete mixture. According to Ramachandran et al. (2001), the use of bacteria in concrete remediation was considered unorthodox. MICCP is highly desirable because the calcite precipitation induced as a result of microbial activities is pollution-free and natural. Biomineralization has unique features and functions that have encouraged innovative high-performance composite applications in construction and in other new materials. The application of microorganisms that lead to biomineralization in concrete is a potential field for further research (Park et al. 2010). Ramachandran et al. (2001) investigated the use of microbiologically induced mineral precipitation for the improvement of the compressive strength of Portland cement mortar cubes at the age of 7 and 28 days. They found that inclusion of microbial biomass enhanced the compressive strength of cement mortar cubes. They used live and killed cells of different concentrations of *Bacillus pasteurii* and found that the live cells, at lower concentrations, increase the compressive strength of cement mortar with a longer incubation period. The overall increase of strength, therefore, resulted from the presence of an adequate amount of organic substances in the matrix due to the microbial biomass.

To achieve greatest improvement in the compressive strength, cell concentrations/microbial doses needs to be optimized. Ghosh et al. (2005) demonstrated the

positive effect of the addition of *Shewanella* on the compressive strength of mortar specimens. They found that the strength of mortar cubes increased at all levels of anaerobic microbe addition. For these samples, the presence of a fibrous material inside the pores could be noticed. As a result, a modification of the pore size distribution was observed. The positive effect of the addition of *Shewanella* improved with increasing curing times. The greatest improvement in compressive strength was reported to occur at cell concentrations of 10^5 cells/ml for all ages (3, 7, 14 and 28 days). For a concentration of 10^5 cells/ml, an increase of the compressive strength of 17% and 25% was observed after 7 and 28 days, respectively. However, no increase of the compressive strength was observed with additions of *Escherichia coli* (non-urease producing microbe) to the mortar mixture. This led the authors to suggest that the choice of the microorganism plays an important role in the improvement of the compressive strength. Jonkers and Schlangen (2007) investigated that the addition of a high number of bacterial spores ($10^8/cm^3$) by spore forming bacteria (*Bacillus pseudofirmus* and *Bacillus cohnii*) resulted in 10% increase of strength.

Park et al. (2010) used four different species of calcite forming bacteria (*Sporosarcina soli*, *Bacillus massiliensis*, *Arthrobacter crystallopoietes* and *Lysinibacillus fusiformis*) for compressive strength improvement in mortar. The 28-day strength test for consolidated mortar showed that the cube treated with *Arthrobacter crystallopoietes* had the greatest improvement in compressive strength, and the observed change in compressive strength from 7 to 28 days was 22%.

Achal et al. (2009a) showed that nutrients to grow bacteria also play an important role when used with construction materials. To economize the overall process, they replaced standard nutrient with some industrial by products such as lactose mother liquor (LML) and corn steep liquor (CSL). They used *Sporosarcina pasteurii* in mortar cubes with LML medium and found 17% improvement in compressive strength at 28 days (26.3 MPa) compared to control (23.2 MPa). There was no significant difference when LML medium was replaced by standard nutrient medium. Further, medium containing CSL resulted in significantly higher compressive strength even when compared with commercially available medium. All the reports suggest significant increase in the compressive strength at the age of 28 days compared to early ages (3- and 7-days). The overall trend of an increase in compressive strength up to 28 days might be attributed to the behavior of microbial cells within the cement mortar matrix. During the initial curing period, microbial cells obtained good nourishment, because the cement mortar was still porous; but growth might not be proper due to the completely new environment for microbes (Achal et al. 2011). It may also be possible that as the pH of the cement remained high, cells were in inactive condition and as curing period was increased, it started growing slowly. Upon cell growth, calcite would have precipitated on the cell surface as well as within the cement mortar matrix. Once many of the pores in the matrix were plugged, the flow of the nutrients and oxygen to the bacterial cells stopped, eventually the cells either died or turned into endospores and acted as an organic fiber, that may enhance the compressive strength of the mortar cubes (Ramachandran et al. 2001).

Despite the importance of urease producing bacteria in remediation of cracks and fissures towards enhancement in the durability of building structures, very few bacteria have been exploited. Microorganisms inhabit all possible locations including extremes and exhibit growth and reproduction in such environments. There is a need to explore extreme alkaline environments to isolate indigenous bacteria that can survive in concrete structures for effective biocalcification. Recently Achal et al. (2011) isolated bacteria from cement, *Bacillus* sp. CT-5 and used to study compressive strength. They reported 36% increase in compressive strength of cement mortar with the addition of bacterial cells. Further, it has suggested that due to the ability to tolerate high pH, *Bacillus* sp. CT-5 enhanced the compressive strength of cement mortar cubes significantly.

To determine whether the increase in compressive strength of the specimens prepared with bacteria could be attributed to the microbial calcite precipitation, MICCP was quantified by X-ray diffraction (XRD) analysis and visualized by SEM (De Muynck et al. 2008; Achal et al. 2009a, 2011). The results indicated the presence of a newly formed layer on the surface of the mortar specimens, consisting mainly of calcite. The sample showed calcite crystals grown all over and precipitated with rod shaped structures (typical shape of *Bacillus* species). From these findings, it can be concluded that compressive strength of cement mortar increases with an addition of urease producing microbes such as *Bacillus*, *Shewanella* and *Arthrobacter* species. This improvement in compressive strength might be due to deposition on the microorganism cell surfaces and within the pores of cement–sand matrix, which plug the pores within the mortar as a result of MICCP (Ramakrishnan et al. 1998).

24.3.4 Microbiologically Enhanced Crack Remediation (MECR)

Use of microbial products as a long-term remediation tool has exhibited high potential for crack cementation of various structural formations such as granite and concrete (Gollapudi et al. 1995; Stocks-Fischer et al. 1999). The participation of *Bacillus pasteurii* in sand consolidation has been demonstrated by Kantzas et al. (1992). Gollapudi et al. (1995) further investigated the use of *B. pasteurii* for the plugging of sand columns. Although the bacteria were mixed with the sand slurry, consolidation mainly occurred near the surface. Stocks-Fischer et al. (1999) showed that microorganisms directly participated in the calcite precipitation by providing a nucleation site and by creating an alkaline environment which favoured the precipitation of calcite. Zhong and Islam (1995) used the consolidation of sand mixtures for the remediation of cracks in granite. Cracks in granite were packed with a mixture of bacteria, nutrients and a filler material. Among the different materials that were mixed with *B. pasteurii*, the silica fume (10%) and sand (90%) mixture lead to the highest compressive strength and lowest permeability.

A novel approach of microbiologically-enhanced crack remediation has been reported by Bang and Ramakrishnan (2001). They used *Bacillus pasteurii* to induce

$CaCO_3$ precipitation. Scanning Electron Micrography (SEM) and X-Ray Diffraction (XRD) analysis has shown the direct involvement of microorganisms in calcium carbonate precipitation. As a further extension to this research, Ramachandran et al. (2001) investigated the microbiological remediation of cracks in concrete. The authors proposed MICCP as an effective way to seal cracks. The appearance of cracks and fissures is an inevitable phenomenon during the ageing process of concrete structures upon exposure to weather changes. If left untreated, cracks tend to expand further and eventually lead to costly repair. Specimens with cracks filled with bacteria, nutrients and sand demonstrated a significant increase in compressive strength and stiffness values when compared with those without cells. The presence of calcite was, however, limited to the surface areas of the crack. The authors attributed this to the fact that *B. pasteurii* grows more actively in the presence of oxygen. Still, the highly alkaline pH (12–13) of concrete was a major hindering factor to the growth of the moderate alkaliphile *B. pasteurii*, whose growth optimum is around a pH of 9. To retain high metabolic activities of bacterial cells at such a high pH, immobilization technology (where microbial cells are encapsulated in polymers) can be applied. Polyurethanes have been widely used as a vehicle for immobilization of enzymes and whole cells because of its mechanically strong and biochemically inert characteristics (Klein and Kluge 1981; Wang and Ruchenstein 1993). Bang et al. (2001) found that physicochemically versatile polyurethane is an effective enhancement tool in microbiologically induced calcite precipitation in concrete cracks. In order to protect the cells from high pH, Day et al. (2003) investigated the effect of different filler materials on the effectiveness of the crack remediation. Beams treated with bacteria and polyurethane showed a higher improvement in stiffness compared to filler materials such as lime, silica, fly ash and sand. According to the authors, the porous nature of the polyurethane minimizes transfer limitations to substrates and supports the growth of bacteria more efficiently than other filling materials, enabling an accumulation of calcite in deeper areas of the crack. No differences could be observed between the overall performances of free or polyurethane immobilized cells in the precipitation of carbonate (Bang et al. 2001). In addition to this work, Bachmeir et al. (2002) investigated the precipitation of calcium carbonate with the urease enzyme immobilized on polyurethane. As an extension to their research on biodeposition on cementitious materials, De Belie and De Muynck (2008) further investigated the use of microbiologically induced carbonate precipitation for the repair of cracks in concrete by using *B. sphaericus*. Ramachandran et al. (2001) studied the effect of microbiological calcite precipitation of cracks of various depths on the compressive strength values in Portland cement mortar cubes and found an increase of the strength in the presence of *B. pasteurii* in the cubes prepared with the deepest cracks (25.4 mm), whose microbial remediation increased the compressive strength by approximately 61% of that of the control concrete.

Recently Qian et al. (2010a) reported that the compressive strength of the treated specimens could be restored to 84%, and demonstrated that this bio-restoration method is effective in repairing surface defects of cementitious materials. Sand particle surfaces were covered by $CaCO_3$ precipitation induced by bacteria which

could cement loose sand and supply conjunction between individual particles. SEM results indicated that $CaCO_3$ crystals were precipitated on crack surface, where sand was consolidated and cemented by $CaCO_3$ crystals resulting in compressive strength recovery.

24.3.5 Permeability Based on MICCP

Research has indicated that a concrete that is low in permeation properties lasts longer without exhibiting signs of distress and deterioration (Nolan et al. 1995). Therefore, the permeation properties have been used principally for the comparison of the effectiveness of different surface treatments enhancing the durability of concrete. Permeation controls the ingress of moisture, ionic and gaseous species into concrete. As the permeation of concrete decreases, its durability performance, in terms of physicochemical degradation, increases. Carbonation is a chemical reaction between carbon dioxide, in the presence of moisture, and calcium hydroxide present in hydrated concrete to form calcium carbonate. Carbonation occurs at the concrete surface including the surfaces of any cracks throughout the life of the concrete.

There are some conventional techniques available to improve impermeability to enhance the durability of building materials. Chemical admixtures such as plasticizers, superplasticisers and water reducing agents help to improve the workability by reducing the intergranular friction ultimately affecting the porosity and distribution of pores. However, these conventional methods of protection have a number of disadvantages, such as (i) an incompatibility of the protective layer and the underlying layer due to differences in their thermal expansion coefficient; (ii) disintegration of the protective layer over time; and (iii) a need for constant maintenance by a treatment that is reversible and repeatable. Such chemical treatments commonly result in the formation of incompatible and often harmful surface films. Additionally, because large quantities of chemical solvents are used, they contribute to pollution (Camaiti et al. 1988; Rodriguez-Navarro et al. 2003) and also such techniques are not long lasting.

The shortcomings of conventional techniques have drawn the attention to alternative treatments to reduce permeation properties for the improvement of the durability of concrete. Many scientists studied the effect of biomineralization in the form of calcium carbonate deposition in sandstone and limestone to reduce the permeation properties. Tiano et al. (1992) and Tiano (1995) proposed the use of organic matrix macromolecules extracted from *Mytilus californianus* shells to induce the precipitation of calcium carbonate within the pores of the stone. The organic matrix was shown to produce a more relevant and durable carbonate precipitation compared to the single use of calcium chloride or hydroxide. This precipitation resulted in a slight decrease in porosity and water absorption by capillarity. Tiano et al. (1999) also observed a reduction of about 60% from the limestone samples treated with biodeposition. Le Metayer-Levrel et al. (1999) studied the bacterial carbonatogenesis for the protection and regeneration of limestone in buildings, monuments

and statues. Their study confirmed the deposition of protective surface due to biocalcification on the stone surface which reduced its permeability for gas without affecting its aesthetic appearance. They further state that biomineralization recreates a material that is remarkably similar to the limestone substrate. It uses natural microbial mediation which follows the same natural process that formed many limestones. Finally, they concluded that biological mortars or cement could be used to affix small pieces broken from statues and to fill small cavities on limestone surfaces. Nemati and Voordouw (2003) noticed a decrease of the permeability of sandstone cores after injecting $CaCO_3$ forming reactants. Dick et al. (2006) studied such deposition on degraded limestone by *Bacillus* species. They concluded that such deposition reduces the water absorption rate of limestone. They studied the capillary water absorption and absorption under vacuum. A decrease in the ability to absorb water will result in a deceleration of the weathering process.

The positive results of MICCP on the sandstone and limestone attracted many researchers to apply this technique on mortar and concrete to reduce permeability. Microbial carbonate precipitation (biodeposition) has also been reported to decrease the permeation properties of mortar and concrete. De Muynck et al. (2007) investigated the effects of microbial biomass on the permeability of concrete and mortar. To determine the increase in resistance towards water penetration, they carried out a sorptivity test. They coated the mortar specimens with bacterial biomass (*Bacillus sphaericus*), oven dried then dipped into 10 ± 1 mm of water. They found that the presence of only bacteria resulted in a significant decrease of the water uptake compared to untreated specimens (a reduction of 45%, 43% and 24% with increasing w/c). When a calcium source was added to the medium an additional significant decrease of the water absorption coefficient was noticed. They also studied the durability of the treated surface by measuring the resistance to carbonation and chloride ingress, as chloride ions are one of the corrosion causing agent. They obtained a 19% decrease of the chloride migration coefficient, by the addition of bacterial biomass, compared to untreated cubes. The addition of bacterial biomass resulted in a significant smaller carbonation rate compared to untreated cubes. The deposition of a layer of calcium carbonate induced by *Bacillus sphaericus* on the surface of the mortar specimens resulted in a decrease of water absorption and gas permeability (De Muynck et al. 2008). According to the authors, the biodeposition treatment on cementitious materials should be regarded as a coating system. This could be attributed to the fact that the carbonate precipitation was mainly a surface phenomenon due to the limited penetration of the bacteria in the porous matrix.

Ramakrishnan et al. (1998) investigated the effect of this technique on the durability of concrete. The presence of bacteria was observed to increase the resistance of concrete towards alkali, sulfate, freeze thaw attack and drying shrinkage; the effect being more pronounced with increasing concentrations of bacterial cells. The authors attributed this to the presence of a calcite layer on the surface, as confirmed by XRD analysis, lowering the permeability of the specimens. Recently Achal et al. (2011) reported that the mortar cubes treated with *Bacillus* sp. CT-5 over a period of 168 h absorbed nearly six times less water than the control cubes. The deposition of a layer of calcium carbonate crystals on the surface resulted in a decrease of the

permeation properties. As a consequence, the ingress of harmful substances may be limited. From these reports, it is clear that the presence of a layer of carbonate crystals on the surface by bacterial isolate has the potential to improve the resistance of cementitious materials towards degradation processes.

24.3.6 Corrosion of Reinforcement and MICCP

Corrosion of steel and rebar in concrete structures is one of the most frequent reasons for civil infrastructure failure. Corrosion and permeation properties are some how correlated. The transport mechanisms normally associated with reinforced concrete deterioration are: absorption, diffusion and permeation. Any combination of these mechanisms may act to transport aggressive species through the concrete. In experimental methods, it is common to limit the flow to one single mechanism, which can be used to derive a transport parameter for the concrete that is being tested. Ingress of chlorides occurs due to the permeation of water through concrete in some form. It was widely recognized that the concrete cover provides protection to the reinforcement against both carbonation and chloride ingress and these are related to different transport characteristics of the concrete. Therefore, in addition to monitoring the advance of the chloride front and the carbonation front, a measure of the transport properties of the concrete cover is important for assessing the durability of reinforced concrete. The permeability of water and pollutants is the major threat to the reinforced concrete sections. Such penetration leads to the ingress of moisture and chlorides which is responsible for early leakages and corrosion of the embedded steel. The corrosion products volume is greater than the uncorroded steel. Hence, after corrosion, it exerts pressure on surrounding concrete leading to cracking of the section. Corrosion initiates due to the ingress of moisture, chloride ions and carbon dioxide through the concrete to the steel surface. After initiation, the corrosion products (iron oxides and hydroxides) develop expansive stresses that crack and spall the concrete cover. This in turn exposes the reinforcement to direct environmental attack that results in accelerated deterioration of the structure (Neville 1995). Thus, by sealing the paths of ingress, the life of the reinforced concrete structures can be improved dramatically. If microbial deposition is able to seal the pores in the cover portion of the concrete in top few millimeters, then the life of the reinforced concrete sections will improve substantially.

Corrosion requires air and water to take place, so the key to durability in concrete design is to have a mix which ensures that the reinforcement is always well protected from these elements. It is likely that the presence of a cementitious cover by microbial induced calcite precipitation would affect the initiation of corrosion and the subsequent low corrosion rate but no detail research of this type has been published. Recently Mukherjee et al. (2010a) studied the role of MICCP in reducing the corrosion rate of reinforcement using the bacteria *Bacillus* sp. CT-5. The authors showed that MICCP resulted in around fourfold reduction in corrosion of reinforced concrete specimens. Microbially induced calcite precipitation also resulted in enhancement of pullout strength and reduction in mass loss of the reinforcing bar. Further Mukherjee

et al. (2010b) reported a search for a low energy binder in concrete using calcite producing strain of *Sporosarcina pasteurii*. In their work, the efficacy of the proposed method in reducing water and chloride ion permeability has been established. Initial demonstration of the corrosion protection offered by the bacterial concrete has also been discussed. They concluded that the reinforced concrete samples exhibited reduction in mass loss of the reinforcing bars and increase in their pull-out strength based on MICCP. Their current work demonstrated that production of biocalcification by *S. pasteurii* can at least partially replace the industrial binders and provide a more sustainable alternative. Qian et al. (2010a) have shown that MICCP on cement specimens induced by *B. pasteurii* can greatly improve their surface permeability resistance and resist the attack of the acid (pH > 1.5). They concluded that MICCP has the potential application in surface protection of building materials, for example spraying or brushing the mixed solution including bacteria, urea and Ca^{2+} to the surface of building materials to generate $CaCO_3$ and can help in corrosion protection of building materials and structures. Furthermore, microbially induced calcite precipitation could also be used in restoration of micro-cracks in concrete, slope maintenance, desert solidification and so on.

24.4 Future Perspectives

The promising results on the use of microorganisms for the improvement of the durability of building materials have drawn the attention of research groups all over the world but until now, work on such bioremediation was mainly confined in some countries. A lot of work needs be done before the technology can be implemented. The temperatures, humidity, type of concrete, control of various parameters such as type of mix, concentration of bacteria vary considerably from place to place and country to country, hence a consolidated recommendation of any one's result can not be arrived. Apart from these, survival of microorganisms isolated from other parts of the world under different environmental conditions also needs to be considered. No published work to date has described a sufficient degree towards enhancement in the durability of building structures using microbes by their MICCP process. Hence, lot of research is necessary before such technology is ready for field applications. Moreover, long term effect of such treatment is not yet reported.

However, the important parameter such as permeation properties was not dealt with. As calcite deposition fills the pores, the extent of permeability reduction must be studied in detail. The carbonation of structures in urban and coastal regions of any country is generally very high. Carbonated concrete looses protective power of steel. Therefore it accelerates steel corrosion and related problems. Hence, the efficiency of calcite deposition in resisting carbonation and reduction in permeability to improve the corrosion resistance must be studied in detail. Most of the studies based on MICCP were carried out to evaluate compressive strength, water absorption and crack remediation of mortars and concretes. The role of MICCP in reducing the corrosion rate of reinforced concretes has not been studied in detail and needed to look further.

It is clear that the work done by several research groups, focusing on different materials, can only improve our understanding on the possibilities and limitations of biotechnological applications on building materials (De Muynck et al. 2010). However, the challenge for the immediate future is to apply some of the promising results obtained in the field of bioremediation of building materials into practical applications. Future investigations should also focus on different types of nutrients and metabolic products used to grow calcifying microorganisms, as they have an influence on the survival, growth and biofilm formation of the microorganisms inside the building materials. The research is still in its infancy, and it will be largely questionable whether bacteria will be able to remain viable for a prolonged time and upon activation be able to repair the cracks in building structures. Further, studies should be carried out to find some economical nutrient sources from industrial by-products to make overall process green and economic.

24.5 Conclusion

The goal of this chapter is to provide an in-depth overview of the different methodologies used to enhance the durability of building materials and structures. An overview has been given on the application of microbially induced calcium carbonate precipitation in the field of construction industry. The introduction of MICCP offers higher quality concretes with adequate impermeability, compressive strength and reduced reinforced corrosion. This study has conclusively established that high strength economical cementation can be achieved using MICCP via urea hydrolysis to "proof-of-concept" stage. The bioremediation technology has many advantages of over existing conventional technologies, including self-healing ability and *in-situ* application. Cementation can be readily achieved without any additional processing of the bacterial culture liquid (*e.g.* concentration, lysis, removal of medium), by directly mixing the culture with the cementation components and direct application into the construction material. The development of the 'Microbial Concrete' will provide the basis for an alternative and high quality concrete sealant that is cost effective and environmentally safe and ultimately lead to enhancement in the durability of building materials and structures.

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Chapter 25
Microbial Degradation of Cyanides and Nitriles

Tek Chand Bhalla, Nikhil Sharma, and Ravi Kant Bhatia

Abstract Cyanide and nitriles are produced by a wide range of microorganisms and plants as part of their normal metabolism. These are ubiquitous at low levels in soil and water including surface and ground water. Cyanide is used in metallurgical operations in mining of gold and chemical synthesis and nitriles are also extensively used in organic synthesis and as agrochemicals. The sources of cyanide and nitrile contamination of environment include emissions from iron and steel production, coal combustion, petroleum refineries, solid waste incinerators, combustion of nitriles, use of agrochemicals, chemical industries, vehicle exhausts and cigarette smoke. The industrial and anthropological activities have resulted in contamination of soil, water and air with toxic levels of nitriles and cyanide in the environment. In some situations nitrile and cyanide pollution becomes a threat to animal and human beings. A large number of microorganisms have been reported to the degrade nitriles and cyanide to corresponding non-toxic acids or amides. Some microbes have cyanide hydratase or dihydratase enzymes to convert cyanide to formamide or formic acid while others are endowed with nitrilase, nitrile hydratase and amidase systems which transform nitriles to acids and amides. In this chapter we will discuss the sources and extent of cyanide and nitrile contamination of soil, water and air and the potential application of nitrile or cyanide metabolizing organisms in the bioremediation of contaminated habitats will be discussed.

Keywords Cyanide • Nitrile • Environmental contamination • Nitrile degradation • Cyanide degradation • Bioremediation

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25.1 Introduction

The compounds containing cyano moiety (cyanides and nitriles) are widely distributed in the environment. The nitriles are found in many higher plants, bone oils, insects and microorganisms such as fungi, bacteria, algae and sponges. Nitriles are converted to the corresponding carboxylic acids by a variety of chemical processes, but these processes typically require strongly acidic or basic reaction conditions and high reaction temperatures which produces unwanted byproducts. Cyanides on the other hand are the inorganic compounds that contain a cyanide group, as they are present in the environment in several forms including; hydrogen cyanide; simple inorganic salts including NaCN and KCN; complex metal cyanides, thiocyanates, and as nitriles where the cyanide is the functional group to an organic compound (Kjeldsen 1999). The toxicity of cyanides depend upon the type and form of cyanides some of which are extremely toxic e.g., hydrogen cyanide and potassium cyanide, while nitriles vary in toxicity according to the degree of cyanide liberation by these compounds (Figueira et al. 1995). This indeed leads to compounds containing cyano moiety that pollute the environment which directly or indirectly affects soil, sediments, water, air, microbial organisms, plants including animals, and human health.

25.2 Distribution of Cyanides and Nitriles

Cyanides and nitriles are widely distributed in the environment and are thought to be in the environment throughout the evolution of life. Cyanides are present in the environment as hydrogen cyanide, simple inorganic salts including NaCN and KCN complex metal cyanides and thiocyanates (Kjeldsen 1999). About 84% of the HCN produced in industry is used to produce organic cyanides, also known as nitriles, the common ones including acrylonitriles, methyl methacrylonitirle and adiponitrile (Towill et al. 1978). Thiocyanates present in water and soil are discharged from coal processing, extraction of gold and silver and mining industries into soil, air or water. Application of cyano group containing herbicides (weed killers), insecticides, rodenticides and disposal of industrial effluents onto the soil result in land pollution with nitriles and cyanides. Cyanides/nitriles are also released from damaged or decaying tissues of certain plants such as mustard and cabbage. The fate of nitriles in the environment is largely unknown and the majority of the data is based upon predicted behaviour of these compounds in soil, water and air. It has been suggested that acrylonitrile, adiponitrile, acetonitrile and 3-cyanopyridine are likely to be degraded in the atmosphere by reaction with photochemically produced hydroxyl radicals (HSDB 2004).

Table 25.1 General categorization of cyanides (Dash et al. 2009)

Types of cyanide		Examples	Remarks
Free		HCN^-,CN^-	Equilibrium depends on pH, (pKa 9.2 at 25°C)
Simple	Soluble	NaCN, KCN, $Ca(CN)_2$	Ionize in aqueous solution at low concentration and mostly present as HCN below pH 8
	Insoluble	$Zn(CN)_2$, $Cd(CN)_2$, AgCN	
Complex	Weak	$K_2Zn(CN)_4$,$K_2Cd(CN)_4$	Ionize easily
	Moderately strong	$K_2Cu(CN)_3$,$K_2Ni(CN)_4$	Ionize moderately
	Strong	$K_2Fe(CN)_6$,$K_3Co(CN)_6$	Don't ionize easily, very stable
Inorganic		SCN^-,CNO^-, HNCO	Cyanate unstable
Organic (Nitriles)	Aliphatic	Acetonitrile, acylonitrile Adiponitrile, propionitrile	Stable
	Aromatic	Benzonitrile	Stable

25.2.1 Various Forms of Cyanide Present in the Environment:

According to Dash et al. (2009) cyanides are divided into five categories (Table 25.1):

Free cyanide: Free cyanide refers to molecular HCN and cyanide anion (CN^-) (usually in aqueous media) irrespective of their origin.

Simple cyanide: Simple cyanides are the compound that dissociates directly into the cyanide ion (CN^-) and a cation (e.g. H^+, Na^+, K^+, Ca^{++}) with no intermediates e.g. NaCN, KCN, $Ca(CN)_2$.

Complex cyanide: Complex cyanides are the compound in which the cyanide anion is incorporated into a molecular inorganic or organic complex or complexes e.g. ferric-ferrocyanides.

Inorganic cyanides: Inorganic cyanides also called as cyanates with the chemical formula written as [OCN]– or [NCO]–. In aqueous solution it acts as a base, forming isocyanic acid, HNCO. Inorganic cyanides form complexes with different metal ions in which nitogen/oxygen atom are electron donor e.g. SCN^-, CNO^- and HNCO.

Organic cyanides: Organic cyanides, called nitriles, are important industrial chemicals used in synthesis and to form polymers such as polyacrylonitrile ("Orlon"). Cyanogenic glycosides in food (cassava, bitter almonds, lima beans) and in laetrile (an anti-cancer agent or Vitamin B_{12}) are examples of this form of cyanide.

Table 25.2 List of common nitriles

Aliphatic nitriles		Aromatic and heterocyclic nitriles
Saturated mononitriles	***Unsaturated nitriles***	Benzonitrile
Acetonitrile	Acrylonitrile	4-Hydroxybenzonitrile
Propionitrile	Methacrylonitrile	2-Aminobenzonitrile
n-Butyronitrile	Allylcyanide	2-Chlorobenzonitrile
Valeronitrile	Crotonitrile	3-Flurobenzonitrile
Isovaleronitrile	Fumaronitrile	2-Nitrobenzonitrile
Capronitrile	Muconononitrile	Cinnamonitrile
Pelagronitrile		Mandelonitrile
Saturated dinitriles		Cyanopyrazine
Malononitrile		Phenylacetonitrile
Succinonitrile		Cyanopyrazine
Glutaronitrile		
Adiponitrile		

25.2.2 Various Forms of Nitriles Present in the Environment

Nitrile compounds are the products, byproducts or waste products of agriculture, pharmaceutical and chemical industries (Martínková et al. 2009). The enzymatic breakdown of nitriles to simpler and non toxic form plays an increasingly important role in organic synthesis and environment remediation, due to the mild reaction conditions, high activities and the high selectivities of the enzymes (Banerjee et al. 2002; Mylerová and Martínková 2003).Various forms of nitriles which occur have been divided into three general categories (Table 25.2).

Aliphatic nitriles: Aliphatic nitriles contain both saturated mono and di-nitriles as well as unsaturated nitriles. e.g. includes acetonitrile (saturated), malanonitrile, acrylonitrile(unsaturated), succinonitrile (di-nitrile), etc.

Aromatic nitriles: Aromatic form of organonitriles which contains benzene ring e.g. benzonitriles, mandelonitriles, phenylacetonitrile, etc.

Heterocyclic nitriles: Common nitriles of this category are 2-cyanopyridine, 3-cyanopyridine, 4-cyanopyridine, etc.

25.2.3 Sources of Cyanide and Nitriles

25.2.3.1 Soil

The contamination of soil with cyanide is usually in the form of iron cyanide complexes $[Fe(CN)_6]^{3-}$ and $[Fe(CN)_6]^{4-}$ is generally found at several types of industrial sites. The hazards for human health or the environment posed by such sites are mostly linked with the chemical behaviour and mobility of complexed cyanide in soil. This behaviour is probably dominated by equilibrium with prussian

blue, $[Fe_4(Fe(CN)_6)]_3$ which is sparingly soluble under acidic conditions and limits dissolved cyanide concentrations and mobility in acidic soil. However, at pH higher than 7 the solubility of $[Fe_4(Fe(CN)_6)]_3$ is very high, that makes it to be more mobile under such conditions. Cyanide is also released into the soil by the cyanide containing road salts or the disposal of cyanide waste by landfills. On other hand nitriles are released into the environment by agricultural discharges in form of nitrile herbicides and pesticides such as bromoxynil and chlorothalonil (2,4,5,6-tetrachloroisopthalonitrile).

Cyanides generally occur as hydrogen cyanide, alkali metal salts, or immobile metallocyanide complexes in the subsurface of the soil. At low concentrations, these are generally biodegradable, however in soil with pH< 9.2, compounds such as hydrogen cyanide are toxic to microorganisms. From the landfills or spills of cyanides, hydrogen cyanide leaches into groundwater. It is highly toxic for most living organisms as it forms stable complexes with transition metals that are essentially required for the protein functioning, e.g. iron in cytochrome oxidase (Luque-Almagro et al. 2005). Therefore, organisms growing in the presence of cyanide need to have a cyanide-insensitive metabolism such as an alternative oxidase in plants (Berthold et al. 2000) or the cytochrome bd (or cyanide insensitive oxidase) in bacteria (Jünemann 1997; Richardson 2000).

25.2.3.2 Water

In water, cyanides occurs in the forms of hydrocyanic acid, simple cyanides and easily degradable complex (e.g. zinc cyanide), and other poorly decomposable complexes of cyanides (e.g. iron and cobalt cyanides). The cyanide complexes of nickel and copper cyanides are intermediate between the easily degradable compounds. Generally cyanide has low persistence in surface waters but may persist for longer period of time in underground water. Volatilization is an easy method for the removal of free cyanide from concentrated solutions and is very effective under higher concentration of dissolved oxygen, high temperatures, and at elevated concentrations of atmospheric carbon dioxide. Loss of simple cyanides from the water column is usually through sedimentation, microbial degradation and volatilization.

Water-soluble cyanide complexes such as ferricyanides and ferrocyanides, do not normally release free cyanide unless subjected to ultraviolet light. Therefore, sunlight may lead to cyanide release in wastes containing iron-cyanide complexes (Ebbs et al. 2003). The ratio of hydrocyanic acid to total cyanide varies frequently due to changes in pH caused by acid wastewater discharges or by photosynthetic and other metabolic cycles of aquatic flora (Irwin et al. 1997). Cyanide is not easily available to higher biological systems in soils because it is either complexed with trace metals, metabolized by various microorganisms, or lost through volatilization. Less is known about the fate of complex cyanides in natural waters, however a number of metallocyanide complex ions are quite stable in aqueous solution unless subjected to ultraviolet and visible light. A photodecomposition of some cyanide complexes with the release of cyanide ions may be observed in the presence of sunlight.

Most of the information on the fate of cyanides in the aquatic environment has been generated from waste mixtures with high concentrations of cyanides. It is, rather difficult and often misleading to apply such observation so far available to the degradation/dissociation of cyanide complexes under natural conditions. From the limited information available under natural conditions, it cannot be concluded that cyanides have a short residence time in the aquatic environment. Few complex metallocyanides have been reported to be degraded in water, generating complex cyanide ions, which may further decompose to release free cyanide ion. It has been noted that, many complex ions are more stable than their parent compounds and hence their subsequent conversion to cyanide ions is relatively low (Hidaka et al. 1992). Cyanide enters air, water, and soil from both natural processes and industrial activities. However, the amount of hydrogen cyanide formed is usually not enough to be harmful to human beings. Some cyanide in water will be converted to less harmful forms by microorganisms and lower forms of plants and animals or will be complexed with metals, such as iron. The half-life of cyanide in water has not been precisely worked out. Nitriles on the other hand are organic compounds whose functional groups are polar.

Nitriles with low molecular weight are liquid and soluble in water but there solubility decreases with increase in their molecular weight. The nitriles are extensively used in organic synthesis and their use has been significantly increased in the last several decades. Herbicides such as bromoxynil (3, 5 dibromo-4-hydroxybenzonitrile) is used as selective herbicide in cereal crops while dichlobenil (2,6 dichlorobenzonitrile) is used as total weed controls in orchard as well as an aquatic herbicide (Harper 1985). Nitriles contaminate the soil due to their accidental release from chemical laboratories or release of untreated nitrile containing effluents from industries into the soil which may be harmful for human beings, and flora and fauna. Common examples of these include acetonitrile, acrylonitrile and benzonitrile that are widely used in laboratories and industries as solvents and extractants, or used as an ingredient in the synthesis of pharmaceuticals, plastics, synthetic rubbers, drug intermediates (chiral synthons), herbicides and pesticides .

25.2.3.3 Air

Cyanide in the air is mostly present as HCN and metallocyanides. Hydrogen cyanide being colourless and pale blue liquid is produced by the oxidation of ammoniamethane mixtures under controlled conditions and by the catalytic decomposition of formamide. Cyanide emissions into the air have been estimated to be 44 million pounds/year based on data obtained during 1970s. More than 90% of these emissions were attributed to releases from automobile exhaust. The second largest source of cyanide emission into the air is from the manufacture of methyl methacrylate, acrylonitrile and hydrogen cyanide (EPA 1981). Hydrogen cyanide gas comes as a by-product from the coke-oven and blast-furnace operations. The particles of HCN eventually are removed by rain and snow from the air settle over land and water. However, gaseous form of hydrogen cyanide is not easily removed from the air by settling rain or snow. The half-life of hydrogen cyanide in the atmosphere is about 1–3 years. Various other forms of cyanide include gaseous, water-soluble potassium

and sodium cyanide salts, and poorly water-soluble mercury, copper, gold, and silver cyanide salts in the air. In addition to this a number of cyanide-containing compounds known as cyanogens tend to release cyanide during metabolic degradation in the living organism Most cyanide in surface water forms hydrogen cyanide that vaporizes into the air. Cyanide is also found in cigarette smoke to which humans are frequently or occasionally exposed. The burning of nitriles or their metabolism following absorption by the skin or gastrointestinal tract release HCN, whereas, cyanogen chloride and cyanogen bromide (gases with potent pulmonary irritant effects), nitriles (R-CN), and sodium nitroprusside, which may cause iatrogenic effect i.e. an adverse condition in patient resulting from treatment by physician or surgeon which leads to cyanide poisoning from prolonged or high-dose of intravenous therapy (>10 mcg/kg/min) (Akyildiz et al. 2010). A number of synthetic (e.g., polyacrylonitrile, polyurethane, polyamide, urea-formaldehyde, melamine) and natural products (e.g., wool, silk) release HCN upon burning .The resulting smoke due to combustion is highly hazardous .The extraction of gold from low-grade ores involve trickling of solution of sodium cyanide over pads of crushed ore, and while doing so small quantities of hydrogen cyanide gas escapes into the air. The combustion or pyrolysis of certain materials under oxygen-deficient conditions may also leads to release HCN into the air. The HCN in the exhaust of internal combustion engines and tobacco smoke also enters into the air. Certain plastics, especially those derived from acrylonitrile, release hydrogen cyanide when these are heated or burnt (Anon 2004).

Most of the cyanide in the atmosphere generally exists as hydrogen cyanide gas, and a small amount of metal cyanides may be present as particulate matter in air. The half-life for the reaction of hydrogen cyanide vapor with hydroxyl radicals in the atmosphere has been observed to be about 334 days. A relatively slow rate of degradation of hydrogen cyanide normally means that this compound has the potential to be transported over long distances before its removal by physical or chemical processes. Since hydrogen cyanide is miscible in water, its wet deposition on the surface may be the normal fate of this cyanide, as metal cyanide particles appear to be removed from air by both wet and dry deposition. Volatilization appears to be an important fate of hydrogen cyanide. At pH< 9.2, most of the free cyanide exists as hydrogen cyanide i.e. a volatile form of cyanide. The rate of volatilization of cyanide is affected by a number of parameters i.e. like temperature, pH, wind speed, and cyanide concentration.

25.3 Cyanide and Nitrile Pollution

Cyanide/nitrile compounds are produced as by-products/wastes in a number of industrial processes. Major sources of cyanide discharges include petrochemical refining, synthesis of organic chemical and plastics, electroplating, aluminum works, gas industry, metal mining and processing industries. Cyanide can be present in environmental matrices and waste streams as simple cyanides (e.g. HCN, CN^-, NaCN), metal cyanide complexes, cyanates and nitriles. The release of cyanide from these industries has been estimated to be more than 14 million kg $year^{-1}$ (Ebbs 2004). The Environmental Protection Agency of U.S. regulates the levels of cyanide that are allowable in drinking water. The highest level of cyanide allowed in drinking

water is 0.2 ppm (parts per million).The Occupational Safety and Health Administration (OSHA) has set a limit of 10 parts per million (10 ppm) for hydrogen cyanide and most cyanide salts in the workplace (ATSDR 2006).

The organic and inorganic cyanide compounds are found in many areas around the world. As predicted these compounds are mostly produced by human activities such as gold and silver mining, steel manufacturing and polymer synthesis. Large amount of cyanide in these industries is used and every year many accidental spillage of cyanide is reported. Main source of cyanides in soil and water is industry. The effluent discharge from some metal mining processes, organic chemical industries, iron and steel plants and publicly owned wastewater treatment facilities is the major cause of cyanide pollution of water. Significant amount of cyanide is released in/by vehicle exhaust, chemical industries, burning of municipal waste, and indiscriminate use of cyanide-containing pesticides. Some cyanide may enter water through runoff rain water from the road where salts containing cyanide are used. Cyanide in landfills is likely to contaminate underground water. Hydrogen cyanide, sodium cyanide, and potassium cyanide are the forms of cyanide that enter the environment from the industrial activities. Cyanide can form complex with Fe, Au, Cd, Co, Cu and Ni, and the cyanide with Fe and Au are highly complexes being the most stable ones. Iron cyanides are the predominant cyanide species in soil and groundwater (Meeussen et al. 1992) and the total cyanide concentrations in contaminated media as high as 4% by weight (Barclay et al. 1998a; Theis et al. 1994) As iron (Fe) is a ubiquitous element in soils and aquifers, and therefore formation of complexes such as ferrocyanide $[Fe(CN)_6^{-4}]$ and ferricyanide $[Fe(CN)_6^{-3}]$ is quite common in these habitats . The strong affinity of cyanide complexes to form complexes with gold has been utilized for decades in ore heap leaching operations to selectively extract gold from low grade ores. This approach creates large volumes of cyanide-laden solutions that need be subsequently treated to remove the cyanide. These metal cyanide complexes show greater resistance to biodegradation than simple cyanides.

Thiocyanates are other group of compounds formed by the combination of sulfur, carbon and nitrogen. Thiocyanates are found in some foods and plants as they are produced from the reaction of free cyanide with sulfur. This reaction occurs in the environment and in the human body after cyanide is swallowed or absorbed. Thiocyanate is the major product formed from cyanide that passes into the body as the body attempts to get rid of cyanide. Thiocyanates present in water are mainly from the effluents of discharges from coal processing, extraction of gold and silver, and mining industries. The direct application of herbicides (weed killers), insecticides, and rodenticides and from disposal of byproducts from industrial processes also lead to the formation of thiocyanates in soil. The damaged or decaying tissues of certain plants, such as mustard, kale and cabbage also add some thiocyanates into soil and water. Although thiocyanates are less harmful than cyanide in humans yet they are known to affect the thyroid glands, which may reduce the synthesis of hormones that are necessary for the normal functioning of the body. Ammonium thiocyanate is used in antibiotic preparations, pesticides, liquid rocket fuels, adhesives, and matches. It is also used in photographic processes, improvement of silk strength and as a weed killer.

25.4 Microbial Metabolism of Cyanide and Nitriles

Nitrile compounds are abundant in the natural environment and are synthesized by plants and microbes as intermediates in chemical biosynthesis and degradation (Legras et al. 1990). The widespread occurrence of nitrile compounds in nature may explain the prevalence of nitrilases in prokaryotes and eukaryotes. Nitrile-metabolizing enzymes are found in a wide variety of bacterial, fungal and plant species. Nitrilases and nitrile hydratases transform both aliphatic and aromatic nitriles to the corresponding acid or amide, respectively, but exhibit less substrate specificity than cyanide hydratase and cyanidase. In bacteria, nitriles may be formed during the detoxification of endogenous and exogenous cyanide. Bacteria produce cyanide in a process termed cyanogenesis, in which HCN and CO_2 are synthesized from glycine by the enzyme HCN synthase. Bacteria that are exposed to actively producing cyanide may protect themselves from cyanide toxicity using cyanide-degrading enzymes, which may have cyanide hydratase, cyanide dihydratase or rhodanase activity. Alternatively, bacteria may convert cyanide into nitrile compounds, such as β-cyano-L-alanine, which can then be used by the bacteria as a carbon and nitrogen source through the activity of a nitrilase, nitrile hydratase and amidase (Yoshikawa et al. 2000; O'Reilly and Turner 2003; Baxter and Cummings 2006).The microbial degradation of many toxic nitriles proceeds through two different enzymatic pathways Nitrile hydratase and amidase catalyzes the hydration of a nitriles to corresponding amides and further to acid and ammonia, whereas nitrilase catalyze the direct hydrolysis of nitriles to corresponding acid and ammonia (Fig. 25.1).

Large part of the absorbed cyanide is converted to thiocyanate ion by sulfurtransferases. Minor metabolic pathways include combination with cystine to form

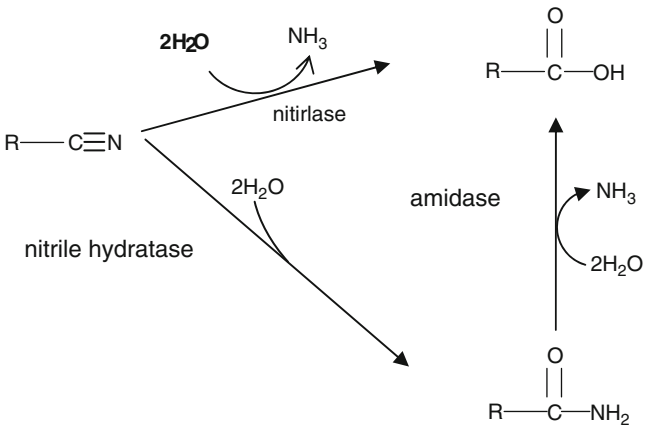


Fig. 25.1 Microbial degradation of nitriles (R = aliphatic, aromatic or aryl group)

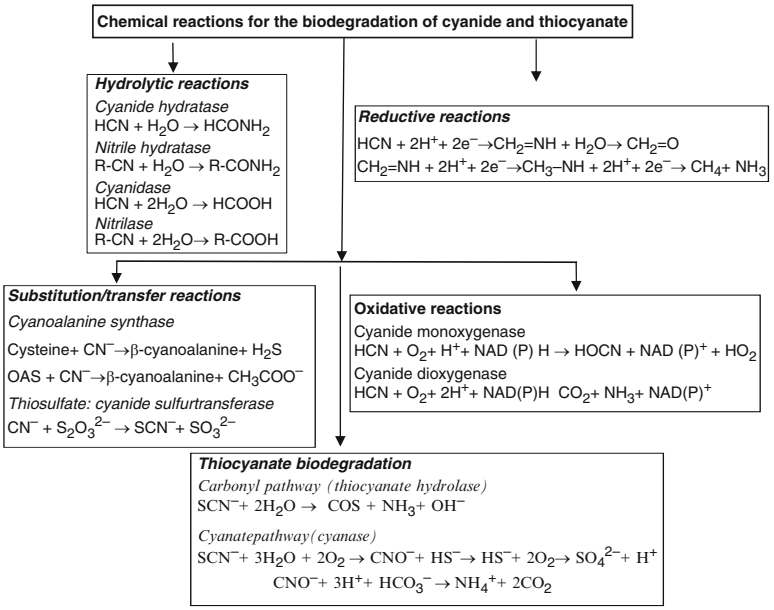
2-imino-thiazolidine-4-carboxylic acid, oxidation to carbon dioxide and formate, to cyanocobalamin in some organism (Goodman and Gilman 1975). The enzymatic decomposition of thiocyanate into cyanide by a thiocyanate oxidase has been found in mammals, but only in erythrocytes. A very small fraction of the total cyanide probably less than 1% is bound by hydroxocobalamin (Irwin et al. 1997).

25.5 Microbial Degradation of Cyanide and Nitriles

Cyanide/nitrile containing waste is an increasingly prevalent problem in the environment. Many chemical processes that utilize cyanide are gold mining and electroplating, and these processes generate effluents with varying amount of cyanide. Cyanide/nitrile-converting microbes are considerable interest from the view points of treating toxic nitrile and cyanide-containing wastes and as agents for the synthesis of organic chemicals for a wide range of applications (Trott et al. 2001). Disposal of cyanide/nitrile wastes or remediation of sites containing with cyanide /nitrile is necessary to prevent contamination of soil and water. Among the various options to deal with cyanide/nitrile with contamination, bioremediation is a more attractive and cost effective alternative. The biodegradation of cyanide seems very simple as it ultimately breaks to formic acid and ammonia. The bacteria oxidize the cyanide, breaking it down into harmless compounds. In many cases, cyanide-oxidizing bacteria are naturally present in processing ponds and waste piles. The environmental nitrile pollution is mainly among the major source of nitrile contamination to the environment are due to release of certain nitrile compounds such as acrylonitrile, acetonitrile, which occurs in various form of natural products in microorganisms. Acetonitrile and acrylonitrile are the most prominent contaminants under nitriles. Acrylonitrile which is mainly used for the manufacture of polyacrylonitrile polymer is the prominent pollutant among the nitrogen containing organic compounds. As many as four general pathways for the degradation of cyanide are reported: hydrolytic oxidative, reductive and substitutive (Fig. 25.2).

25.5.1 Biodegradation of Cyanide

Cyanides are present in the environment and waste streams in the form of cyanates and nitriles (Ebbs 2004). Cyanides are extensively used in the industries and the effluent of such industries badly pollutes the environment (Desai and Ramakrishnan 1998). It is not economic to employ chemical or physical methods for degradation of cyanide and their other forms, therefore microbial degradation (Table 25.3) seems to be more attractive and less expensive method for treatment of cyanides contaminated habitats(Akicl 2003). In the mining industry cyanide serves as an ore extractant and its wastewater often has high concentration of cyanide salts of Ni, Cu, Zn, and Fe. The cyanide degrading bacteria occur in nature and these utilize cyanide as sole source of carbon and nitrogen.



The chemical reactions responsible for the biodegradation of cyanide and thiocyanate. For the hydrolytic reaction involving nitriles, R represents either an aliphatic or aromatic group. The substitution/transfer reaction catalyzed by cyanoalanine synthase can also use O-acetylserine (OAS) as a substrate. The cyanate formed by cyanide monoxygenase is converted to NH_4^+ and CO_2 by the same pathway as the cyanate from thiocyanate. The reductive pathway is derived from the action of nitrogenase and the products resulting from the transfer of pairs of electrons.

Fig. 25.2 Various reactions for the degradation of cyanides (Ebbs 2004)

Table 25.3 List of some cyanide degrading bacteria (Ubalua 2010)

Name of organisms	Enzymes involved
Pseudomonas sp.	Cyanide mono-oxygenase
Pseudomonas fluorescens, Bacillus cereus, Bacillus pumillus	Cyanide dioxygenase
Escherichia coli, Rhodococcus rhodochrous	Cyanase
Pseudomonas, Corynebacterium, Brevibacterium	Cyanide hydratase, Nitrile hydratase
Alcaligenes xylosoxidans	Cyanidase
Klebsiella ozaenae, Arthrobacter sp., Pseudomonas aeruginosa, Norcadia sp.	Nitrilase

There are some other enzymes capable of cyanide transformation. Cyanase is an enzyme that catalyzes the hydrolysis of cyanate to ammonia and bicarbonate. *Escherichia coli* and *Flavobacterium sp*. also utilize cyanate as sole source of nitrogen. Ammonia and carbon dioxide are the major end products of aerobic cyanide metabolism. The primary end products from anaerobic cyanide transformation are formate and bicarbonate, but the mechanisms of adaptation to and degradation of cyanide in anaerobic systems are still being explored. It may be

pointed out that microbial degradation combined with oxic/anoxic processes may prove more beneficial for the detoxification of cyanides (Ebbs 2004; Desai and Ramakrishnan 1998). Addition of microbes in nitrile/cyanide wastes significantly increases the phosphates requirement of the system and thus addition of phosphate encourages the bacterial population *vis-a-vis* break down of the cyanide/nitrile down into carbon and nitrogen.

Biodegradation of cyanide occur under both aerobic and anaerobic conditions. Under aerobic condition some microorganisms consume hydrogen cyanide and generate hydrogen cyanate which is further hydrolyzed into ammonia and carbon dioxide (given below).

(i) $2HCN + O_2 \xrightarrow{\text{Cyanide monooxygenase}} 2HCNO$

(ii) $HCNO + H_2O \rightarrow NH_3 + CO_2$

The direct oxidation of cyanide requires metal, bacteriological, or photochemical catalyst, and produces cyanate ions (CNO_2):

(iii) $2CN_2 + O_2 \xrightarrow{\text{sunlight}} 2CNO_2$

Anaerobic biodegradation of cyanide and hydrogen cyanide is restricted to the moderately to strongly reduced portions of the heap of garbage/waste and can only occur if HS_2 or H_2S are present. The type of sulphur species depends on pH i.e. if pH is greater than 7, it makes HS_2 dominant species and if the pH is lower than 7, H_2S is prevalent.

(iv) $CN_2 + H_2S \rightarrow HCNS + H_2$

(v) $HCN + HS_2 \rightarrow HCNS + H_2$

The HCNS will then hydrolyze to form NH_2, H_2S and CO_2. As compared to the aerobic biodegradation, anaerobic biodegradation of cyanide is much slower and cyanide toxicity threshold are 2 and 200 mg/L for an aerobic and anaerobic bacteria respectively (Smith and Mudder 1991).

25.5.2 Biodegradation of Nitrile

Comparatively more work has been carried out on the biodegradation of nitrile (Nagle et al. 1995; Manolov et al. 2005) as nitriles are readily biodegraded by several strains of bacteria common in sewage sludge, natural waters and soil. The aerobic biodegradation of nitriles leads to the formation of metabolic products like acetamides, acetic acid and ammonia. Microbial degradation of organonitrile compounds (and their derivatives) has been extensively explored by many researchers using a number of bacteria (Table 25.2).From the information available hitherto, it seems very promising rather practical to degrade organonitriles in industrial effluents or nitrile contaminated habitats using bacterial systems (Table 25.4).

Table 25.4 List of some nitrile degrading bacteria

Name of the organisms	Enzyme system	Substrate affinity	References
Acidovorax facilis ATCC 55746	Nitrilase	Alkylnitriles, benzonitrile	Cooling et al. (2001), Gavagan et al. (1998), and Gavagan et al. (1999)
Agrobacterium sp. DSM 6336	Nitrilase	Heterocyclic nitriles	Kiener (1992)
Agrobacterium tumefaciens B-261	Nitrile hydratase	Arylnitriles, arylalkyl nitriles	Kobayashi et al. (1995)
Bacillus cereus	Nitrile hydratase	Acrylonitrile	Saroja et al. 2000
Bacillus pallidus Dac521	Nitrilase ,nitrile hydratase	Alkylnitriles,arylnitriles, heterocyclic nitriles, alkylnitriles	Almatawah et al. (1999), Almatawah and Cowan (1999) and Cramp and Cowan (1999)
Candida guilliermondii CCT 7202	Amidase	Propionoamide	Dias et al. 2001
Comamonas testosteroni ATCC 55744	Nitrile hydratase	Alkylnitriles	Gavagan et al. (1998)
Gordona terrae FERM BP-4535	Nitrilase	Arylalkylnitriles	Tamura (1998) and Hashimoto et al. (1996)
Pseudomonas putida NRRL-B-18668	Nitrile hydratase	Arylalkylnitriles	Payne et al. (1997)
Rhodococcus sp. 409 (NOVO SP 409)	Nitrile hydratase	Arylnitriles, heterocyclic nitriles,arylalkylnitriles, cycloalkylnitriles	Deigner et al. (1996)
Rhodococcus ruber	Nitrilase	Acrylonitrile	Hughes et al. (1998)
Rhodococcus rhodochrous PA 34	Nitrilase ,nitrile hydratase	Acrylonitrile,benzonitrile	Bhalla et al. (1992)

25.6 Bioremediation of Cyanide and Nitrile Contaminated Sites

In situ application of microbes in removal of hazardous compounds to remediate the contaminated soil/water is gaining importance in as it is cost-effective and ecofriendly and requires lesser energy input. The bacterium or consortium of bacteria that are involved in the degradation of the hazardous compound occur naturally at the site. Cyanide can be degraded into simpler substances by microorganisms in the wastewater, piles and in the soil. Many bacterial and fungal species are capable of decomposing nitriles and highly toxic cyanides. The biological treatment methods do not depend on the supply of electricity or other energy sources. The bacterial detoxification is found to be safer, quicker, and cost effective than the chemical treatment for the handling of cyanide and nitrile wastes.

Cyanide has been found in at least 29% of the 1,430 EPA's National Priorities List sites. Studies have shown that cyanide solutions placed in ponds or tailings impoundments undergo natural attenuation reactions which result in the lowering of the cyanide concentration. These attenuation reactions are dominated by natural volatilization of hydrogen cyanide, but other reactions such as oxidation including biooxidation, hydrolysis, photolysis and precipitation also occur. Natural cyanide attenuation occurs with all cyanide solutions exposed to the atmosphere, however it occurs at slower in anoxic environments. In general, biological cyanide degradation is accomplished by stimulating indigenous bacteria through nutrient addition and optimizing growth conditions (i.e. pH, temperature, oxygen, etc.). Aerobic bacteria have a relatively high free cyanide tolerance threshold of 200–280 mg/L up to >600 mg/L in protected environments pH ~ 7.0 to ~9.5 optimum for cyanide degradation. Improved bioprocess technology allows cyanide to be rapidly treated in a bioreactor environment with the cyanide-free effluents used to rinse pads to achieve rapid cyanide removal (Fig. 25.3).

However, in spite of the fact that an isolated species of microorganisms may show a promising potential in degrading these toxic organonitriles compounds, it is probably of greater practical importance to be able to use a mixed culture (a consortium microorganisms rather than one single species), if possible, should be used for achieving better results in degrading of these toxic compounds. Information on biodegradation of organonitrile compounds with a mixed culture is scanty. In addition to this many other factors such as pH, substrate concentration, process temperature and oxygen content affects the biodegradation performance. The biodegradation of organonitriles has been commonly carried out under aerobic condition, there have been only a few cases of anaerobic decomposition of acetonitrile by a single species. However, the anaerobic process was rather slow and severely limited by the acetonitrile toxicity (Nagle et al. 1995; Munoz et al. 2005). So far, not much information is available on the anaerobic degradation of acetonitrile or any other organonitrile compounds by a mixed culture.

The biodegradation of cyanide under anaerobic conditions has recently demonstrated the feasibility for concomitant biogas generation, a possible economic

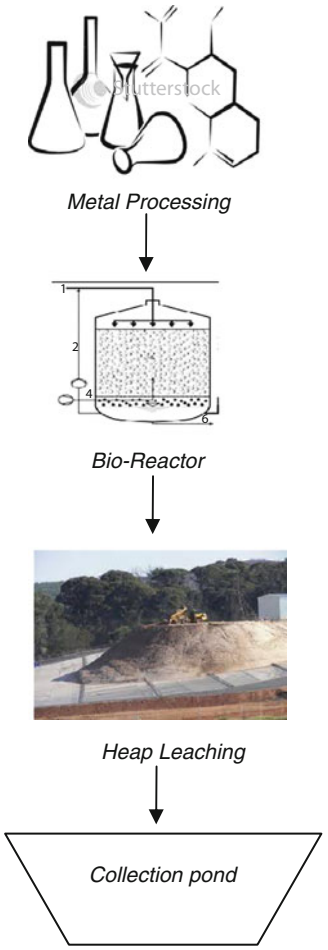


Fig. 25.3 Biotechnological approach for removal of cyanide from industrial effluent or contaminated sites

benefit of the process. *Pseudomonas putida* isolated from contaminated industrial wastewaters and soil sites is able to utilize acetonitrile and sodium cyanide as a sole source of carbon and nitrogen. The *Pseudomonas putida* cells are immobilized in calcium alginate and the rate of degradation of acetonitrile (160 mM) and sodium cyanide (40 mM) by the immobilized cells of *Pseudomonas putida* has been very well explained by determining the products such as NH_3 and CO_2. The product-dependent kinetics of sodium cyanide and acetonitrile indicate that the rate of degradation of these compounds follow a first order kinetics. Many hydrolytic reactions are catalyzed by cyanide hydratase, producing formamide, or cyanidase, which forms formate and ammonia. Cyanide hydratase is primarily a fungal enzyme and is highly conserved among several fungi (Barclay et al. 2002). Cynases and Cyanidase (cyanide dihydratase) are principally bacterial enzymes and have amono acid sequence and structural similarity with nitrilase and nitrile hydratase enzymes

(O'Reilly and Turner 2003). The cyanide hydratase gene from *Fusarium lateritium* has been introduced into *Escherichia coli* which allowed its growth on nitriles as the sole source of nitrogen. The diversity of enzymes in nitrilases superfamily and their variation in catalytic activity and substrate specificities presents opportunity for development of the biotechnological processes, including the bioremediation of industrial nitrile wastes (Rezende et al. 2000; Dias et al. 2001). Two yeast strains, *Cryptococcus sp.* UFMG-Y28 and *Candida guilliermondi* CCT 7207, were able to use nitriles such as benzonitrile as the sole nitrogen source for growth. The immobilized cells of *Candida guilliermondii* CCT 7207 have been reported to be efficient for the biodegradation of nitriles. It has been shown that immobilized organisms or enzymes provide an effective platform for cyanide biodegradation and the biodegradation is further influenced by the cyanide concentration in the system. Biodegradation of acrylonitrile by *Bacillus subtilis* increased levels of cyanide in the medium, which in turn initiate the autolysis of the cell of this bacterium (Reyes et al. 2000). It has been observed that whenever the rate of nitrile hydrolysis exceeds the subsequent rate of cyanide biodegradation then a sustained biodegradation system is not maintained unless additional organisms are included in the system to biodegrade the cyanide formed. This leads to combine nitrilase activity with that of β-cyanoalanine synthase, by one of the substitution/transfer pathways where the cyanoalanine is converted to asparagine by cyanoalanine hydrolase or asparaginase. Efforts have been made to develop this combined approach or an approach based solely upon the substitution/ transfer reactions.

Cyanide monoxygenase converts cyanide to cyanate, with cyanase that catalyzes the bicarbonate-dependent conversion of cyanate to ammonia and carbon dioxide. Cyanases, from numerous bacteria, fungi, plants and animals (Guilloton et al. 2002), are the principal focus of researchers in the area of cyanide degradation. Cyanases have been found to be a protection against cyanate poisoning (Raybuck 1992) as cyanate is not a common metabolite and more precise roles of cyanases in bicarbonate/ carbon dioxide and nitrogen metabolism have been proposed.

As industrial activities are likely to escalate thiocyanate and selenocyanate levels in the environment and therefore biodegradation of these compounds need to be explored. Essentially a second oxidative pathway utilizes cyanide dioxygenase to form ammonia and carbon dioxide directly (Fig. 25.1). The requirement for a pterin (heterocyclic compound) cofactor in this reaction has been proposed (Kunz et al. 2001). In *Escherichia coli* strain BCN6 and *Pseudomonas fluorescens* NCIMB 11764, the formation of cyanohydrin complexes has been reported to be necessary for oxygenase-mediated cyanide degradation (Kunz et al. 1998; Figueira et al. 1996). Whether or not complexation is essential for cyanide biodegradation via oxygenase activity has yet to be confirmed. Biodegradation of metal cyanide complexes is perhaps the most urgent need with respect to cyanide biodegradation.

Cyanide complexes with iron (Fe) dissociate slowly in the dark, but are rapidly lysed in the presence of UV light to yield free cyanide. This makes remediation of metal cyanide complexes in soil and water more complicated and renders biodegradation studies of metal complexes of cyanide more difficult under laboratory condition. Metal cyanide complexes in waste streams can be treated by current

biotechnological approaches, yet there is little emphasis on the remediation of iron cyanides in the environment because of their lower toxicity and stability. The earlier and recent studies on biodegradation of metal cyanide complexes have shown that microorganisms such as *Pseudomonas fluorescens, Fusarium solani* are efficient in degradation of cyanide as well as Fe and Ni complexes of cyanide (Kwon et al. 2002; Yanese et al. 2000; Barclay et al. 1998b).

Many investigations regarding the control of light have revealed that biodegradation products of metal cyanide complexes include ammonia or formamide while in the absence of proper controls it is difficult to conclude whether the organisms present used the metal cyanides directly or whether biodegradation agent *i.e.* microorganisms acted solely upon the free cyanide. However some studies suggested that the biodegradation of metal cyanide complexes is a function of the dissociation of the complex, rather than the biological degradation of the cyanide complex. However, it has been reported that *in situ* remediation of simple cyanide and iron–cyanide complexes is feasible by the of microbes (Trapp et al. 2001).

25.7 Conclusions

The level of cyanide and nitriles in the environment is increasing due to their uses in metallurgical operations, organic synthesis and as agrochemicals. The contaminated soil, water and air poses health hazards and therefore, some chemical, physical and biological approaches have been explored in the past to reduce the level of cyanide and nitrile in the environment. A number of microorganisms have been reported and utilized in certain situations to degrade the cyanide and nitriles to nontoxic metabolites. The metabolic pathways and enzymes involved in microorganisms for degradation of these chemical pollutants have been documented. Since the structure and properties of various forms of cyanides and nitriles vary therefore, more focused research efforts are needed to find out specific microorganisms that have affinity for large number of cyanides and nitriles and also there is a need to develop and design consortia of microorganisms for bioremediation of contaminated habitats. Studies pertaining to *in situ* degradation of these environmental pollutants and nitriles in the contaminated habitats by microorganisms will be more interesting and fruitful.

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Chapter 26
Bioremediation of Petroleum Hydrocarbons in Soils

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Abstract Crude oil is a complex mixture of hydrocarbons, basically composed of aliphatic, aromatic and asphaltene fractions along with nitrogen, sulfur and oxygen containing compounds. Major causes of oil contaminated soils include: leakage of storage tanks and pipelines, land disposal of petroleum wastes and accidental spills. Apart from damaging crops and affecting the fertility of soil, crude oil on the fields also affects livestock. The loss of soil fertility and toxicity of petroleum hydrocarbon at higher concentrations have been linked to displacement of nutrients and nutrient linkage, reduction in phosphorus and nitrogen availability, and anoxic conditions. Moreover, it can cause microbial community structure changes and a decrease in microbial diversity. The aromatics in crude oils such as α-pinene, limonene, camphene, and isobornyl acetate were observed to be inhibitory to the microorganisms. In recent years, there has been increasing interest in developing on site and *in situ* techniques for remediation of oil-contaminated soils. Bioremediation can be achieved by natural attenuation, biopiling, bioaugmentation, phytoremediation or rhizoremediation, singly or in combination. Numerous genera of bacteria are known as good hydrocarbon degraders. Most of them belong to *Aeromonas, Alcaligenes, Acinetobacter, Arthobacter, Bacillus, Brevibacterium, Mycobacterium, Pseudomonas, Rhodococcus, Sphingomonas,* and *Xanthomonas* species. Similarly white-rot fungi like *Bjerkandera adusta, Irpex lacteus, Lentinus tigrinus* and *Pleurotus tuberregium* are reported to degrade polyaromatic hydrocarbons. Other filamentous fungi include: *Aspergillus, Cladosporium, Fusarium, Geotrichum, Mucor* and *Peniciliium,* to name a few. The yeasts such as *Candida, Debaryomyces, Leucosporidium, Lodderomyces, Metschnikowia, Pichia, Rhodosporidium, Rhodotorula, Sporidiobolus, Sporobolomyces, Stephanoascus, Trichosporon* and *Yarrowia* were also reported to be effective hydrocarbon degraders. The microbial

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diversity in the different soil layers can be studied using functional diversity (community-level physiological profile, via Biolog) and genetic diversity using mainly PCR-DGGE (density gradient gel electrophoresis) technique. The details regarding microbial diversity changes due to oil contamination, biochemical pathways for hydrocarbon degradation, use of different microorganisms, singly and in combination, for bioremediation, possible ways to restore original diversity and soil fertility have been discussed.

Keywords Bioremediation • Microbial diversity • Petroleum hydrocarbons • Soil fertility

26.1 Introduction

Every year approximately 128 million metric tons of crude oil is being processed in 18 refineries in India (Sood and Lal 2009). The most visible source of petroleum pollution is the oil-tanker spills. The accidents with tankers, pipelines and oil wells release huge quantities of petroleum in to the land and marine eco-systems. Furthermore, land disposal of petroleum wastes is also a major concern. The largest oil spills in history by volume were in the range of 140–800 thousand tons and most of them were due to tanker accidents (www.aspenpublishers.com/environment.asp). In August 2010 two ships collided off the Mumbai coast leaking >2,000 ton of oil into the sea. The large oil spill in the open ocean may do the less harm to marine ecosystem than the relatively small spill near the shore. The consequences of these oil spill include serious, widespread and long-term damage to marine ecosystems, terrestrial life, human health and natural resources. Most of the lands and shorelines in the oil producing communities are important agricultural resources and under continuous cultivation. Any oil spill would usually damage soil properties, microbial and plant communities due to the associated changes in soil conditions. For instance, the soil pH was reported to be increased up to 8.5 and the levels of both carbonate and bicarbonates were also more which hampered plant growth (Ogri 2001; Agbogidi et al. 2007; Osuji and Nwoye 2007). The toxicity of petroleum hydrocarbon at higher concentrations was linked to displacement of nutrients and nutrient linkage and insufficient soil aeration (Mahmood et al. 2006; Peretiemo-Clarke and Achuba 2007).

The damage to the environment due to petroleum hydrocarbon spills mainly depends on the composition of the polluting petroleum product. It may be crude oil or refined petroleum products. Most of the products are fuels: gasoline, jet fuel, diesel fuel, kerosene and propane, are the major ones. The crude oil was also used to make asphalt and lubricant grease, plastics, pesticides, cleaning fluids, etc. (Yamane et al. 2008). Petroleum hydrocarbon constituents mainly the aromatic compounds were described to be toxic, mutagenic or carcinogenic (Balba et al. 1998). The sections below discuss the issues on potential and challenges in bioremediation of hydrocarbon contaminated soil.

26.2 Crude Oil Constituents

Petroleum crude oil, along with natural gas, is a complex mixture of hydrocarbons, basically composed of aliphatic, aromatic, resins and asphaltene fractions along with nitrogen, sulfur and oxygen containing compounds. The aliphatic fraction includes linear or branched-chain alkanes and cycloparaffins. The alkanes from pentane (C_5H_{12}) to octane (C_8H_{18}) are refined in to petrol, while nonane (C_9H_{20}) to hexadecane ($C_{16}H_{34}$) in to diesel and kerosene. The aromatic fraction contains mono-, di-, and poly-nuclear/cyclic aromatic hydrocarbons (PAH) containing alkyl side chains and/or fused cycloparaffins. The resins and asphaltenes contain more polar compounds, consisting of heterocyclic, oxygenated hydrocarbons and high molecular weight aggregates. The constituent hydrocarbon compounds are present in various proportions resulting in great variability in crude oils from different sources. The relative proportions of these fractions are dependent on many factors such as the source, geological history, age, migration and alteration of crude oil. The properties of petroleum hydrocarbon products which affected their biodegradation were chemical structure, degree of substitution, solubility, viscosity and concentration of its various components (Atlas. 1981; Jain et al. 2005; Seo et al. 2009).

26.3 Biochemistry of Petroleum Hydrocarbon Degradation

Several hundred thousand gallons of high-octane gasoline was reported to contaminate ground water in Pensylvania, USA for two consecutive years 1971–1972. The physical methods to remove gasoline were not efficient. For the first time the potential of microorganisms to degrade petroleum hydrocarbons was tested (Chapelle 1999). The metabolic pathways that hydrocarbon-degrading heterotrophs use can be either aerobic or anaerobic. The aerobic degradation usually proceeds more rapidly and is considered to be more effective than the anaerobic one. The Table 26.1 shows some genera of microorganisms which metabolize hydrocarbons other than methane.

26.3.1 Aerobic Degradation

Aerobic degradation of hydrocarbons is a long known and well-studied process. Oxidation of medium chain or long chain alkanes by aerobic microorganisms occurs *via* monoterminal (CH_3-CH_2-$(CH_2)_n$-CH_2-CH_3 to CH_3-CH_2-$(CH_2)_n$-CH_2-CH_2OH), biterminal (CH_3-CH_2-$(CH_2)_n$-CH_2-COOH to $HOCH_2$-CH_2-$(CH_2)_n$-CH_2-COOH) or subterminal (CH_3-CH_2-$(CH_2)_n$-CH_2-CH_3 to CH_3-CH_2-$(CH_2)_n$-CH_2-$CHOH$-CH_3) pathways as suggested by Berthe-Corti and Fetzner (2002). The enzymes involved in the aerobic degradation of aliphatic hydrocarbons are: alkane 1 monooxygenase

Table 26.1 Oxygenases involved in *n*-alkane oxidation

Enzymes	Alkane (chain length)	Genera	Reference
Methane monooxygenases	C_1–C_8	*Methylosinus* *Methylococcus* *Methylobacter*	Fox et al. (1989), Smith et al. (1997), and Hakemian and Rosenzweig (2007)
AlkB family of alkane monooxygenases	C_5–C_{16}	*Pseudomonas* *Rhodococcus* *Acinetobacter* *Mycobacterium* *Geobacillus*	Whyte et al. (1997), Binazadeh et al. (2009), Geißdörfer et al. (1999), Smits et al. (2002), and Feng et al. (2007)
Bacterial P450 (CYP153, Class I)	C_5–C_{16}	*Mycobacterium*	van Beilen et al. (2005)
Eukaryotic P450 (CYP52, Class II)	C_{10}–C_{16}	*Yarrowia* *Candida, Lodderomyces*	Iida et al. (2000) and Mauersberger et al. (1984)
Dioxygenases	C_{10}–C_{30}	*Acinetobacter*	Maeng et al. (1996)

(EC 1.14.15.3), alcohol dehydrogenase [EC 1.1.1.1(NAD$^+$) or EC 1.1.1.2 (NADP$^+$)] and aldehyde dehydrogenase (EC 1.2.1.3) and acyl CoA synthetase (EC 6.2.1.3). The monooxygenases initiate the alkane degradation (Table 26.1).

$$n\text{ - Octane} + \text{reduced rubredoxin} + O_2 + 2H^+ = 1\text{ - octanol} + \text{oxidized rubredoxin} + H_2O$$

The general degradation pathway for an alkane involves sequential formation of an alcohol, an aldehyde and a fatty acid. The fatty acid is cleaved by beta-oxidation, releasing carbon dioxide and forming a new fatty acid that is two carbon units shorter than the parent molecule (Watkinson and Morgan 1990).

The general pathway for aromatic hydrocarbons involves cis-hydroxylation of the ring structure forming a diol (e.g. benzene) using dioxygenase. Benzene 1,2–dioxygenase (EC 1.14.12.3) for instance catalyzes following reaction:

$$\text{Benzene} + \text{NADH} + H^+ + O_2 = \text{cis-cyclohexa-3,5-diene-1,2-diol} + \text{NAD}^+$$

The ring is oxidatively cleaved by dioxygenases, forming a dicarboxylic acid. Oxidation of substituted aromatics generally proceeds by initial beta-oxidation of the side chain, followed by cleavage of the ring structure (Diaz 2004; Van Hamme et al. 2003; Husain 2008).

Aerobic degradation in soil is associated with a variety of microorganisms, including bacteria and fungi. *Pseudomonas* appears to be the most ubiquitous

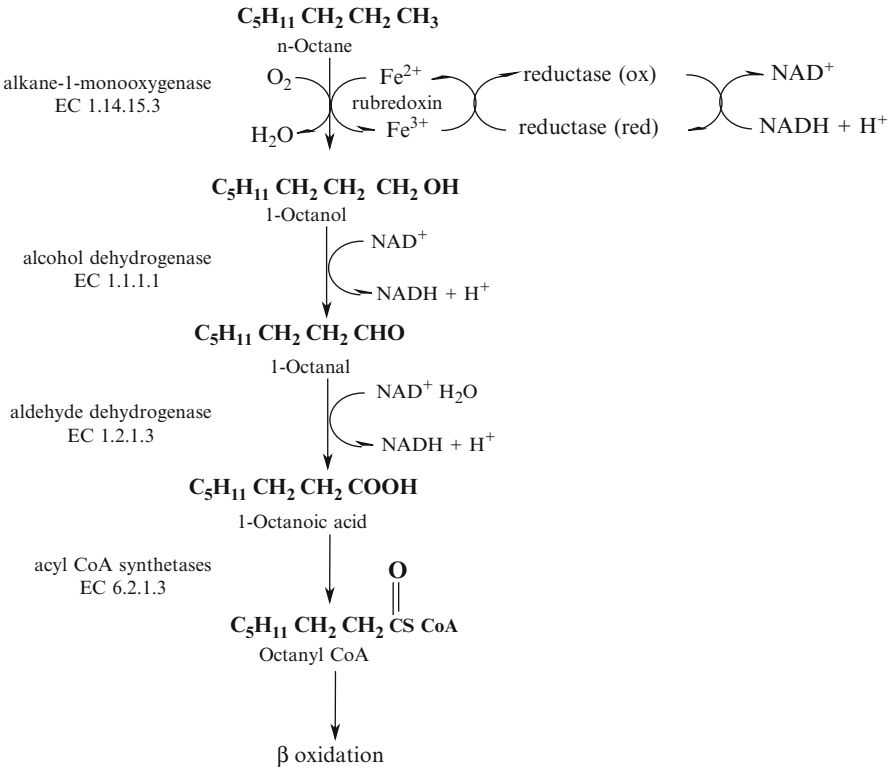

Fig. 26.1 Possible *n*-octane degradation pathway in *Pseudomonas putida* (van Beilen et al. 2001; Van Hamme et al. 2003)

bacterium found in soil, and also in oil contaminated soil. Pseudomonads have high degradative potential e.g. *Pseudomonas putida* and *Pseudomonas fluorescens*. These have ability to adapt to many different hydrocarbons not solely with catabolic enzymes but also on metabolic regulation. Certain species of *Pseudomonas* have the capacity to degrade particular aliphatic hydrocarbons. For instance, one strain of *Pseudomonas aeruginosa* was found to degrade C_6, C_8 and C_{10} compounds but failed to degrade longer aliphatic compounds while another strain showed degradation of longer chain compounds. These differences in the degradative capabilities prompted researchers to engineer novel strains genetically (Chapelle 1999). The Fig. 26.1 depicts the terminal *n*-octane oxidation pathway in *P. putida* encoded by the OCT plasmid.

Acinetobacter and *Rhodococcus* were two bacterial strains often associated with petroleum contaminated habitats. *Acinetobacter* utilized an alkane monooxygenase (terminal oxidation) to convert the hydrocarbon to a primary alcohol to allow the subsequent breakdown and utilization of the hydrocarbon (Fig. 26.2) (Geißdörfer et al. 1999). *Rhodococcus* possessed an alkane monooxygenase as *Acinetobacter*,

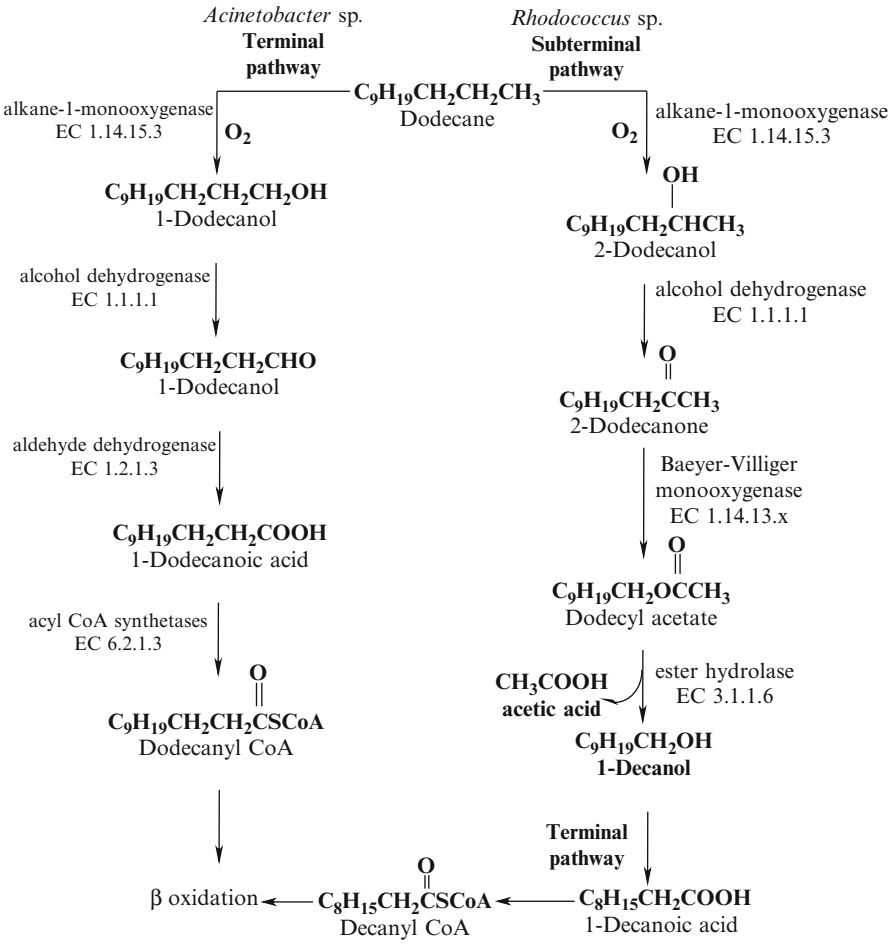

Fig. 26.2 Dodecane degradation pathways for *Acinetobacter* species and *Rhodococcus* species (Binazadeh et al. 2009; Geißdörfer et al. 1999)

but exhibited subterminal oxidation leading to the production of a secondary alcohol and the subsequent ketone was further metabolized to a primary alcohol for further breakdown (Fig. 26.2) (Binazadeh et al. 2009).

Filamentous fungi were also reported to degrade long chain alkanes. Hadibarata and Tachibana (2009) studied the degradation of n-eicosane (C_{20}) by *Trichoderma* sp. They observed that the fungus converted the alkane to the fatty acid nonadecanoic acid. While Zinjarde et al. (1998) reported hexadecane (C_{16}) degradation by a marine isolate *Yarrowia lipolytica*. This marine isolate was further evaluated for the degradation of a diesel range *n*-alkanes (C_{10}–C_{29}) (Kulkarni et al. unpublished data).

Naphthalene is the most common contaminant found in hydrocarbon polluted soils. Davies and Evans (1964) were the first to study the enzymes involved in naphthalene degradation by soil Pseudomonads. The degradation proceeds with the addition of two atoms of oxygen to the naphthalene ring to form cis-1,2-dihydroxy-1,2-dihydronaphthalene by 1,2-naphthalene dioxygenases (EC 1.14.12.12). The second step is catalyzed by naphthalene(+)-cis-dihydrodiol dehydrogenase (EC 1.3.1.29) which forms 1,2-dihydroxynaphthalene using NAD^+ as an electron acceptor. The next steps involve series of reactions to form salicylic acid which is further oxidized to catechol by salicylate hydroxylase (EC 1.14.13.1) (You et al. 1988). The catechol thus formed may undergo ring cleavage by a *meta* pathway to form pyruvate and acetaldehyde, or an *ortho*- pathway to form succinyl-CoA and acetyl-CoA as end products (Grimm and Harwood 1997). *Sphingomonas* sp. VKM B-2434 was reported to degrade naphthalene using a similar pathway (Baboshin et al. 2008).

Annweiler et al. (2000) reported a different naphthalene breakdown pathway in *Bacillus thermoleovorans*. This thermophile produced some different metabolites apart from the ones reported in mesophiles such as 2,3-dihydroxy naphthalene, 2-carboxycinnamic acid, phthalic acid and benzoic acid. *Mycobacterium* sp. metabolized naphthalene by monooxygenation and dioxygenation with the formation of both cis- and trans-1,2-dihydrodiols in the ratio 25:1 (Kelley et al. 1990). The reaction to trans-1, 2-dihydrodiol via naphthalene 1,2-oxide was catalyzed by cytochrome P-450 monooxygenases. Dioxygenases were involved in formation of cis-1, 2-dihydrodiol. Fungi also have the ability to degrade aromatic hydrocarbons. Cerniglia and Gibson (1977) studied naphthalene degradation in *Cunninghamella elegans* and observed that naphthalene breakdown yielded the major metabolites 1-naphthol and 4-hydroxy-1-tetralone and 1,4-naphthoquinone, 1,2-naphthoquinone, 2-naphthol and trans-1,2-dihydroxy-1,2-dihydronaphthalene. The initial reaction was catalyzed by a cytochrome P-450 monooxygenase. The Fig. 26.3 shows the pathways for the naphthalene degradation by *P. putida*, *B. thermoleovorans*, *Mycobacterium* species and *C. elegans.*

The involvement of plasmids in *Pseudomonas* sp. in hydrocarbon degradation was extensively reported. Many plasmids were characterized which encode pathways for petroleum hydrocarbon degradation. Plasmids NAH, OCT and TOL were reported to be involved in naphthalene, octane and toluene, degradation respectively (Chakrabarty et al. 1973, 1978; Yen and Gunsalus 1985). Some of the plasmids related to naphthalene degradation in the genus *Pseudomonas* are listed in Table 26.2.

The catabolic pathways for three- and four-ring PAHs in *P. putida* were also examined. Phenanthrene was degraded by *Pseudomonas* sp. *via* a dioxygenase-initiated pathway that converged with the naphthalene degradation pathway (Prabhu and Phale 2003). *P. putida* was also reported for the study on the substrate interactions during cell growth on carbazole-containing mixtures with *p*-cresol and sodium salicylate. Both *p*-cresol and sodium salicylate could be utilized by the bacterium as the sole carbon and energy sources (Loh and Yu 2000).

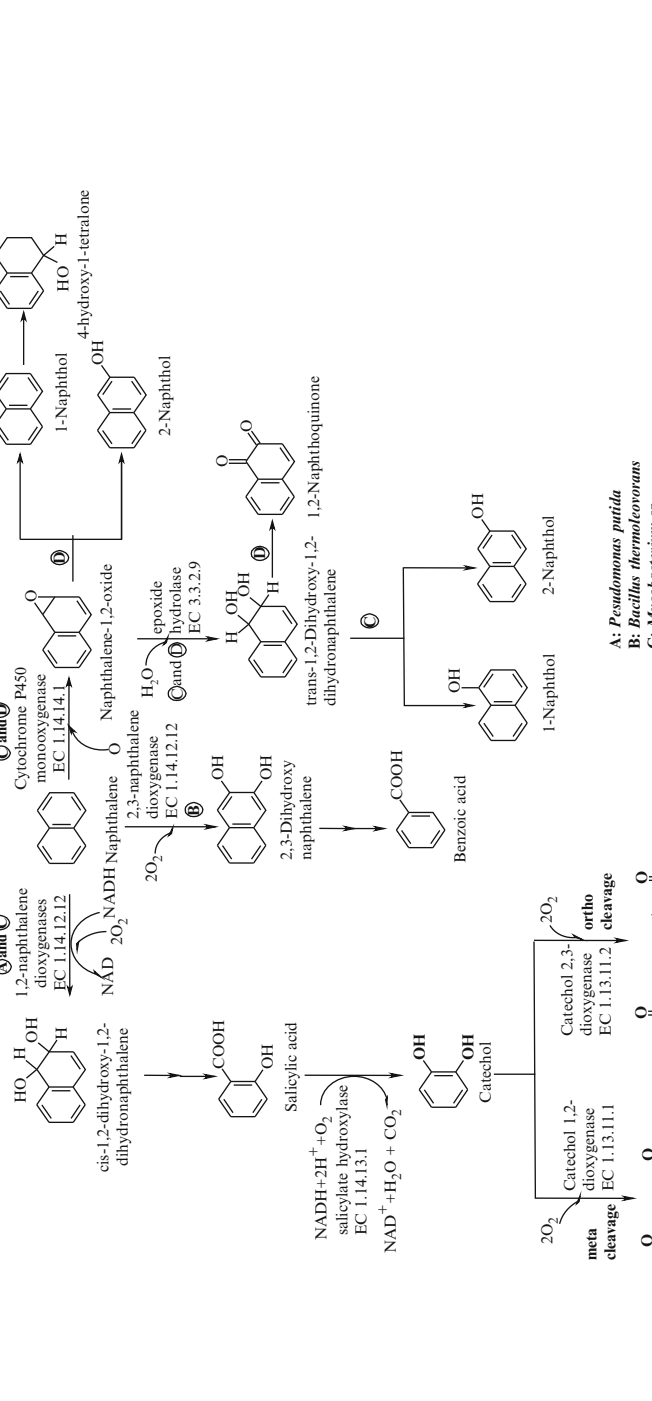

Fig. 26.3 Pathways for the naphthalene degradation by *Pseudomonas putida, Bacillus thermoleovorans*, *Mycobacterium* species and *Cunninghamella elegans* (Annweiler et al. 2000; Cerniglia and Gibson 1977; Davies and Evans 1964; Kelley et al. 1990; You et al. 1988)

Table 26.2 Some of the plasmids related to naphthalene degradation in the genus *Pseudomonas*

Plasmid	Microorganism	Reference
pWW60	*Pseudomonas sp.* NCIB 9816	Cane and Williams (1982)
NAH7	*Pseudomonas putida*	Yen and Gunsalus (1985)
NPL1, pBS2	*Pseudomonas putida*	Boronin et al. (1993)
pLIB119	*Pseudomonas stutzeri* 19SMN4	Rossello-Mora et al. (1994)
pDTG1	*P. putida* strain NCIB 9816-4	Dennis and Zylstra (2004)
pND15	*Pseudomonas* sp. strain NGK 1	Subba Rao et al. (2010)

26.3.2 Anaerobic Degradation

In contrast to the fact that aerobic microbial hydrocarbon metabolism was extensively investigated, the same was not true for anaerobic hydrocarbon degradation (Townsend et al. 2004; Foght 2008). In fact under number of conditions such as in deep sediments, flooded soils, eutrophic lagoons, stagnant fresh and ocean waters and in oil reservoirs oxygen is not available. Several alkanes, alkenes and alkylbenzenes were anaerobically utilized by denitrifying, ferric iron- reducing and sulfate-reducing bacteria (Heider et al. 1999). It was not until the late 1980s that photosynthetic *Blastochloris sulfoviridis,* denitrifying bacterium *Thauera aromatica,* plant associated *Azoarcus tolulyticus*, halotolerant *Halomonas,* ferric iron-reducing *Geobacter metallireducens*, to name a few were found to degrade *n*-alkanes, branched alkanes, and cycloalkanes under anoxic conditions (Aitken et al. 2004; Heider et al. 1999; Widdel and Rabus 2001). In case of anaerobic toluene degradation, for instance, benzylsuccinate synthase (EC 4.1.99.11) catalyzed the first step of addition of toluene to fumarate to form benzylsuccinate (Heider et al. 1999).

Naphthalene degradation proceeds via carboxylation to form 2-naphtoate in sulfate-reducing bacteria. The identification of other metabolites in a sulfate-reducing enrichment culture indicated the further metabolism of 2-naphthoate via subsequent reduction of the two rings to yield decahydro-2-naphthoic acid (Zhang and Young 1997; Meckenstock et al. 2000). While Safinowski and Meckenstock (2006) reported that sulfate reducing culture(s) initiated the reaction in the naphthalene degradation pathway by methylation to 2-methylnaphthalene which then underwent oxidation to the 2-naphthoic acid.

Anaerobic hydrocarbon degradation can technically be used in the bioremediation of some oil polluted sites which have limited aeration such as contaminated groundwater aquifers (Heider et al. 1999).

26.4 Diversity of Petroleum Hydrocarbon Contaminated Soils

There are many approaches towards studying the diversity of oil contaminated soil. Liquid enrichment cultures are routinely used in laboratory (Greene et al. 2000; Vinas et al. 2002). Vinas et al. (2002) reported that the enrichment with a specific

hydrocarbon increased only those organisms, from a consortium, that could utilize that hydrocarbon as a sole source of carbon. In other words, the community structure obtained from the same soil after enrichment using two different hydrocarbons could be different (Baek and Kim 2009). Use of molecular genetic techniques like Denaturing Gradient Gel Electrophoresis (DGGE) and Terminal Restriction Fragment Length Polymorphism (T-RFLP) were proved to be beneficial for judging the community structure of contaminated soil, which were independent of the ability of bacteria to grow in the culture media (Jung et al. 2005; Kaplan and Kitts 2004; Macnaughton et al. 1999).

Nutrient balance (C and N), pH and moisture content of soil were usually affected as a result of contamination by hydrocarbons (Bundy et al. 2002). As a result organisms that can withstand and utilize hydrocarbons could only survive and grow affecting whole community structure (Evans et al. 2004; Bordenave et al. 2007; Popp et al. 2006; Baek et al. 2007). For example, *γ-proteobacteria* mainly *Pseudomonas* species were found to be selectively enriched after the contamination by hydrocarbons such as alkanes, and PAHs followed by *α-proteobacteria* and *β-proteobacteria* (Popp et al. 2006). Yamane et al. (2008) characterized diversity of bacteria obtained directly from crude oil. They found presence of *Acinetobacter, Propionibacterium, Sphingobium* and *Bacillus*. Baek et al. (2007) studied crude oil contaminated sandy loam soil (pH 7.1) and found presence of *Mycobacterium, Nocardia, Thiocalovibrio, Pseudomonas citronellosis* and sulfur oxidizing bacteria. The change in microbial diversity of agricultural soil may have adverse effects on fertility and thus, on the crop yield. This necessitates the need for restoration of either useful, similar or same diversity of agricultural soil, which can possibly be achieved by various bioremediation techniques.

Ogino et al. (2001) studied community structure of contaminated soil by comparing it with untreated control soil. Hydrocarbon contamination increased the diversity of oil degraders especially *Pseudomonas*, initially and subsequently it became similar to that of uncontaminated soil. Similarly, Kaplan and Kitts (2004) reported increase in the population of *Flavobacterium* and *Pseudomonas* at the end of first 3 weeks of oil contamination thereafter their abundance decreased. Earlier Macnaughton et al. (1999) reported similar observations for the coastal soils with experimental crude oil spill. Using phospholipid fatty acid (PLFA) analysis and 16S rDNA PCR-DGGE to monitor *in situ* microbial community structures they reported that contaminated plot was dominated by *α-proteobacteria* which were undetected for the uncontaminated plot.

Microbial mat is made up of very complex microbial communities which show diverse genetic and metabolic potential. Hence change in such communities after contamination is more pronounced. Bordenave et al. (2007) conducted a study on microbial mat contaminated with heavy (C_{23}–C_{40}) fuel oil. Immediately after contamination *γ-proteobacteria* mainly *Pseudomonas* species were abundant, while after 90 days bacilli and staphylococci increased in abundance to more than 50%. A cynobacterium, *Microcoleus chthonoplastes* formed consortium with bacteria which were found to degrade aromatic hydrocarbons from crude oil contaminated microbial mat (Sanchez et al. 2005). Abed et al. (2002)

performed a study on microbial mat contaminated with diesel and obtained enrichment of *Phormidium*- and *Oscillatoria*-like cynobacteria, γ- and β-*proteobacteria*. Al-Hasan et al. (1998) demonstrated *n*-alkane oxidation by cyanobacteria. Bundy et al. (2002) found that the community structure following diesel contamination on three different soil types was not similar. Hence each soil type bears a unique indigenous microbial community which needs to be studied before implementation of bioremediation technique.

Different indices are used to quantify diversity from soil (Kennedy and Smith 1995). Diversity, *per se*, after contamination usually was changed and subsequently gradually it was decreased as easily biodegradable substrate was diminished (Popp et al. 2006). Oil-degrading mixed microbial flora was found to be better than the isolated ones which could be due to their complementary degradative capabilities (Jacques et al. 2008; Vinas et al. 2002).

26.5 Techniques of Bioremediation

Number of techniques can be employed to restore useful, similar or same diversity to get the desired level of soil fertility and crop yield. The cost for the clean-up of the contaminated sites with conventional physical and chemical techniques such as incineration, land filling, chlorination, ozonation and surfactant washing were enormous (Liu et al. 2010). Moreover, for these approaches the possible environmental hazards and their effect on soil diversity were of a major concern. Therefore, alternative methods to restore polluted sites in a less expensive, less labor intensive, safe and environmental friendly way were found to be necessary (Kuiper et al. 2004). Bioremediation can be achieved by natural attenuation, biopiling, bioaugmentation, phytoremediation or rhizoremediation.

26.5.1 Natural Attenuation

Rarely, oil contamination occurs suddenly (e.g. by tanker accidents or explosions), but more often it is a creeping contamination for long periods due to leakage of a pipeline, or a storage tank. Sanchez et al. (2000) described natural attenuation or self –cleaning as a collection of biological, chemical and physical processes that occur naturally resulting in the containment, transformation, or destruction of undesirable chemicals in the environment. This can be effectively bioremediated by the indigenous microflora which was adapted over the long period. The indigenous microflora used the pollutants as a carbon and energy source (Watanabe 2001). Although indigenous microflora in the soil degraded a wide range of target constituents of the oil, their population and efficiency were affected when toxic contaminant was present at relatively high concentrations (Mishra et al. 2001).

Serrano et al. (2008) provided evidence for restoration of diesel contaminated soil by natural attenuation. They simulated a diesel fuel spill at a concentration of

1 L/m^2 (*i.e.* concentration of total hydrocarbons below the legal limit) soil on a plot of agricultural land and monitored natural attenuation of aliphatic hydrocarbons over a period of 400 days. According to the data on soil quality parameters *viz.* soil microbial mass and dehydrogenase activity, after 200 days original levels were regained. These observations were attributed to the presence of majority of aliphatic hydrocarbons at the surface (up to 10 cm deep) which were volatilized initially after spill and subsequently remaining were degraded by natural microbial flora.

26.5.2 Biostimulation

Microbial degradation of hydrocarbons in soil is usually affected by many factors, such as nutrients, pH, temperature, moisture, oxygen, soil properties, major contaminant and of course by the potential of the microorganism. In a process of biostimulation, a natural attenuation by microorganisms was improved by optimizing conditions such as aeration, pH and temperature and by the addition of nutrient, manure and crop residue addition (Liu et al. 2010; Margesin and Schinner 2001; Molina-Barahona et al. 2004; Mukherji et al. 2004). For example, the manure addition increased hydrocarbon degraders and thus helped to reclaim the oily sludge-contaminated soil in Shengli oilfield in China (Liu et al. 2010).

26.5.3 Biopiling

Ex situ bioremediation methods include slurry-phase remediation, treatment –bed remediation and biopiles. Many organic pollutants especially petroleum hydrocarbons were successfully bioremediated using biopiles *i.e.* accelerated microbial bioremediation, at pilot and field level (Jorgensen et al. 2000). As the name indicated Jorgensen and co-workers (2000) used piling of contaminated soil with bark chips and nutrients with sufficient aeration for growth of microorganisms and found 71% decrease of contamination due to microbial activity within 5 months. The addition of specific mixed culture of oil-degrading bacteria enhanced the bioremediation process (Jorgensen et al. 2000).

26.5.4 Bioaugmentation

In this method the relative proportion of specific microorganisms, indigenous or exotic is increased for the effective bioremediation. Mishra et al. (2001) reported use of a indigenous bacterial consortium grown in a laboratory as an inoculum to stimulate *in situ* bioremediation of oily-sludge contaminated soil. Bento et al. (2005) used a consortium of *Bacillus* species such as *B. cereus, B. sphaericus, B. fusiformis,*

B.pumilus, Acinetobacter junii and *Pseudomonas* species for the degradation of hydrocarbons in soil. The microbial flora degraded light fraction (C_{12}–C_{23}) more efficiently than heavy (C_{23}–C_{40}) fractions of total petroleum hydrocarbon (TPH). Furthermore they compared the effectiveness of other methods namely, natural attenuation, biostimulation with bioaugmentation and found the latter treatment most effective.

The microbial species that degrade hydrocarbons are widely distributed in nature and are not restricted to only a few genera. Many microorganisms such as bacteria, fungi and microalgae have the ability to utilize petroleum hydrocarbons as the sole source of energy in their metabolism. The ability of microbes to degrade phenols and related aromatic compounds was recognized since the beginning of the twentieth century (Czekalowski and Skarzynski 1948). According to Van Beilen and Funhoff (2007) work of SÖhngen N.L. published in 1913 on bacteria responsible for the disappearance of oil slicks on water has opened up the new area of tackling of oil-pollution. Interestingly the work was initiated to find out the pathogenic nature of bacteria, if any. Eventually the focus shifted to the use of these organisms for bioremediation.

Numerous genera of bacteria are known as good hydrocarbon degraders. Most of them belong to *Aeromonas, Alcaligenes, Acinetobacter, Arthobacter, Bacillus, Brevibacterium, Geobacillus, Mycobacterium, Pseudomonas, Rhodococcus, Sphingomonas, Thermus* and *Xanthomonas* species (Atlas 1981; Plaza et al. 2008; van Beilen and Funhoff 2007). Auffret et al. (2009) reported two strains, *R. wratislaviensis* and *R. aetherivorans* that could degrade more than 15 petroleum compounds from a mixture of benzene, toluene, ethylbenzene, *m*-xylene, *p*-xylene, *o*-xylene, octane, hexadecane, 2,2,4-trimethylpentane [isooctane], cyclohexane, cyclohexanol, naphthalene, methyl *tert*-butyl ether [MTBE], ethyl *tert*-butyl ether [ETBE], *tert*-butyl alcohol [TBA], and 2-ethylhexyl nitrate [2-EHN]. The co-culture degraded 13 compounds completely. Interestingly, these strains had broad degradation capacities toward the more recalcitrant compounds like MTBE, ETBE, isooctane, cyclohexane, and 2-EHN. Some of the compounds hampered degradation of other in two-substrate systems (Auffret et al. 2009). Ryu et al. (2006) isolated a psychrotrophic *Rhodococcus* sp. from an oil-contaminated ground water which could degrade various petroleum hydrocarbons (C_9–C_{12} and C_{19}–C_{32} alkanes 90% within 20 day) and was able to grow in 7% NaCl at 4°C.

White-rot fungi like *Pleurotus tuberregium, Pleurotus ostreatus, Bjerkandera adusta, Irpex lacteus and Lentinus tigrinus* were reported to degrade polyaromatic hydrocarbons (Schutzendubel et al. 1999; Isikhuemhen et al. 2003). Further Isikhuemhen et al. (2003) examined the ability of *P. tuberregium* to bioremediate crude oil polluted soils and used it for the seed germination and seedling growth of *Vigna unguiculata*. The effect of various concentrations of crude oil on fungal populations of soil was investigated by Obire and Anyanwu (2009). These included *Alternaria, Aspergillus, Candida, Cephalosporium, Cladosporium, Fusarium, Geotrichum, Mucor, Penicillium, Rhizopus, Rhodotolura, Saccharomyces, Torulopsis* and *Trichoderma*. The authors reported the decreasing order of occurrence of a variety of fungal genera (fungal diversity) of both total fungi and petroleum-utilizers

with increase in crude oil concentrations. They showed that higher concentrations of crude oil has adverse effect on fungal diversity while enhancing the population of a fewer fungi. The yeast species described in literature as being able to use hydrocarbons as carbon sources belong especially to the genera *Candida, Clavispora, Debaryomyces, Leucosporidium, Lodderomyces, Metschnikowia, Pichia, Rhodosporidium, Rhodotorula, Sporidiobolus, Sporobolomyces, Stephanoascus, Trichosporon* and *Yarrowia* (Csutak et al. 2010). Sood and Lal (2009) isolated a *Candida digboiensis* from soil samples contaminated with acidic oily sludge (pH 1–3) which could degrade 73% of the total petroleum hydrocarbons present in the medium at pH 3 in a week. Miranda et al. (2007) investigated the potential of the diesel degrading yeasts, *Rhodotorula aurantiaca* and *Candida ernobii.* They reported complete degradation of tetradecane, 5 methyl-octane and octadecane by *C. ernobii* and 93% for decane, 38.4% for nonane and 22.9% for dodecane with *R. aurantiaca*.

26.5.5 Phyto-/Rhizo-Remediation

A method used to extract, sequester, or detoxify pollutants with the help of plants. A number of plants which have extensive fibrous roots such as common grasses, corn, wheat, soyabean, peas and beans were studied for their rhizoremediation potential (Glick 2003). Several trees of family *Salicaceae* (poplar and willow) which can grow fast and possess a deep rooting ability were planted to bioremediate soil polluted with 20,000 mg/kg gasoline and diesel compounds to a depth of 3 m (Trapp et al. 2001). However, there use for bioremediation, *per se*, was restricted and localized and for low hydrocarbon contamination.

After phytoremediation plants can then be subsequently harvested, processed and disposed. In most of the cases it was difficult to distinguish between the roles of plant and bacteria from rhizosphere. The plants usually influence rhizosphere microbial community (Lee et al. 2007). However, number of plants also reported to produce enzymes involved in the metabolism of *n*-alkanes (Hardman and Brain 1971; Vega-Jarquin et al. 2001). The cell cultures of *Cinchona robusta* and *Dioscorea composita* were found to take up and metabolize *n*-hexadecane with the formation of hexadecanol and hexadecanoic acid (Vega-Jarquin et al. 2001). It was further reported that the levels and activities of cytochrome P450 and peroxidase were increased suggesting their role in biotransformation of *n*-hexadecane. Diab (2008) evaluated potential of broad bean (*Vicia faba*), Maize(*Zea mays*) and wheat (*Triticum aestivum*) plants for their abilities to stimulate the microbial degradation of soil pollutants in desert soil contaminated with crude petroleum oil. The rhizosphere soil of *V. faba* as compared to rhizosphere soils of *Z. mays* and *T. aestivum* exhibited effective degradation of petroleum hydrocarbons. Earlier, Pradhan et al. (1998) used phytoremediation technology for the treatment of soil contaminated with PAHs. Three plant species, alfalfa (*Medicago sativa),* switch grass *(Panicum virgatum)* and little bluestem grass *(Schizachyrium scoparium)* were found successful remediation

of PAH within 6 months. Tanee and Akonye (2009) examined the ability of cow pea (*Vigna unguiculata*) for the remediation of crude oil polluted soil for the cultivation of an economic crop cassava. They observed that *V. unguiculata* remediated the polluted soil by reducing the Total Hydrocarbon Content (THC) by 54% within 2 months along with an improved growth and yield of the cassava crop in the phytoremediated soil.

One of the main advantages of phytoremediation is the maintenance of structure and texture of soil more or less undisturbed and with an original microbial diversity. During this process plant exudates can stimulate the survival, dispersal and action of microorganisms, which subsequently results in a more efficient degradation of pollutants. Microbes in turn positively influence plant growth through a variety of mechanisms, including fixation of atmospheric nitrogen, phosphate solubilization and production of plant growth promoting hormones.

26.6 Future Perspectives and Conclusions

The main challenge in the bioremediation of agriculture soil, in particular, is the restoration of soil fertility. In other words, restoration of useful, similar or same microbial diversity is necessary. The profile of oil contamination with respect to depth and the distance from the source of contamination, *i.e.* from heavy contamination to uncontaminated soil can be correlated with the microbial diversity. The distances of qualitative and quantitative changes in the diversity from the uncontaminated end can prompt to design the treatment for bioremediation. In case of accidental spillage, usually a large area is covered by the oil. On the other hand, contamination due to seepage may cover smaller area but to a greater depth. The depth of penetration is affected by factors like soil characteristics such as texture, structure, composition, and water saturation capacity (Massaud et al. 2000). Usually TPH quantity was found to be decreased as depth of penetration increased (Al-Sarawi et al. 1997). Raymond et al. (1976) reported the oil contamination up to 30 m deep. However, the bioremediation using biostimulation method was achieved up to 15 m. The localized contamination of petroleum hydrocarbons deep in the soil up to 3 m can be reduced using tree species which can withstand low level of diesel/gasoline contamination (<1,000 mg/kg soil) (Trapp et al. 2001). In the case of biopiling, the soil layers up to 0.5 m can be removed and further processed. While rhizo-remediation with grasses and the use of microorganisms having constitutive or induced capabilities to degrade hydrocarbons were found to be effective to remove the contaminants in the upper layers of soil up to 0.2 m. Furthermore, enzyme-tailoring and recombinant organisms can also be effectively used. In this regard, the studies will be useful to pin point the effect of oil contamination on un-culturable diversity and the possibility to restore it too. It is indeed possible to restore useful and/or similar diversity using bioremediation techniques singly or in combination. But the translation of pilot level observations in to a technology that can be used in fields is a major challenge.

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Chapter 27
Hairy Roots: A Promising Tool for Phytoremediation

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Abstract Environmental pollution caused by natural processes or anthropogenic activities is a major global problem. Although several physical and chemical strategies have been used for environmental remediation, these methods are expensive and associated with certain limitations. Phytoremediation is an alternative, biological approach where different plant species are used for the removal of pollutants from the environment or for converting toxic molecules to non toxic forms. Both organic and inorganic compounds of various types are the targets of phytoremediation. The technique is cheaper than other established methods and has several advantages like soil stabilization, production of biofuels, non invasiveness etc. Plants readily absorb certain compounds, otherwise considered contaminants, through the root system and utilize for their normal growth and development. Hairy roots of plants are among the several experimental systems which have been employed to improve the efficiency of phytoremediation. Hairy root disease, characterized by a massive production of adventitious roots with numerous root hairs at the wound site, is caused by *Agrobacterium rhizogenes*, a gram negative soil bacterium. The bacterium transfers a segment (the transferred DNA or T-DNA) of its root inducing (Ri) megaplasmid into the plant genome, modifying the normal hormonal metabolism of the plant; this hormonal imbalance induces the formation of hairy roots at the site of infection. Apart from being suitable for the production of a wide array of valuable secondary metabolites, hairy roots possess tremendous potential for phytoremediation. Hairy roots of different plant species have been investigated for the accumulation and biodegradation of hazardous compounds. The present review summarizes the role of plant hairy roots in phytoremediation research.

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27.1 Introduction

Contamination of soil and water caused naturally through geological erosion or through anthropogenic activities is a global problem. Contaminants both organic and inorganic can be present in toxic levels in the environment or they may be present in trace amounts which after long periods of exposure can become deleterious to both human beings and animals. Several physical and chemical methods have been employed for the purpose of environmental remediation, but the processes are expensive and have got certain other limitations. Biological methods on the other hand are cheaper and appear to be attractive alternatives to the non-biological techniques. Phytoremediation refers to the ability of plants to clean-up environmental pollution. Plants are autotrophic, using sunlight and carbon dioxide as sources of energy and carbon and take up water and nutrients from the soil through the root system. Plants also absorb a variety of natural allelochemicals released by competing organisms (*viz.* microbes, insects and other plants) and xenobiotic compounds which they detoxify through diverse natural mechanisms (Cole 1983; Sandermann 1994; Singer 2006). In fact, many compounds otherwise considered as contaminants, are vital for the normal growth and development of plants. The concept that plants can breakdown xenobiotic compounds was developed as early as the 1940s when they were shown to metabolise toxic pesticides (Sandermann 1994). Diverse plant species, both monocots and dicots, are able to uptake, tolerate and sequester pollutants from hazardous waste sites and transform them to nontoxic forms. As suggested by Van Aken (2009) and Doran (2009), mechanism of detoxification of hazardous wastes by plants involves three different phases. In the first phase the compound is oxidized, reduced or hydrolysed by enzymes like P450 mooxygenases, peroxidases, reductases, dehydrogenases and esterases. Some level of toxicity is retained by the end products of phase I. This is followed by detoxification of compounds i.e. conjugation of metabolites using transferases with a molecule of plant origin *viz.* sugar residues, glutathione or amino acids forming stable water soluble compounds. Lastly, the conjugated derivatives are exported from the cytosol by ATP or proton dependant membrane transporters and compartmentalized in the vacuole or apoplast. Once taken up by plants, a compound can be stored in the plant tissue or can be volatilized, metabolized or mineralized to generate carbon dioxide, water and chlorides. The enzymatic and chemical procedures that detoxify harmful compounds in the plant are in many cases similar to those that occur in mammalian livers and thus plants have been aptly referred to as ‘green livers’ in some cases (Sandermann 1992).

Coined in 1991, the term phytoremediation has its origin from ‘phyto’ meaning plant and ‘remediation’ meaning correct evil. It relies on the natural ability of plants to uptake chemicals from polluted soil, water and air using solar energy.

Phytodecontamination or the removal of contaminants from the polluted area and stabilization of the contaminant to prevent its off-site movement and harmful effects are the two most important aspects of phytoremediation (Sadowsky 1999).

Initially developed for the removal of heavy metals from the soil (Van Aken 2009), the technology has proven to be efficient for the clean-up of diverse types of pollutants, both organic and inorganic, from the environment *viz.* heavy metals, chlorinated solvents, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, pesticides, excess nutrients (nitrate, ammonium, phosphate), munitions, explosives, radionuclides etc. (Salt et al. 1998; Sadowsky 1999; Suthersan 1999). Some of the compounds are more readily degraded or detoxified than the others. The compounds most amenable to phytoremediation include the pollutants which move into the vicinity of plant roots by means of mass flow or diffusion (Schnoor et al. 1995; Cunningham et al. 1996).

27.2 Various Forms of Phytoremediation

Depending on the type of contaminant present, phytoremediation treatments can be diverse (Doty 2008). Metals uptaken by plants cannot be metabolized but instead are translocated to the harvestable regions *viz.* stems and leaves from where they can be removed by harvesting those parts or they may be volatilized (Doty 2008). The harvested parts are finally treated as hazardous wastes or the metals are recovered as ore (Sadowsky 1999). Removal of contaminants from the soil *via* roots and bioaccumulation in foliage is referred to as phytoextraction (Suthersan 1999; Doty 2008). Different types of metals *viz.* Cd, Pb, Zn, Cu, Cr, Ni, Se, Hg, Co, As, Cs can be removed from contaminated soil by the method of phytoextraction (Cunningham et al. 1996; Chaney et al. 1997; Doty 2008). For phytoextraction, 'hyperaccumulating' plants have played a crucial role. Plants capable of accumulating and tolerating high concentrations of toxic heavy metal contaminants (0.01% for Cd, 0.1% for Ni, Co, Cu, Pb and 1.0% for Zn) in the foliage are termed as 'hyperaccumulators' (Baker et al. 2000). These species can accumulate about 100 times more concentration of heavy metals than non accumulator plants growing in the same locality (Suthersan 1999), without causing any deleterious effects on the plants. Common examples of hyperaccumulator plants include *Pteris vittata, Thlaspi caerulescens, Alyssum* sp., *Phyllanthus* sp., *Leucucroton* sp., *Senecio* sp., *Pentacalia* sp., *Brassica juncea*, *Alyssum* sp., *Silene vulgaris*, *Apocynum* sp., *Aeollanthus biformifolius, Aeollanthus subcaulis* var. *lineris, Paspalum notatum* (Suthersan 1999; Suresh and Ravishankar 2004). Hyperaccumulation occurs by several sequestration and detoxification strategies, the details of which are still not known. Complexation with phytochelatin peptides synthesized from glutathione, coordination of the heavy metal with histidine, binding to the cell wall or localization in apoplast have been suggested as some of the mechanisms leading to their hyperaccumulation in plants (Vázquez et al. 1992; Krämer et al. 1996, 2000; Cobbett 2000). However, these hyperaccumulating species are too small and slow growing

to be used for direct practical applications (Ebbs et al. 1997). Moreover they are physiologically not adapted to the climatic conditions at many polluted sites (Suthersan 1999). Thus research is focused on high biomass crops, which have deep roots systems like *Populus* spp. and *Salix* spp. for the phytoremediation of metal contaminants. Poplars and willows are not hyperaccumulating species in true sense as they do not bioaccumlate metals at a high concentration. But because of their greater biomass and deep root systems, they are effective in phytoextraction (Doty 2008). Also willow plants can regrow readily after the shoots have been removed- a trait necessary for phytoremediation of metal contaminated areas (Pulford and Watson 2003).

Phytodegradation refers to the metabolic degradation of organic pollutants such as trichloroethylene, perchloroethylene, polycyclic aromatic hydrocarbons, herbicides and explosives through either internal enzymes of plants or secreted enzymes (Suresh and Ravishankar 2004; Doty 2008). Pollutants like carbon tetrachloride are degraded anaerobically by soil microbes leading to the production of chloroform, a carcinogen, while the same compound can be metabolized aerobically by plants using a cytochrome P450 enzyme (Wang et al. 2002). Hybrid poplars (*Populus trichocarpa* x *P. deltoides*) (Newman et al. 1997; Gordon et al. 1998), the tropical leguminous tree *Leucocaena leucocephala* (Doty et al. 2003) and *Ipomoea batatas* (Khan and Doty, unpublished) are some of the plants frequently used for phytodegradation.

Removal of wastes from a contaminated site is always not feasible. In such cases, stabilization provides a logistical alternative. Stabilization of contaminated soil by plants is termed as phytostabilization or phytosequestration (Suthersan 1999; Sadowsky 1999). Plants inhibit erosion of contaminated soil by wind and water and also decrease water infiltration. A variety of alkalizing agents, phosphates, organic matter and biosolids are added so that the metals become insoluble and this prevents leaching (Suthersan 1999). Thus phytostabilization eliminates the availability of toxic metals in the soil.

Phytovolatilization denotes yet another form of phytoremediation where contaminants taken up by roots translocate to leaves from where they are volatilized through the stomata (Vroblesky et al. 1999). Contaminants uptaken by plants may either be immobilized in a mineral amended soil or a geomat (mineral containing mat) – a process known as phytoimmobilization (Arthur et al. 2005).

Rhizodegradation refers to transformation of contaminants with the help soil microorganisms residing in the rhizosphere (Suthersan 1999; Suresh and Ravishankar 2004; Arthur et al. 2005). Plant root exudates represent a potential source of nutrients and energy to the microbial population present in the root zone. The microbes in turn degrade the organic contaminants near the roots. Rhizodegradation represents a form of *explanta* phytoremediation where contaminants are detoxified outside the plant. The decaying fine roots of plants can also add organic carbon to the soil which increases the rate of microbial mineralization (Suthersan 1999).

Use of plants for the removal of contaminants from the water bodies is known as phytofiltration or rhizofiltration. Several plants like *Eichhornia crassipes, Hydrocotyle umbellata, Lemna minor, Scirpus lacustris, Phragmites karka, Bacopa*

monnieri, Azolla pinnata can effectively remove metal contaminants from water bodies (Salt et al. 1995; Chandra et al. 1997). Rhizofiltration involves the use of hydroponically cultivated plant roots for remediation of contaminated water and the method is also advantageous for recovery and reuse of metals which are toxic, yet valuable. Pilon-Smits et al. (1999) reported that *Myriophyllum brasiliense, Juncus xiphioides, Typha latifolia, Scirpus robustus* are effective for phytoremediation of Se from wetlands. Phytofiltration of Cr from aquatic areas by hydrophytes like *Nasturtium officinale, Veronica beccabunga, Mentha longifolia* and *Cardamine uliginosa* has been evaluated by Zurayk et al. (2001). Other metals like Pb, As, U, Sr, Cs have been effectively phytofiltered by *Hemidesmus indicus, Pteris vittata,* certain sunflower species, tomato and tobacco roots from field grown plants and plants from the Asteraceae family (review by Arthur et al. 2005). Field experiments with alfalfa, smooth bromegrass, orchardgrass and soybean were conducted by Russelle et al. (2004) to remove nitrates, which are very common contaminants present in drinking waters in agricultural areas.

27.3 Advantages and Limitations of Phytoremediation

Compared to other clean-up approaches, phytoremediation has several advantages. The method is cheap, being ten times less expensive than conventional non-biological strategies (Chappell 1998). Phytoremediation requires low installation and maintenance costs, involving only the use of fertilizers and watering to maintain plant growth. Cleaning up of contaminated sites using standard strategies is expensive. $6.0–8.0 billion is spent per annum to remediate waste sites in the USA alone whereas on a global level, the cost is as high as $25–50 billion (Glass 1999; Tsao 2003). Excavation, transport, soil washing, extraction, pumping and treating contaminated water, addition of reactants like hydrogen peroxide and potassium permanganate and incineration are some of the basic steps of engineering methods for clean-up which lead to the huge cost and also damages the environment (Hannink et al. 2002; Doty 2008). As a result, contaminated commercial properties are often left untreated; in the USA there are over 500,000 such 'brownfields' lying untreated (Doty 2008). Phytoremediation on the other hand is cost effective; the cost was estimated to be $10,000–30,000 per acre, while it was $25–100 for treatment of 1 ton soil and $0.6–6.0 per 1,000 gal of aquatic waste (Suresh and Ravishankar 2004). It is a passive, solar driven, ecofriendly process bypassing soil excavation and transport, thereby retaining the fertile topsoil and causes less disruption of the ecosystem (Doty 2008; Doran 2009). Plants used in phytoremediation also stabilize contaminated soil and facilitate the growth of microbes colonizing the rhizosphere, which symbiotically degrade and detoxify hazardous compounds (Suthersan 1999; Doty 2008; Doran 2009). The method generates less secondary wastes (Doty 2008) and minimizes leaching of contaminants (Suthersan 1999). It offers certain other beneficial side effects *viz.* carbon sequestration and production of biofuel, wood, pulp that can act as an additional

aid in financing the clean-up process (Stanton et al. 2002; Dietz and Schnoor 2001; Doty et al. 2007). The method is aesthetically beneficial and is highly accepted by public and regulatory agencies (Suthersan 1999; Sadowsky 1999; Doty 2008). Also plant roots can penetrate the microscopic pores in the soil matrix and uptake the contaminants trapped in the pores, which otherwise cannot be remediated by conventional pump and treat systems (Suthersan 1999). Lastly, the technology also helps in providing habitats to wildlife; *Populus* spp. plantations provides shelter to a variety of birds and small mammals (Moser et al. 2002) while *Salix* spp. can act as stopover sites for several species of migratory birds (Kuzovkina and Quigley 2005).

Although phytoremediation appears to be promising, there are certain limitations associated with the technique. As plants are used in environmental clean-ups, it takes a long period of time than other remediation approaches, requiring a long term commitment for maintenance or the process may be effective seasonally. Moreover, contaminants present beyond the rooting depths of plants cannot be remediated using this technology (Suthersan 1999; Doran 2009). Plants are also sensitive to types and concentrations of pollutants present at the waste sites (phytotoxicity) (Doty 2008) and are not able to remove all types of contaminants (Sadowsky 1999), particularly hydrophobic contaminants which tend to remain adsorbed to soil particles (Suthersan 1999). Plants lack the catabolic enzymes needed for metabolizing the organic compounds, normally present in bacteria and mammals (Van Aken 2009). Thus phytoremediation often results in slow and incomplete detoxification, leading to the accumulation of parent compounds and toxic metabolites in the plant which eventually return back to the environment (Van Aken 2008). Often site for disposing toxic vegetation is required (Sadowsky 1999) and the contaminants may also enter the food chain (Doty 2008; Van Aken 2009). Some of the contaminants may also form complexes with plant exudates and are subsequently transported by ground water (Suthersan 1999). Limited knowledge of plant metabolic pathways and the mechanisms involved in tolerance are certain other drawbacks which restrict the use of plants as a potential alternative for environmental remediation. Also, little is known about the enzymes, which transform xenobiotic compounds. A thorough understanding of the primary biochemical and physiological mechanisms is required to genetically modify plants with better ability of pollutant hyperaccumulation. Although much progress has been made in this area, elucidation of interactions between plant cells and toxic chemicals present in the environment is a field that still remains largely unexplored.

27.4 Methods for Improving the Effectiveness of Phytoremediation

To improve the efficiency of phytoremediation techniques, several experimental systems have been employed, which are outlined below.

27.4.1 Plant Tissue Culture

Plant tissue culture has played a crucial role in elucidating the ability of plant cells in detoxification of contaminants. *In vitro* culture is used as model plant systems to study the responses of plant cells to hazardous compounds, the metabolic processes involved in their degradation and the end products formed. The information obtained from plant tissue culture experiments can be used to direct field trials for phytoremediation. In contrast to whole plants, *in vitro* cultures can be maintained indefinitely under axenic conditions. As a result, tissue culture represents a promising tool for differentiating the responses and intrinsic enzymatic capabilities of plant cells from those of the microbes present in the rhizosphere or within the plant tissue (Chaudhry et al. 2005; Lebeau et al. 2008). In fact, the use of plant tissue culture has proved that plant cells are capable of metabolizing a variety of xenobiotic compounds independent of the enzymatic action of the microorganisms residing in the root zone (Fletcher et al. 1987; Goel et al. 1997; Hughes et al. 1997; Van Aken and Schnoor 2002). It also helps in assessing the genetic capabilities of plants in detoxifying compounds. If a compound is metabolized by *in vitro* grown plant cells, it indicates that the parent plant from which the cell is derived possess the genetic information required for transforming the same compound. As callus and plant cells in suspension lack various barriers (*viz.* leaf wax, bark, cuticle, epidermis, endodermis etc.) which otherwise regulate the penetration of compounds in whole plants, *in vitro* culture can result in greater and uniform uptake of compounds than whole plants (Camper and Mcdonald 1989; Lucero et al. 1999). This in turn acts as an added advantage for biochemical and metabolic research as significant quantities of metabolites and intermediates suitable for analysis by mass spectrometry and nuclear magnetic resonance (NMR) can be recovered (Laurent et al. 2007). Also, *in vitro* culture allows experiments to be designed using tissues obtained from the same mother plant, thereby avoiding variability between specimens (Pollard and Baker 1996). The uniformity of *in vitro* cultures and the ease of standardization of culture conditions help in improving the reproducibility of results compared to that of field grown plants. In whole plants, photosynthesis helps in reassimilating the released carbon dioxide into the plant biomass; this masks the efficiency of plants in mineralization reactions (Van Aken et al. 2004). The absence of photosynthesis in tissue cultured cells thus helps in assessing their ability to mineralize organic contaminants to carbon dioxide. Plant tissue culture also represents a vital step in the incorporation of foreign genes into plants for the development of transgenic plants with improved phytoremediation capabilities. *In vitro* culture plays an important role in selecting transformants with improved traits before the tedious process of plant regeneration is carried out. Lastly, plant tissue culture can be tools of immense importance in future genomic, transcriptomic, proteomic or metabolomic analyses related to phytoremediation research (Doran 2009).

Despite being a powerful tool, cultured cells and tissues cannot be directly applied in large scale phytoremediation treatments. *In vitro* cultures need to be maintained aseptically; contamination of cultures by bacteria or fungi leads to the

loss of cell viability and are thus not suitable for phytoremediation of contaminated soil or aquatic areas, which naturally contain various microbes. Being heterotrophic, plant tissue culture requires sugars in the culture medium so that plant cells are outnumbered by faster growing microflora under non-sterile conditions. Maintenance of cultured plant cells in bioreactors in large scales required for environmental clean-ups is also expensive compared to methods where cheap agricultural waste products are used for removal of environmental pollutants. In addition, the outcome of *in vitro* studies may be dependant on the culture conditions applied, time of addition and exposure to a particular contaminant, tissue morphology, degree of cellular differentiation, aggregation and may vary between newly established cultures and those that have been maintained for a long time (Cole and Owen 1987; Canivenc et al. 1989; Schmidt et al. 1997; Kučerová et al. 1999). Thus, plant tissue culture systems mainly act as auxiliary models helping us understand the metabolic and tolerance mechanisms operative in whole plants (Doran 2009).

27.4.2 Transgenic Plants

The efficacy of plants in removing environmental pollution can be enhanced by overexpressing genes involved in the metabolism, uptake or sequestration of specific contaminants. Transgenic plants expressing these heterologous genes from microorganisms, other plants or animals are of potential importance in improving the efficiency of phytoremediation. Bacteria and mammals are heterotrophic organisms possessing enzymes needed for the complete mineralization of organic compounds. Genes encoding these enzymes have been transferred to plants to complement their normal metabolic processes. Transgenic plants harbouring these genes have been shown to possess greater tolerance and enhanced metabolism of hazardous compounds compared to the wild type plants, which can be attributed to a steeper gradient concentration inside the plant and the ability of the plants to overcome phytotoxicity (French et al. 1999; Doty et al. 2000). Reports indicate that transgenic plants also enhanced the activity of microflora in the rhizosphere (Travis et al. 2007). Since phytoremediation involves exposure of plants to contaminants for a long period of time, development of stable transformants is an appropriate approach than using transient expression systems *viz.* plant viral vectors (Doran 2009).

27.4.3 Hairy Roots

27.4.3.1 What Are Hairy Roots?

Agrobacterium rhizogenes, a soil borne gram negative bacterium, incites hairy root disease in higher plants, characterized by an extensive formation of adventitious roots with numerous root hairs at or near the site of infection (Armitage et al. 1988;

Nilsson and Olsson 1997; Sevón and Oksman-Caldentey 2002). The pathogenicity of *A. rhizogenes* is determined by a megaplasmid – the Ri plasmid or the root inducing plasmid (Armitage et al. 1988). The plasmid contains distinct segment(s) of DNA (the transferred DNA or T-DNA) which is transferred to the plant genome (Huffman et al. 1984; De Paolis et al. 1985; Saito et al. 1992; Sevón and Oksman-Caldentey 2002). Infection of wound sites in plants by *A. rhizogenes* is followed by an inter-kingdom transfer, stable integration and expression of T-DNA genes from the Ri plasmid and subsequent development of the hairy root phenotype. The T-DNA genes also direct the transformed plant tissues to produce unusual metabolites known as opines which are condensation products of sugars and amino acids (Armitage et al. 1988; Nilsson and Olsson 1997). These opines are not metabolized by the plants themselves but instead are excreted to the environment where they are consumed by *A. rhizogenes* as sources of carbon and nitrogen (Chilton et al. 1982; Armitage et al. 1988; Nilsson and Olsson 1997). These give *Agrobacterium* a selective advantage over other soil bacteria.

27.4.3.2 The Ri Megaplasmid

The Ri plasmids are large (more than 200 kb) (Riva et al. 1998; Sevón and Oksman-Caldentey 2002) and carry one to several different T-DNAs (Huffman et al. 1984; De Paolis et al. 1985; Nilsson and Olsson 1997). The T-DNA carries a cluster of oncogenes (genes encoding enzymes involved in the synthesis of plant hormones) whose expression in plants lead to neoplastic outgrowths and genes responsible for opine synthesis (Nilsson and Olsson 1997; Tzfira and Citovsky 2006). The T-DNA genes help the plant cell to grow in the absence of exogenous phytohormones (Sinkar et al. 1987). The T-DNA fragments (15–20 kb) [Sevon and Oksman-Caldentey (2002)] are flanked by 25 bp *cis*-acting direct repeats (Armitage et al. 1988; Jouanin et al. 1989) (termed as left and right T-DNA borders) and are separated from each other by ~15 kb non integrated plasmid DNA (Sevon and Oksman-Caldentey 2002). During infection, the different T-DNA fragments can be transferred independent of each other (Vilaine and Casse-Delbart 1987) and get integrated into the plant genome. Any foreign DNA placed between the T-DNA border sequences can be transferred to the plant cell and transcription of T-DNA genes takes place only in the plant tissue. The genes are of bacterial origin but have eukaryotic regulatory sequences allowing their expression in plant cells (Giri and Narasu 2000). The process of T-DNA transfer from the bacteria to the plant cell is mediated by proteins encoded by genes in the 30 kb virulence region (*vir* genes) of the Ri plasmid (Huffman et al. 1984; De Paolis et al. 1985) which do not enter the plant and by a set of bacterial chromosomal (*chv*) genes (Tzfira and Citovsky 2006). The vir region is organized into six operons – *virA, virB, virC, virD, virE* and *virG*. The Ri plasmid also carries genes involved with conjugative transfer and the replicative maintenance of the plasmid within the *Agrobacterium* (Armitage et al. 1988).

The Ri plasmids are mainly classified on the basis of opines synthesized by the hairy roots. Strains *viz.* A4, 15834, LBA9402, 1855 etc. induce roots to synthesize opines like agropine, mannopine and related acids and are known as 'agropine' type strains whereas the 'mannopine' type strains (8196, TR7, TR101 etc.) induce roots to synthesize mannopine and related acids (Rhodes et al. 1990). The agropine type Ri plasmids carry two distinct T-DNA regions designated as TL-DNA and TR-DNA (left T-DNA and right T-DNA) (White et al. 1985) while there is a single T-DNA region in the mannopine type Ri plasmids which is mostly homologous to the TL-DNA of the agropine type Ri plasmid (Gelvin 1990). The TR-DNA of the agropine type Ri plasmid harbours genes encoding synthesis of auxin (*tms1* and *tms2*) and agropine (*ags*) (White et al. 1985; Cardarelli et al. 1985). This segment does not play an essential role in the induction of hairy roots but contains the *aux1* gene that provides an additional source of auxin to the transformed cells (Sevon and Oksman-Caldentey 2002). Mutational analysis of the TL-DNA has revealed the presence of four genetic loci (*rolA, rolB, rolC, rolD*) which affect the development of hairy roots (White et al. 1985; Palazón et al. 1997). The complete nucleotide sequence of the TL-DNA led to the identification of eighteen open reading frames (ORFs); four of these ORFs – 10, 11, 12, 15 correspond to the *rolA, rolB, rolC,* and *rolD* respectively (Slightom et al. 1986; Sevon and Oksman-Caldentey 2002). The *rolB* gene plays the central role in the differentiation process leading to the development of hairy roots while *rolA* and *rolC* have accessory functions (White et al. 1985; Palazón et al. 1997).

27.4.3.3 Molecular Mechanism of Interaction Between *A. Rhizogenes* and the Plant Cell

The earliest step in the transformation process is the attachment of the bacteria to the surface of a wounded plant cell (Matthysse 1986). The wounded cell releases certain signal compounds *viz.* small phenolics like acetosyringone and other monosaccharides acting synergistically with phenolic compounds which are detected by the VirA sensory protein (Cangelosi et al. 1990; Winans 1992; Pan et al. 1993). VirA subsequently transphosphorylates the VirG protein (Jin et al. 1990a, b) which in turn activates the other *vir* genes. The activation of *vir* genes depends largely on external factors like temperature, pH and are not expressed at temperatures greater than 32°C due to a conformational change of VirA leading to its inactivation (Jin et al. 1993). The activated *vir* genes generate single stranded (ss) molecules of DNA representing the bottom strand of the T-DNA. Proteins VirD1 and VirD2 recognize the T-DNA border sequences and nick the bottom strand at each border by endonuclease activity generating the 'T-strand'. VirD2 molecule remains covalently attached to the 5′ end of the T-strand forming the 'T-complex'; this attachment prevents exonucleolytic attack to the 5′ end of the T-strand and makes this end the leading end during the transfer process. A VirB/D4 type IV secretion system transfers this T-complex associated with several other Vir proteins to the host cell (Christie 2004). The entire length of the T-complex is then coated inside the host

cell cytoplasm by VirE2 protein to generate the mature T-complex which prevents the complex from nuclease attack and confers the structure needed for the transport this complex to the host cell nucleus (Citovsky et al. 1989; Abu-Arish et al. 2004). Once inside the nucleus, the T-complex travels to its point of integration into the host genome and before integration all the accessory proteins are stripped off (Tzfira and Citovsky 2006). The exact mechanism leading to the integration of the T-strand into the host genome is not yet characterized (Tzfira et al. 2004). However, it has been assumed that the host DNA repair machinery converts the T-strand to double stranded T-DNA intermediates and incorporates the fragments to the host genome (Tzfira and Citovsky 2006).

The exact physiological basis for the expression of hairy root phenotype is still unclear; alteration of auxin metabolism in the transformed cells has been proposed to play a crucial role in the developmental process (Zambryski et al. 1989; Gelvin 1990).

27.4.3.4 Distinctive Features of Hairy Root Cultures

Hairy root cultures are highly branched and are capable of growing fast on hormone free medium (Sevon and Oksman-Caldentey 2002). The roots are plagiotropic and remain genetically and biochemically stable over long periods of time (Sevon and Oksman-Caldentey 2002). These roots have proven to be an effective means of producing compounds that are normally biosynthesized in the roots of plants at levels comparable to or higher than that of whole plants (Alvarez et al. 1994; Lorence et al. 2004). Whole plants can be easily regenerated from hairy roots, thereby transmitting the Ri T-DNA genes to the progeny (David et al. 1984; Tepfer 1984).

27.4.3.5 Role of Hairy Roots in Phytoremediation

As mentioned above, the ability of hairy roots to grow fast in hormone free, aseptic conditions and their genetic and biochemical stability make them excellent tools for phytoremediation. Because of their fast growth rates and branched nature, these roots provide a large surface area of contact between the contaminant and the tissue (Eapen et al. 2003; Suza et al. 2008) compared to wild type roots. The organized nature of the roots makes them amenable to scale up in bioreactors (Doran 1997; Suresh et al. 2005) and the biomass can be used for environmental clean-up. Hairy root cultures are important means for selecting plant species for contaminant uptake. They also help in assessing the effect of environmental factors on growth of plant tissues and accumulation of the pollutant. The absence of shoots helps in understanding the mechanisms present only in the roots for remediation of contaminants without the effects of translocation. As they are genetically stable, foreign genes introduced in hairy roots by transformation can be expressed for a long term and the resultant functional proteins can metabolize environmental pollutants (Banerjee et al. 2002). These proteins are preferred over bacterial and yeast proteins as they

are fully functional and do not need post translational modifications (Fischer et al. 1999). Use of hairy roots for phytoremediation offers certain other advantages also. The enzymes and metal chelating compounds found in the exudates of hairy roots can be used to detoxify or sequester harmful compounds (Gujarathi and Linden 2005; Doty 2008), thereby the roots can be recycled for future use. Hairy roots thus provide useful information for the use of selected enzymes or extracts containing these enzymes in environmental clean-up processes. These roots are also potential tools to better understand the enzymatic machinery involved in the bioconversion of toxic pollutants to non-toxic metabolites (Macek et al. 2000) and the mechanism involved in metal tolerance and hyperaccumulation; this in turn will help in designing novel transgenic plants with improved detoxification traits. In addition, hairy roots help in quick screening of cultures harbouring the gene of interest involved in the metabolism of environmental contaminants before they are regenerated into whole plants.

Reports indicated that hairy root cultures of several plant species have been used successfully to study uptake and degradation of various environmental pollutants (Table 27.1). Phenols, chlorophenols and polychlorinated biphenyls are categorized as major classes of hazardous pollutants (Wentz 1989; Harvey et al. 2001; Rezek et al. 2007). Phenols are present as contaminants in aqueous effluents from coal conversion processes, coke ovens, petroleum refineries, in waste waters emanating from industries manufacturing fiberglass, fungicides, herbicides, insecticides, slimicides, pesticides, disinfectants, antiseptics etc. (Singh et al. 2006 ; Araujo et al. 2006 ; González et al. 2006 ; Coniglio et al. 2008) at concentrations ranging from 10 to 1,000 mg/l (Buchanan and Nicell 1997). Phenolic compounds can also be released via the partial degradation of aromatic contaminants *viz.* polycyclic hydrocarbons, certain surfactants etc. (Huang et al. 2005). Being toxic, carcinogenic, mutagenic and teratogenic (Autenrieth et al. 1991) phenols pose a major threat to human health. Exposure to phenols may also cause liver damage and haemolytic anaemia (Araujo et al. 2006) and they are listed as priority pollutants in the US EPA list (US EPA 2004). Physical and chemical methods like adsorption on activated charcoal, steam distillation, irradiation, oxidation, incineration etc. have been employed for the removal of phenol from contaminated areas; but the methods have certain limitations like low efficiency, high cost of implementation or generation of metabolites that are even more toxic than the starting phenols. Thus phytoremediation strategies were considered as potential alternatives for the detoxification of these hazardous pollutants from the environment. In order to fully exploit the technology it is necessary to screen plant species capable of removing phenolic pollutants and thus hairy root cultures of different plant species have been studied by researchers.

Singh et al. (2006) screened the effect of hairy roots of four plant species namely *Brassica juncea, Beta vulgaris, Raphanus sativus* and *Azadirachta indica* on the removal of phenol from the culture medium. Among the four species tested, hairy roots of *B. juncea* showed the highest (97%) potential for removal of phenol followed by *B. vulgaris* (70%), *R. sativus* (54%) and *A. indica* (51%). On being exposed to phenol, peroxidase activity (enzymes involved in oxidative polymerization of

Table 27.1 Hairy roots of plant species utilized to study phytoremediation

Plant species	Pollutant	References
Catharanthus roseus	TNT, RDX	Hughes et al. (1997) and Bhadra et al. (2001)
Medicago sativa	Anthracene	Paul and Campanella (2000)
Hyptis capitata	Cu	Nedelkoska and Doran (2000a)
Thlaspi caerulescens	Cd	Nedelkoska and Doran (2000b)
Nicotiana tabacum, *Atropa belladona*		
Solanum nigrum	Polychlorinated biphenyls	Kučerová et al. (2000) Rezek et al. (2007)
Adenophora lobophylla, *A. potaninii*	Cd	Wu et al. (2001)
Alyssum bertolonii, A. tenium, A. troodii	Ni	Nedelkoska and Doran (2001)
Atropa belladona	Trichloroethylene	Banerjee et al. (2002)
Chenopodium amaranticolo, *Brassica juncea*	Uranium	Eapen et al. (2003)
T. caerulescens, A. bertolonii	Cd, Ni 2,4-dichlorophenol	Boominathan and Doran (2003)
Brassica napus	Phenol	Agostini et al. (2003), and Coniglio et al. (2008)
Cichorium intybus, B. juncea	DDT	Suresh et al. (2005)
Helianthus annuus	Oxytetracycline	Gujrathi and Linden (2005)
B. juncea, Beta vulgaris, *Raphanus sativus*, *Azadirachta indica*	Phenol	Singh et al. (2006)
Daucua carota, Ipomoea batatas, *Solanum aviculare*	Phenols, chlorophenols	Araujo et al. (2006)
Lycopersicon esculentum	Phenol	Wevar Oller et al. (2005); González et al. (2006)
Alyssum murale	Ni	Vinterhalter et al. (2008)

phenols) was enhanced in the roots. Also addition of hydrogen peroxide (H_2O_2) (a prerequisite for peroxidase activity) to the culture medium was not required as H_2O_2 was synthesized *in situ* and the levels of H_2O_2 was increased in presence of phenol. But when phenol was added in four repetitive cycles over a period of 12 days, a gradual decrease in phenol removal efficiency, from 97% in the first use to 34% in the third use, was noted.

Araujo et al. (2006) investigated the efficacy of hairy roots of some other species of plants *viz. Daucus carota, Ipomoea batatas* and *Solanum aviculare* in uptaking phenols and chlorophenols from the liquid media. Roots of *D. carota* could tolerate

1,000 μmol/l phenol in the culture medium showing normal growth rates while hairy roots of *I. batatas* and *S. aviculare* could grow normally when phenol was present upto a concentration of 500 μmol/l. In contrast to phenols, chlorophenols were highly toxic for root growth and roots grew in presence of only 50 μmol/l chlorophenols in the media. The roots were also highly efficient in removing phenols and chlorophenols from the culture media. Among the three species tested, *S. aviculare* appeared to be the most efficient in the uptake of phenol, removing 98.6% from the medium in a span of 72 h followed by *I. batatas* (90.7% phenol removal) and *D. carota* (72.7% phenol removal). Whereas hairy roots of *D. carota* could remove 83.0% initial concentration of 2,6-dichlorophenol (2,6-DCP) in 72 h, roots of *S. aviculare* and *I. batatas* removed 73.1% and 57.7% of the compound respectively. At the end of the experimental period (240 h) almost 100% of the initial concentrations of phenol and more than 90% 2,6-DCP were removed from the medium in presence of the hairy roots. Inherent peroxidase activity was highest in *I. batatas*, being twofold higher than *S. aviculare* and sevenfold higher than in *D. carota* in presence of phenol. Labeling of phenols with ^{14}C indicated that after absorption they were stored in the roots in a highly polar conjugated form and were probably incorporated into the cell walls and membranes.

Hairy roots of tomato (*Lycopersicon esculentum*) derived from inoculation of sterile leaf explants with *A. rhizogenes* strain LBA 9402, proved to be another promising candidate for the removal of phenol from water (González et al. 2006). The authors optimized conditions for maximum efficiency in phenol removal and to minimize inactivation of enzymes likely to be involved. Phenol present at a concentration of 100 mg/l of the solution was removed by the roots in presence of 5 mM H_2O_2. Rates of removal were high within the first hour of incubation, reaching 95% of efficiency at the end of 5 h, over a wide pH (4.0–9.0) and temperature range (20–60°C). Removal efficiencies and kinetic studies of enzyme extracts and purified isoenzymes revealed that basic peroxidase isoenzymes were catalytically more efficient than the acidic ones in the removal process. Tomato hairy roots have also been used for the overexpression of peroxidase, which increased phenol removal by ~20% (Wevar Oller et al. 2005).

In yet another study, Coniglio et al. (2008) obtained hairy roots of *Brassica napus* following infection with *A. rhizogenes* strain LBA 9402, capable of producing peroxidases in the roots and culture medium. Roots grew normally in presence of low concentrations of phenol (10–50 mg/l). Addition of 100 mg/l phenol induced symptoms of phenol toxicity and root growth was inhibited. The authors suggested that disruption of membrane due to hydrophobic interactions with phenol might have led to toxicity. Yet, the roots could remove 90% phenol at concentrations upto 500 mg/l in the presence of exogenously added H_2O_2 (10 mM). The authors also observed a negative effect of higher H_2O_2 concentration on phenol removal. However, high removal efficiency was reached with 1 h of treatment. The fast rate of removal of pollutants is advantageous when compared with other methods like biological oxidation by bacteria which requires long periods of treatment (weeks or even months) due to adverse effects of the contaminants on microbial ecosystems (Nakamoto and Machida 1992) However, similar to the results of

Singh et al. (2006), when reused for five consecutive cycles, a gradual decrease in phenol removal efficiency of *B. napus* hairy roots (99% in the first use to 20% in the fourth use) was noted. The authors suggested that the reduction in peroxidase activity could be due to inactivation caused by H_2O_2 concentrations, irreversible reactions between peroxidases and phenyl or phenoxy radicals, due to adsorption of the final polymer on the peroxidases making the access of the substrate difficult to the enzyme's active site or due to adsorption of the polymer over the roots, limiting the reuse of biomass.

The above mentioned studies indicated that isolation of enzymes might not be necessary for decontamination processes and hairy root masses might be used as inexpensive sources of enzymes for dephenolization of waste waters.

Chlorophenols are particularly recalcitrant to degradation and are highly toxic. 2,4-dichlopophenol (2,4-DCP), used in the manufacture of industrial and agricultural products like herbicides (*viz.* 2,4-dichlorophenoxyacetic acid), pesticides, germicides, resins, antiseptics (Agostini et al. 2003) is discharged as a contaminant in industrial effluents. Long term exposure to this compound may permanently damage liver, skin, eyes, kidneys and are suspected to be carcinogens (Edwards and Santillo 1996). As mentioned earlier, hairy root cultures of *Brassica napus* are capable of producing peroxidases in the roots and culture media. These roots were used as an inexpensive system for the removal of 2,4-DCP (100–1,000 mg/l) from aqueous solutions in the presence of external H_2O_2 (Agostini et al. 2003). In a relatively short incubation period of 1 h, 97–98% removal of 2,4-DCP was achieved and over a pH range of 4.0–8.0, 98–99% of the pollutant was removed. In contrast to the results of Singh et al. (2006) and Coniglio et al. (2008), the roots could also be reused successively for six cycles for the removal of 2,4-DCP (with an efficiency of ~90% after six cycles) which is an important feature for the large scale use of these roots for continuous detoxification purposes.

Polychlorinated biphenyls (PCBs) are another class of recalcitrant pollutants frequently encountered in the soil. PCBs are used in different industrial applications *viz.* in the production of electrical transformers, capacitors, in hydraulic systems, gas turbines, vacuum pumps, paints, plasticwares etc. The PCBs enter the food chain and affect human beings after being accumulated in the fat tissue (Skaare et al. 2002; Petrik et al. 2006). Kučerová et al. (2000) and Rezek et al. (2007) demonstrated that hairy root cultures of *Solanum nigrum* (clone SC90) could successfully transform a wide range of PCB congeners examined.

Hairy roots cultures have also been used for the uptake and degradation of insecticides. DDT [1,1,1-trichloro-2,2-bis-(4′-chlorophenyl)ethylene] is widely used for controlling pests because it is relatively cheap, has a broad spectrum of activity, is easy to formulate, and has high residual biological activity (Turusov et al. 2002). However, exposure to DDT through the food chain can cause cancer and endocrine abnormalities in human beings. Because of its negative impact on human health, environmental persistence and water insolubility it is necessary to develop methods for its remediation. Endogenous enzymes from hairy root cultures of *Cichorium intybus* and *Brassica juncea* were used for the efficient uptake and degradation of this persistent insecticide to various other products like DDD [1,1-dichloro-2,

2-bis-(4′-chlorophenyl)ethane], DDE [1,1-dichloro-2,2-bis-(4′-chlorophenyl)ethylene] and DDMU [1-chloro-2,2-bis-(4′-chlorophenyl) ethylene] (Suresh et al. 2005).

An interesting study was conducted by Banerjee et al. (2002) who showed that *Atropa belladona* hairy roots expressing a heterologous protein were able to metabolize the pollutant trichloroethylene (TCE). Cytochrome P450 2E1 is a mammalian liver enzyme known to metabolize a wide range of xenobiotic compounds; among these is TCE, a major environmental pollutant (Costa et al. 1980 ; Guengerich et al. 1991). *A. rhizogenes* strain A4 harbouring a binary vector with rabbit P450 2E1 was used to transform *A. belladona.* The hairy roots expressed the mammalian protein which was capable of oxidizing TCE to chloral and trichloroethanol (TCOH). But the protein was not exuded in the culture medium and remained associated with the root biomass, indicating that further research in this field may open up perspectives in isolating pollutant decontaminating proteins from the culture medium, thus recycling roots for future use.

Till date, the mechanisms of metal accumulation and the metabolic responses of hyperaccumulating plants are poorly understood. Since current research is focused on genetically modifying high biomass plants with genes which could improve tolerance to metals or increase their metal accumulating potential, the molecular and physiological basis of hyperaccumulation and tolerance to heavy metals must be known. Hairy roots have proven to be effective tools in the study of metal hyperaccumulation as well.

Cadmium (Cd) is a heavy metal found in fertilizers, sewage sludges, compost and derived from processes like mining, smelting and electroplating. It is phytotoxic, disrupts aquatic and terrestrial ecosystems and causes health hazards in human beings and animals (Prasad 1995; Oliver 1997). Hairy root cultures of the hyperaccumulating plant *Thlaspi caerulescens* derived from infection of seedling explants with *A. rhizogenes* strain 15,834 were used to study the effectiveness of such cultures to uptake Cd from liquid solution (Nedelkoska and Doran 2000b). When compared with hairy roots of non hyperaccumulator species (*Nicotiana tabacum* and *Atropa belladona*) Cd accumulation in *T. caerulescens* hairy roots was 1.5–1.7 fold greater than in hairy roots of *N. tabacum* or *A. belladona* exposed to the same initial Cd concentration. *T. caerulescens* hairy roots remained healthy and grew in presence of upto 100 ppm Cd, whereas roots of *N. tabacum* turned brown and growth was inhibited when exposed to 20 ppm Cd. Hairy roots of *T. caerulescens* accumulated upto 10,600 ± 300 μg/g dry weight Cd which was well above the threshold value of 100 μg/g for Cd hyperaccumulation status (Baker et al. 2000). Analysis of cell wall fractions and Cd levels in whole roots revealed that in *N. tabacum* most of the metal taken up was transported directly into the symplasm within 3 days of exposure to Cd whereas Cd was stored in the wall fraction for the first 7–10 days in *T. caerulescens* hairy roots before passing to the symplasm. This delay in transport helped in the activation of detoxification mechanisms, thereby protecting *T. caerulescens* from Cd poisoning.

Hairy roots of *T. caerulescens* were also utilized by Boominathan and Doran (2003) in another study to demonstrate that even though growth is unaffected in the presence of heavy metals, metal-induced oxidative stress occurs in hyperaccumulating plants. Also, antioxidative defense mechanisms play a crucial

role in the response of hyperaccumlating plants to heavy metals. The study also revealed that in order to improve Cd tolerance in high biomass plants like *N. tabacum* overexpression of catalase and/or superoxide dismutase with other molecular approaches might be important.

Reports indicate that organic acids are associated with tolerance, transport and storage of heavy metals like Al, Cd, Fe, Ni and Zn (Godbold et al. 1984; Krotz et al. 1989; Ma et al. 2001; Nigam et al. 2001). In hyperaccumulator species, increase in concentration of Ni or Zn in the biomass have been correlated with levels of citric, malic, malonic and oxalic acids (Tolrà et al. 1996). High constitutive levels of citric, malic and malonic acids were found in hairy roots of *T. caerulescens* and *Alyssum bertolonii* accumulating Cd and Ni respectively (Boominathan and Doran 2003). But after being exposed to 20 ppm Cd and 25 ppm Ni, only 13% of total Cd and 28% of total Ni were associated with organic acids of the respective roots. As reported earlier (Nedelkoska and Doran 2000b), Cd was mostly localized in the cell walls of *T. caerulescens* whereas 85–95% Ni was associated with symplasm in *A. bertolonii* hairy roots. When treated with diethylstilbestrol (DES), an inhibitor of plasma membrane H^+-ATPase, roots of *T. caerulescens* remained viable and the concentration of Cd in the symplasm was increased by about sixfold, whereas Ni transport across the plasma membrane and root viability were reduced in *A. bertolonii*. These results demonstrated that *T. caerulescens* hairy roots can tolerate and hyperaccumulate Cd in spite of the effects of plasma membrane depolarization but mechanisms of Ni tolerance and hyperaccumulation are negatively affected in the hairy roots of *A. bertolonii*.

Uptake of Cd by hairy root cultures has been further investigated by Wu et al. (2001) who used hairy root cultures of two species of *Adenophora viz. A. lobophylla,* which is endangered and *A. potaninii* which has a wide distribution in the same habitat. When exposed to Cd concentrations higher than 50 μM, Cd accumulation was higher in the hairy roots of *A. lobophylla*. Their study demonstrated that Cd detoxification strategies were different in the two species. While *A. lobophylla* synthesized higher levels of phytochelatin, an exclusion system for Cd and a tight homeostasis mechanism to maintain cellular GSH (reduced glutathione) might have evolved in *A. potaninii* together with its ability to synthesize phytochelatins. The studies thus provided a further insight into the process of Cd tolerance and hyperaccumulation.

Nedelkoska and Doran (2000a) demonstrated that Cu uptake in hairy root cultures of *Hyptis capitata* was biphasic showing a rapid accumulation in the initial phases till day 14 after which there was a steady increase of Cu content in the root biomass. Uptake of Cu continued to increase reaching 800 ± 73 μg/g dry weight, representing a concentration factor of 40; $99.7 \pm 1\%$ Cu was recovered from the experiment. At the end of 28 days, hairy roots of *H. capitata* could remove 54% of the initial concentration of Cu added.

Reactive oxygen species (ROS) generated as a part of the defense response in plants subjected to stress is also used to degrade organic contaminants. Gujrathi and Linden (2005) reported that oxytetracycline was oxidized through the reaction of ROS released in the culture medium by hairy root cultures of *Helianthus annuus*.

Nedelkoska and Doran (2001) transformed several species of *Alyssum viz. A. tenium, A. bertolonii, A. troodii* and showed that the hairy roots could hyperaccumulate Ni. Interestingly, plants regenerated from hairy roots of *A. tenium* accumulated more Ni and tolerated higher concentrations of the metal than the hairy roots of the same plant. *A. rhizogenes* strain A4M70GUS was used to develop hairy roots of another species of *Alyssum*, *A. murale* (Vinterhalter et al. 2008); upto 24,700 μg/g dry weight Ni was accumulated by shoots regenerated from the hairy root cultures.

Brassica juncea and *Chenopodium amaranticolor* are capable of uptaking heavy metals from aqueous solutions. Hairy roots of these two species were used to remove uranium from solutions (Eapen et al. 2003). Ninety percent uranium was taken up by the roots within 10 h of treatment. Among the two species tested, hairy roots of *B. juncea* could take up 2–4 times more uranium from solution at a concentration of 5,000 μM and thus proved to be a promising candidate for the removal of this heavy metal and radionuclides from contaminated solutions.

Hairy root cultures of *Catharanthus roseus* have also been exploited for the study of degradation of explosives like TNT (2,4,6-trinitrotoluene), RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine or the royal demolition explosive) to other metabolites (Hughes et al. 1997; Bhadra et al. 2001) and *Medicago sativa* roots were used in the biotransformation of anthracene (Paul and Campanella 2000).

27.5 Conclusions and Future Perspectives

Over the past several years phytoremediation has emerged as a proven technology that has been effectively used for treating hazardous waste sites. A survey of literature demonstrates that significant progress has been made in the field of phytoremediation research to improve the efficiency of the process. Especially, genetic engineering has opened up new perspectives for phytoremediation. Hairy roots, generated by infection of plants by *A. rhizogenes*, are versatile experimental systems that have been exploited to study detoxification of xenobiotics. They have been used as biological models to assess the physiological characteristics of plants suitable for phytoremediation. The roots have proven to be excellent tools to screen plant species with superior traits for environmental clean up – a critical step in the process. The plants can now be collected, cultivated and a germplasm can be established for mass production for future use. In some cases, the roots have also helped in elucidating the mechanisms underlying pollutant uptake and degradation. Yet, to fully exploit phytoremediation for cleaning up hazardous wastes, a thorough understanding and identification of the rate limiting steps involved in uptake of pollutants, their transport and transformation are required. As hairy roots are products of transformation, introduction of multiple genes which can act simultaneously on a wide range of pollutants present at a waste site and on different bioremediation processes might further improve the technology. Also, specific genes affecting pollutant uptake and degradation have to be identified. The roots can act as potential tools for

the overexpression of plasma membrane transporters and heavy metal binding proteins. A better knowledge of the interaction between plants, microbes and the pollutants might also help in a wider application of the technology to the remediation of contaminated sites. Designing of experiments more judiciously might further lead to the utilization of results for practical large scale applications. Despite the tremendous potential of hairy root cultures in phytoremediation research, the challenge remains to utilize the results of laboratory experiments to actual field trials.

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Chapter 28
Biosurfactant-Assisted Bioaugmentation in Bioremediation

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Abstract Surface active compounds (SACs) are basically amphipathic in nature, which alter the properties of fluid interfaces, partition at interface between fluid phases leading to formation of micro-emulsion and impart better wetting, spreading, foaming and detergent traits, thereby rendering them as most versatile process chemicals to be utilized in surfactant-enhanced bioremediation practices. Use of chemical surfactants as an additive, however, warrant (i) toxicity, (ii) carcinogenicity, (iii) non-biodegradibility, (iv) bioaccumulation and (v) inconsistent performance with slow desorption kinetics. Therefore, attention has been focused on alternative amphiphilic surfactants of biological origin, which have predilection for interfaces of dissimilar polarities (liquid-air/liquid-liquid) and are soluble in both organic (non-polar) and aqueous (polar) milieu. The mechanisms of biosurfactant-assisted bioaugmentation in bioremediation include: (i) lowering of interfacial tension, (ii) biosurfactant solubilization of hydrophobic contaminants, and (iii) the phase transfer of pollutants from soil-sorbed to *pseudo*-aqueous phase. Hence, microbial surfactants have potential attributes as alternative to synthetic surfactants.

This article reviews key aspects of microbial tensioactives for applications in bioremediation and biodegradation of environmental pollutants with focus on properties and physiological roles, followed by its laboratory, field demonstrations and full-scale applications. Finally, it is concluded with a concise appraisal on *in situ* and *ex situ* biosurfactant-assisted bioaugmentation, along-with impediments and future challenges.

Keywords Biosurfactant • Bioaccumulation • Bioaugmentation • Biosparging

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28.1 Introduction

Pollution is an inadvertent introduction of elements, compounds, or energy into the environment at concentrations that impair its biological functioning or present an unacceptable risk to humans or other target species in the environment. In tandem with phenomenal growth in manufacturing, processing, handling of chemicals and human interference have instigated tremendous pollution of the environment with wide variety of recalcitrant/persistent, hazardous and toxic anthropogenic organic pollutants. Several of these hydrocarbon chemicals have been obtained from petroleum; some synthesized in large volume and substantially differs in chemical structure from natural organics. Many of these chemicals have high degree of carcinogenic potential to bio-accumulate in living system, recalcitrant to biodegradation and persistent in the eco-system for longer duration to cause severe environmental problems. Due to possible potential threats, pollution have now been the most serious environmental concerns in the world and yet, in many respects, least understood. Such concerns have realized the necessity of advanced approach to de-contaminate the affected environment. For this reason, pollution has received the wider consciousness as a part of the burgeoning 'greening' of society (Kundu et al. 2010a, b).

A wide variety of hazardous and toxic hydrocarbons appeared to be the most ubiquitous and priority pollutants due to its application as (i) petroleum derivative fuels and (ii) synthetic precursors in large volumes. Each year, about 1,680,000 gal (~40,000 barrels) of crude oil is spilled from pipeline failure and more than 200,000 from underground storage tanks in the US causing major environment hazards (Huesemann 2004). Oil spill alone accounts for ~ 15% of all pollution incidents in England with about nine incidents per day (Environment Agency 2006) resulting in one million tonnes of oil spillage into terrestrial ecosystems every year (Stroud et al. 2007; Ripley et al. 2002).

Several other hydrocarbons including trichloroethylene (TCE), poly-chlorinated bi-phenyls (PCB), poly-cyclic aromatic hydrocarbons (PAH), benzene, nitro-aromatics, etc. are synthesized in large volumes, released into the environment deliberately, persist in the environment for prolong time, and identified as most hazardous priority pollutants. It is followed by trace metals contamination of the environment. The local concentrations of these pollutants depend on the rate at which the compound is released, its stability, mobility in the environment and its rate of biotic and abiotic removal. Despite of stringent enforcement of regulations, several countries have simply ignored it in order to keep pace with the economic and industrial growth (Dua et al. 2002). Technologies from physical to biological removal approaches for contaminants have been discussed in earlier reviews about their feasibility and economic aspects. However, conventional approaches for treatment of pollutants are not only technically challenging but also cost-intensive and hence, increasing consideration has been focused on development of alternative, economical and reliable biological amelioration.

28.2 Bioamelioration: Restoring Eco-habitats

Microbial communities with great biodiversity, catabolic potential, significant role in nutrient cycling have been explored for the biodegradation of toxicants through (i) catabolic genes and enzymes (Khomenkov et al. 2008) and (ii) acclimation strategies, viz. (a) capacity to tailor the cellular membrane for necessary biological functions (de Carvalho et al. 2009), (b) production of surface-active biosurfactants (Ron and Rosenberg 2002) and (c) potential efflux pumps to overcome passively internalized toxicants (Van Hamme and Urban 2009; Van Hamme et al. 2003). However, the magnitude of microbial degradation of toxicants depend on (i) environment (pH, temperature etc.), (ii) nutrient availability and oxygen, (iii) microbial interactions, (iv) cellular transport properties, (v) degree of acclimation, (vi) chemical complexicity of toxicants and (vii) chemical partitioning in medium. Knowledge pertinent to these bottlenecks of bioremediation can provide tools to (i) optimize, (ii) control key parameters and (iii) make the process more reliable. Hence, fresh spurt in bioremediation processes has recognized microbial potential for decontamination of toxicants. Now, bioremediation is considered as a safe, less expensive approach for removal of toxicants.

Amelioration technologies can be divided into two groups based on the physical location of the remedial action: (i) *in situ* remediation, where treatment of the contaminated media takes place by actions in its actual location in the subsurface, and (ii) *ex situ* remediation, wherein contaminated media is removed from the site for subsequent treatment in an above ground treatment facility (on-site) or disposal elsewhere (off-site) (Gerhardt et al. 2009). Ultimate objectivity of both technologies is to degrade organic chemicals to concentrations below the permissible limits established by regulatory authorities and preferably to undetectable levels (Kulkarni and Chaudhari 2007). Amelioration technologies for *in situ* removal of contaminants include: soil washing, soil vapor extraction, landfarming, composting, biopiles, bioventing, bioslurping and biosparging which are time tested and generally cost-effective. In case of *in situ* technologies viz. (i) natural attenuation (NA) and enhanced natural attenuation (ENA), (ii) biostimulation, (iii) bioaugmentation and (iv) phytoremediation are being heralded for biodegradation (Bombach et al. 2010). The choice of a remedial strategy for a contaminated site entails economical and environmental consequences for the local, regional, and global environment.

Microbial remediation has distinct merits over physico-chemical removal methods which include: (i) least expensive, (ii) flexibility and adaptability to edaphic conditions, (iii) environmentally benign and eco-friendly, (iv) better public acceptability as achieve complete degradation of pollutants without collateral damage to the eco-system, and (iv) on site implementation, indeed often *in situ*, and with dilute or widely diffused contaminants. These environmentally compatible features rendered remediation services market represent 4% of the US$ 213 billion annual environmental industry market, which supports the expansion in near future (Ward 2004).

28.3 Bioaugmentation: An Emerging Trend

Bioaugmentation, involve the use of degradative microbial consortia (Dejonghe et al. 2001) or the augmentation of catabolically-relevant organisms to hasten remediation (Thompson et al. 2005). On the contrary, biostimulation encompasses application of indigenous microbe(s) adapted to the contaminated environment of the site that is being treated (Tyagi et al. 2010). Such approach is not always effective and may need a much longer time because of the scarcity of indigenous microbe(s) capable of degrading high concentrations of the pollutants. Hence, bioaugmentation treatment has been regarded as a promising technology (El Fantroussi and Agathos 2005) for sites that (i) do not have sufficient microbial cells or (ii) the native population do not possess the metabolic apparatus necessary to catabolize the toxicants under concern (Tyagi et al. 2010).

Thematically, bioaugmentation entails two approaches: (i) allochthonous, wherein foreign microbes (single/consortia) are introduced into the target site (soil, sand, and water) and (ii) autochthonous bioaugmentation i.e. use of microbes indigenous to the sites to be decontaminated (Vogel and Walter 2001; Ueno et al. 2007). Practical utility of allochthonous technology is limited because of (i) complex environments to potent microbes, particularly when isolated from sites other than the ones being decontaminated, (ii) effective bioaugmentation in the early phases due to high population density of allochthonous microbes, (iii) rapid loss of dominance of single strains and (iv) poor public acceptability for foreign and genetically engineered microbes (Hosokawa et al. 2009). On the other hand, autochthonous makes use of indigenous microbial consortia or, better adapted isolates to the historically/artificially contaminated environments (Hosokawa et al. 2009).

Inspite of success, application of allochthonous/ autochthonous bioaugmentation (Hosokawa et al. 2009) appeared limited due to problems like (i) adaptation of inoculated microorganisms, (ii) insufficiency of substrate, (iii) competition between introduced and indigenous biomass, (iv) use of other organic substrates in preference to the pollutant, and (v) predation protozoa in food chain (Tyagi et al. 2010).

Alternative bioaugmentation approaches to increase the prevalence and activity of exogenous microorganisms and/or genes following introduction into the contaminated environment include: (i) bioaugmentation with cells encapsulated in a carrier, (ii) gene bioaugmentation where added inoculants transfer remediation genes to indigenous microorganisms, (iii) rhizosphere bioaugmentation where the microbial inoculant is added to the site along with a plant that serves as a niche for the inoculant's growth, and (iv) phyto-augmentation, where the remediation genes are engineered directly into a plant for use in remediation without a microbial inoculants (Gentry et al. 2004). These approaches are still at experimental level. For efficient use of bioaugmentation, due consideration to variety of parameters are required.

Hence, seeding alone should be accompanied by suitable physical and environmental alterations so as to enhance the bioavailability of polllutants (Leahy and Colwell 1990; Gentry et al. 2004).

28.4 Chemical Surfactants: A Solution to Pollution?

Based on the initial realization of enhanced benefit of biodegradation rate, synthetic surfactant enjoyed the coveted role as an external biostimulation factor. Chemical surfactants are basically amphipathic compounds, which partition at interface between fluid phases leading to formation of micro-emulsion, to impart better wetting, spreading, foaming and detergent traits, rendering them as most versatile process chemicals (Mulligan 2005). Use of petrochemical-based surfactants as mobilizing agents for soil flushing and washing, mostly employs synthetic surfactants with hydrophobic parts of paraffins, olefins, alkylbenzenes, alkylphenols and alcohols; the hydrophilic part is usually a sulphate, a sulphonate, or a carboxylate (anionic surfactants), a quaternary ammonium (cationic surfactants), polyoxyethylene, sucrose, or polypeptide (non-ionic) group.

More often than not, from environmental and industrial perspectives, applications of these compounds are discouraged due to the following reasons: (i) disruption of cellular membranes by interaction with lipid components, (ii) reactions of surfactant molecules with proteins essential to the functioning of the cell (Helenius and Simons 1975), (iii) inhibitory effect especially in concentrations above the critical micelle concentration (CMC) due to reduced availability of micellar substrates (Volkering et al. 1998), (iv) negative effects caused by (a) depletion of minerals or oxygen, (b) toxicity of surfactant intermediates than the parent compounds (Holt et al. 1992), (c) preferential degradation of the surfactant, slowing the pollutant degradation (Tiehm 1994), (v) decreased microbial mobility, and (vi) lowered bioavailability by inhibiting bacterial attachment, dispersing soil colloids causing clogging of pores, or interfering with the natural interactions of microbes with the pollutant.

In order to alleviate potential risks and increased environmental awareness among the consumers, cost and public-regulatory perception of sustainable harmony with global environment, oleochemical surfactants viz. natural biosurfactants are seen as a better alternative to the existing chemical surfactants for bioaugmentation tool (Hazra et al. 2010a).

28.5 Biosurfactant: A Balancing Act

Threaded through the theme of molecular commonality, amphipathic molecules characterized by hydrophobic and hydrophilic, or non-polar and polar regions, are common and essential due to life's aqueous milieu. At the cellular and physiological level, single and multi-cellular life forms evolved amphipathic lipid bilayers, transmembrane sensory proteins, electron transport chain proton and sodium motive pumps, flagellar motors and internal membranes to create a controlled environment for biomolecular synthesis which is, among other things, the basis of heredity. These signature features extend to what may be described as biosurfactant/bioemulsifier

produced to modulate and facilitate diverse physical, chemical and behavioural activities within and without the cell.

Biosurfactants are amphiphilic (amphipathic) surface-active agents of biological origin, which comprise both hydrophilic (head) and hydrophobic groups (tail), have predilection for interfaces of dissimilar polarities (liquid-air/liquid-liquid) and are soluble in both organic (non-polar) and aqueous (polar) milieu. Several unique properties like (i) reduction in the surface tension (ST), interfacial tension (IFT) and CMC, (ii) stabilization of emulsions, (iii) promotion of foaming, (iv) induction of flocculating action, (v) increasing wetting, spreading and penetrating action(s), (vi) enhancement of microbial growth and metal sequestration, (vii) rapid biodegradability, (viii) lower toxicity and (ix) environment-friendly 'green' characteristics render them as most possible alternatives to chemical counterparts (Mukherjee et al. 2006; Singh et al. 2007; Rahman and Gakpe 2008; Vardar-Sukan and Kosaric 2009; Roane et al. 2009; Mulligan 2009; Abdel-Mawgoud et al. 2010; Banat et al. 2010; Hazra et al. 2010a, b; Satpute et al. 2010a, b).

28.5.1 Basis of Biosurfactant: A Lucrative Background

Bergström et al. (1946a, b) reported an oily glycolipid produced by *Pseudomonas pyocyanea* (now *P. aeruginosa*) after growth on glucose that was named pyolipic acid and whose structural units were identified as L-rhamnose and β-hydroxydecanoic acid (Hauser and Karnovsky 1954; Jarvis and Johnson 1949). Jarvis and Johnson (1949) further elucidated the structure of a rhamnolipid isolated from *P. aeruginosa* and showed that it was composed of two β-hydroxydecanoic acids linked through a glycosidic bond to two rhamnose moieties, with the two β-hydroxy fatty acid portions linked through an ester bond while the disaccharide portion contained a putative 1,3-glycosidic linkage. Further, Edwards and Hayashi (1965) identified α-1,2-glycosidic linkage between the two rhamnose moieties through periodate oxidation and methylation. On this basis, they chemically described this rhamnolipid as 2-O-α-1,2-L-rhamnopyranosyl-α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate (di-rhamnolipid). This was the first discovered glycolipid containing a link between a sugar and a hydroxylated fatty acid residue (Shaw 1970).

Among glycolipid surfactants, the best-known compounds are rhamnolipids, trehalolipids, sophorolipids and mannosylerythritol lipids (MELs), which contain mono- or disac-charides, combined with long-chain aliphatic acids or hydroxyaliphatic acids. Rhamnolipids from *Pseudomonas aeruginosa* are currently commercialised by Jeneil Biosurfactant, USA, mainly as a fungicide for agricultural purposes or an additive to enhance bioremediation activities. Sophorolipids, on the other hand, are produced mainly by yeasts, such as *Candida bombicola* (also known as *Torulopsis bombicola*), *Centrolene petrophilum, Candida apicola* and *Rhodotorula bogoriensis*, while MELs are produced by *Pseudozyma* yeasts, *Pseudozyma aphidis, Pseudozyma antarctica* and *Pseudozyma rugulosa* (Banat et al. 2010). Besides, cyclic lipopeptides (mainly surfactin and iturin) are produced by a number of *Bacillus* species

as antibiotic molecules. High-molecular-weight polymers RAG-1 emulsan, an amphiphilic polysaccharide produced by *Acinetobacter calcoaceticus* RAG-1, is the only commercially available bioemulsifier at present (Suthar et al. 2008).

28.5.2 Classification and Types

Primarily, biosurfactants include low- and high-molecular weight compounds (Table 28.1); the former are generally glycolipids or peptidyl-lipids (lipo-peptides), while the latter are (lipo) polysaccharides, lipo-proteins or their combinations (Desai and Banat 1997). Alternatively, low or high molecular weight biosurfactants are categorized into (i) glycolipids, (ii) lipo-amino acids and lipo-peptides, (iii) lipo-proteins and lipo-polysaccharides and (iv) phospholipids, mono- and di-glycerides and free fatty acids (Kulkarni et al. 2007). As per Neu (1996), low-molecular-weight compounds are called biosurfactants, such as lipopeptides, glycolipids, proteins and high-molecular-weight polymers of polysaccharides, lipopolysaccharides proteins or lipoproteins are collectively termed as bioemulsans (Rosenberg and Ron 1997) or bioemulsifiers (Smyth et al. 2010).

28.5.3 Why Biosurfactant?

Biosurfactants are gaining prominence by virtue of commercial applicability due to unique attributes like (i) feasible fermentative production using economical renewable resources, (ii) functionality in ppm quantities under extreme conditions (temperature, pH, and salinity), (iii) specificity of application, (iv) potential for tailoring to suit specific applications and (v) better foaming useful in mineral ore processing, besides the above-mentioned attributes. Due to this wide array of applications, ranging from biotechnology to environmental clean-up, biosurfactants have become versatile commodity in technical applications (Banat et al. 2010; Hazra et al. 2010a).

In recent years, biosurfactants have emerged as key metabolites in the rapidly growing biotechnology industry, owing to their multi-faceted functions as utility commodity in wide array of industrial applications (Banat et al. 2010; Satpute et al. a, b). They are potentially useful for (i) emulsification, (ii) emulsion polymerization, (iii) phase dispersion and (iv) de-emulsification. Many of these biosurfactants also possess therapeutic and biomedical properties viz. (i) antibacterial, (ii) antifungal, (iii) algicidal, (iv) antiviral activities, (v) anti-fibrin clotting and (vi) anti-adhesivity action against several pathogens (Cameotra and Makkar 2004; Singh and Cameotra 2004; Rodrigues et al. 2006). Environmentally, substitution of biosurfactant for chemical surfactants reduces the life-cycle of CO_2 emissions by 8%. On this basis, it is estimated that 1.5 million tonnes of CO_2 emissions were avoided (Patel 2004; Rahman and Gakpe 2008). According to Frost and Sullivan, microbial surfactants find most promising applications in oil spill bioremediation of sites contaminated

Table 28.1 Microbial biosurfactants: a brief profile

Microbes	Tensiometric properties: ST (mN m^{-1})	CMC	IFT (mN m^{-1})	Structure
1. **Glycolipid: (a) Rhamnolipid**				
Pseudomonas aeruginosa	29	–	0.25	1. One or two molecules of rhamnose linked to one or two molecules of β-hydroxydecanoic acid
Pseudomonas sp.	25–30	0.1–1	1	
Serratia rubidea	30	10	1–3.5	2. Hydroxyl group of one of the acids forms glycosidic linkage with reducing end of rhamnose; OH group of the second acid forms hydroxyl ester
Teratogenococcus sp.	–	8–10	–	
Rhodococcus erythropolis	32–36	1 4	14–17	
P. putida	–	–	–	3. Predominantly rhamnosyl-L-rhamnosyl-β-hydroxyl decanoate and L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate are referred to as rhamnolipids 1 and 2, respectively
Renibacterium salmoninarum	–	–	–	
Pseudoxanthomonas sp.	–	–	–	
Burkholderia sp.				
Cellulomonas cellulans				
(b) Trehalolipid				
Arthrobacter paraffineus	–	–	–	Disaccharide trehalose linked at C-6 and C-6 to α- branched β-hydroxyl mycolic acid
Corynebacterium sp.	–	–	–	
Mycobacterium sp.	38	3	15	
Neisseria erythropolis	30	20	3.5	
Nocardia sp.	–	–	–	
Rhodococcus ereythropolis	–	–	–	
Torulopsis bombicola	33	–	1.8	
(c) Sophorolipid				
Candida apicola	–	–	–	Carbohydrate sophorose coupled to a long chain hydroxyl fatty acid by glycosidic linkage and occurs as a mixture of macrolactones and free acid
C. bogoriensis	–	–	–	
C. bombicola	31	22	-	
T. bombicola				
C.batistae				
Wickerhamiella domercqiae				
Rhodotorula bogoriensis				
(d) Cellobiose lipids				
Ustilago maydis	–	–	–	–
(e) Polyol lipids				
Rhodotorula glutinus	–	–	–	–
R. graminus				
(f) Diglycosyl diglycerides				
Lactobacillus fermentii	–	–	–	–
(g) Lipopolysaccharides				
Acinetobacter calcoaceticus	–	–	–	–

(continued)

Table 28.1 (continued)

Microbes	Tensiometric properties: ST (mN m^{-1})	CMC	IFT (mN m^{-1})	Structure
RAG1				
Candida lipolytica	–	–	–	
Pseudomonas sp.	–	–	*	
(h) Glucose lipids				
A. borkumensis	29	–	–	Anionic glucose lipids with tetrameric oxyacyl side chain with chain lengths of one/two of the four β-hydroxy fatty acids
C. borgoriensis	–	–	–	
2. Lipopolypeptides- peptide lipids: (a) Serrawettin				
Serratia marcesens	25–28	20	12	Lipid linked to a polypeptide chain
(b) Viscosin				
Pseudomonas fluorescens	25–28	18	–	Lipid attached to a polypeptide chain
(c) Surfactin				
Bacillus subtilis	29	15	12	Cyclic hepta-peptide coupled to a fatty acid chain via lactone ring
B. pumilis	27	10	–	
(d) Lichenysin (Type A and B)				
Bacillus licheniformis	27.9	–	0.36	Molecular weight 1,006 to 1,034 Da; Similar to surfactin
(e) Subtilisin				
Bacillus subtilis	27	–	–	Similar to surfactin
(f) Gramicidins				
Bacillus licheniformes	27	–	–	1. Cyclo-symmetric decapeptide antibiotic 2. Exist as rigid ring with ornithine side chain 3. A two moles of gramicidin form a stable complex with 1 mole of ATP
(g) Polymyxins				
B. polymyxa	27	–	–	Lipodecapeptide antibiotic with amino acid 3 through 10 form cyclic octapeptide
(h) Arthrofactin				
Arthrobacter sp.	–	–	–	–
(i) Ornithine lysine peptides				
Gluconobacter, Streptomyces, Thiobacillus				
(j) Sulfonylipids				
Corynebacterium alkanolyticum	–	–	–	–
Thiobacillus thioxidans	–	–	–	
(k) Protein complex				
M. thermoautotrophium	–	–	–	–

(continued)

Table 28.1 (continued)

Microbes	Tensiometric properties: ST (mN m^{-1})	CMC	IFT (mN m^{-1})	Structure
3. Polymeric surfactants: (a) Emulsan				
Bacillus licheniformes	27.9	–	–	High molecular weight hetero-polysaccharide protein containing repeating trisaccharide of N-acetyl -D-galactosamine, N-acetylgalactosamine uronic acid and an N-acetyl amino sugar.
Bacillus sp.	–	–	–	
A. calcoaceticus BD413	–	–	–	
P. fluorescens				
(b) Liposan				
Acinetobacter calcoaceticus (RAG1)	–	–	–	Poly-anionicamphiphatic hetero-polysaccharides
A. calcoaceticus A2	–	–	–	
C. lipolytica	–	–	–	
(c) Mannosylerythreitol- lipid A				
Candida bombicola	0.1	–	–	Consists of 83% hetero-polysaccharide and 17% protein with carbohydrate portion (glucose, galactose, galactosamine and galacto-uronic acid).
C. lipolytica	–	–	–	
C. tropicalis	–	–	–	
Pseudozyma sp.	–	–	–	
(d) Alasan				
A. radioresistens KA53	–	–	–	Protein polysaccharide (1 MDa); Contains covalent bound alanine
(e) Lipomannan				
Acinetobacter radioresistens	–	–	–	Consists of 44% mannose and 17% protein.
Acinetobacter sp.	–	–	–	
Candida tropicalis	–	–	–	
Saccharomyces cerevisiae	–	–	–	
Myroides				
(f) Streptofactin (Extracellular hydrophobic peptide)				
Streptomyces tendae	39.4	36.4	–	1. Mixture of structurally related peptide (MW 1.003 to 1.127 Da) 2. Allows erection of aerial hyphae
4. Particulate biosurfactants: (a) Vesicles				
Acinetobacter calcoaceticus	–	–	–	–
(b) Whole microbial cells				
Cyanobacteria	–	–	–	–
(c) Waxy matter				
Pseudomonas marginalis	–	–	–	–
(d) Biosur PM				
Pseudomonas maltophila	–	–	–	–

(continued)

Table 28.1 (continued)

Microbes	Tensiometric properties: ST (mN m^{-1})	CMC	IFT (mN m^{-1})	Structure
(e) Fatty acids (Corynomycolic acids, Spiculisporic acids etc.)				
Arthobacter parrafineus	–	–	–	–
	–	–	–	
Capnocytophaga sp.	–	–	–	
Corynebacterium lepus	1	30	–	
	–	–	–	
	–	–	–	
Nocardia erythropolis	–	–	–	
Pennicillium spiculisporum	–	–	–	
Talaromyces trachyspermus				
Acinetobacter sp.				
Thiobacillus thiooxidans				
Halomonas				
Antarctobacter				
Marinobacter				
5. Insecticide emulsifier				
P. tralucida	–	–	–	–
6. Thermophilic emulsifier				
B. stearothermophilus	–	–	–	–
7. Acetyl-heteropolysaccharide				
S. paucimobilis	–	–	–	–
8. Food emulsifier				
C. utilis	–	–	–	–
9. Sulfated polysaccharide				
H. eurihalinia	–	–	–	–
10. PM-factor				
P. marginalis	–	–	–	–
11. Emulcyan				
Phormidium J-1	–	–	–	–

with hydrocarbons and oil-contaminated tanker clean-up, removal of crude oil from sludge, enhanced oil recovery, and recovery of other organic pollutants and heavy metals due to broad range of functional properties (Mulligan 2005). As of now, biosurfactant occupy about 10% of the total world production (nearly ten million ton per year) (Van Bogaert et al. 2007).

28.5.4 Significance of Biosurfactant in Bioaugmentation

Structural uniqueness of biosurfactant resides in the co-existence of a hydrophilic (a sugar or peptide) and a hydrophobic (fatty acid chain) domain in the same molecule,

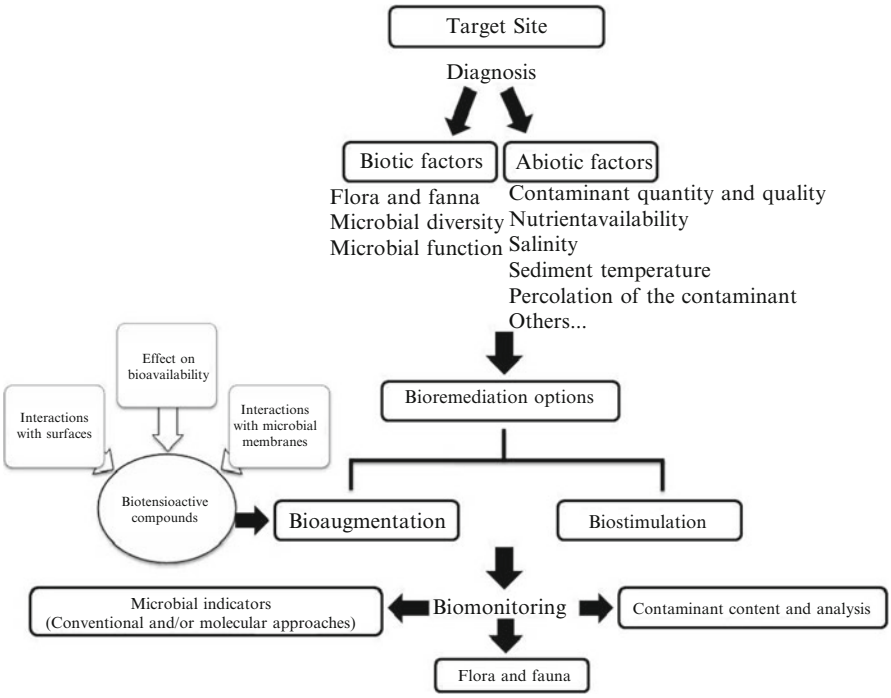

Fig. 28.1 Possible approach of biosurfactants in bioaugmentation: a *bird's eye view*

which allows them to occupy the interface of mixed phase systems (e.g., oil/water, air/water, oil/solid/water) and consequently, to alter the forces governing the equilibrium conditions. It constitutes the pre-requisite for a broad range of surface activities to take place including emulsification, dispersion, dissolution, solubilization, wetting and foaming (Perfumo et al. 2010a, b). Moreover, biosurfactants seem to confer an essential evolutionary advantage allowing microbes to grow under specific conditions as evidenced by their wide distribution across the eubacterial and archeal domains. The natural roles of biosurfactants have been claimed to increase the surface area of hydrophobic, water-insoluble growth substrates, increasing their bioavailability by increasing the apparent solubility or desorbing them from surfaces and regulating attachment and detachment of microorganisms to and from surfaces (Fig. 28.1). Thus, the net effect of a biosurfactant on biodegradation depends on the benefits that result from enhanced solubility of target compounds versus the reduction in direct adhesion of bacteria to those compounds.

Although biosurfactants reportedly enhance bioavailability of hydrophobic organic compounds (HOCs)/persistent organic pollutants (POPs), understanding the term 'bioavailability' is complicated because of a number of interpretations in the literature. For this purpose, Semple et al. (2004) proposed two linked definitions, bioavailability and bioaccessibility. A bioavailable compound is defined as 'a compound which is freely available to cross an organism's membrane from the

medium the organism inhabits at a given point in time'. A bioaccessible compound is described as 'a compound which is available to cross an organisms' membrane from the environment it inhabits, if the organism has access to it; however, it may either be physically removed from the organism, or only bioavailable after a period of time' (Semple et al. 2004). Thus, water-solubility and hence, bioavailability raises several issues pertinent to bioaccessibility and biodegradation of aromatic and aliphatic HOCs/POPs in soil: (i) is the emphasis on the aqueous phase and passive uptake actually relevant to the very low water soluble hydrocarbons? (ii) does the readily desorbed fraction adequately describe the size of a bioaccessible fraction? (iii) are there different modes of biodegradation? The bioavailability of hydrophobic organic pollutants can be enhanced by biotensioactives through the following six mechanisms:

Emulsification of Non-aqueous Phase Liquid Pollutant

As mentioned above, surfactants can decrease the interfacial tension between an aqueous and a non-aqueous phase. This may lead to the formation of micro-emulsions or, with energy input, to the formation of macro-emulsions, resulting into an increase in the contact area, enabling improved mass transport of the pollutant to the aqueous phase and in mobilization of sorbed liquid-phase pollutant.

Enhancement of Apparent Solubility of the Pollutant

The so called 'solubilisation' is caused by the presence of micelles. Hydrophobic organic compounds dissolve mainly in the core of the micelles, whereas more hydrophilic molecules, such as mono-aromatic compounds, may be present in the core and the shell of the micelles. The transport of micellar hydrocarbon to the aqueous phase can be very rapid due to the small size of the micelles, but it is not clear whether 'solubilised' hydrocarbons are directly available to the degrading microorganisms.

Facilitated Transport of the Pollutant

This term covers several different processes, such as the interaction of a pollutant molecule with single surfactant molecules, the interaction of surfactants with separate-phase or sorbed hydrocarbons (both as single biosurfactant molecules and as micelle-like aggregates at surfaces), mobilization of pollutant by swelling of the organic matrix, and mobilization of pollutant trapped in soil ganglia caused by lowering the surface tension of the pore water in soil particles.

Solubilization

It involves the production of biosurfactants by microbes, which increase the concentration of hydrocarbons in the aqueous phase (Fig. 28.2). The solubilization of hydrocarbons by biosurfactants is widely reported (Banat et al. 2010; Perfumo et al. 2010a, b), where higher concentrations of hydrocarbons were found in the aqueous phase than was expected. Bouchez-Naitali and Vandecasteele (2008) noted the importance of solubilization while examining the biodegradation of hexadecane by a variety of bacteria strains. Similarly, Bai et al. (1997) found that the solubility of hexadecane in a 500 $mg \cdot L^{-1}$ rhamnolipid solution was

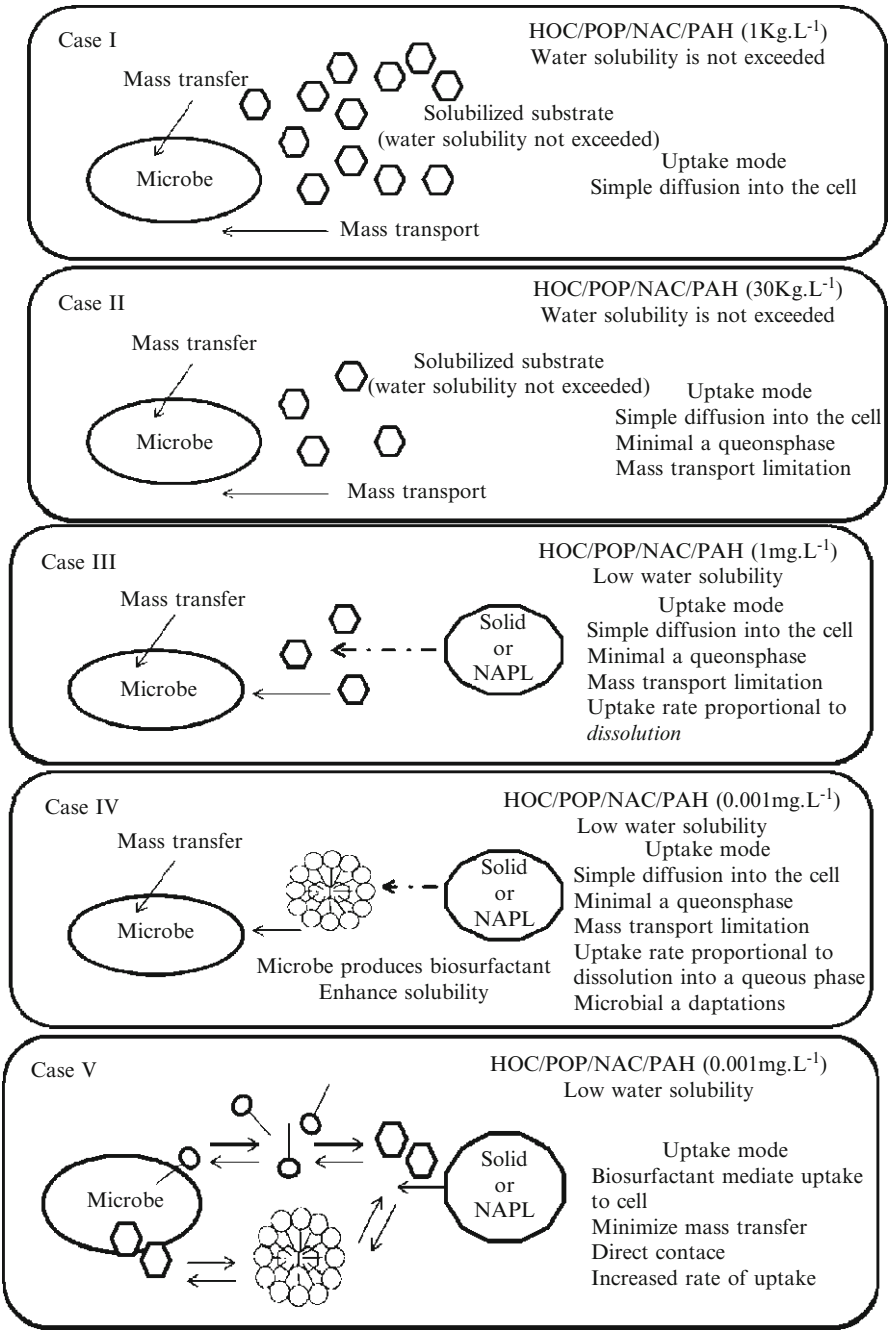

Fig. 28.2 Proposed involvement of biotensioactives for solubilization in bioremediation

19 mg·L^{-1} thereby, increasing hydrocarbon concentrations in the aqueous phase. Whyte et al. (1999) reported that invagination of hydrocarbons occurred, where inclusions of hydrocarbons in cells formed, followed by the uptake. Noordman et al. (2000) reported that the role of rhamnolipids was to mediate the mass transfer of hexadecane into cells, causing biodegradation.

Micellarization

Above the CMC, the formation of micelles s can partition hydrocarbon into the hydrophobic micellar core with increased apparent aqueous solubility. Supplementation of rhamnolipids above CMC, enhanced the apparent aqueous solubility of hexadecane, favoured biodegradation of hexadecane, octadecane, n-paraffins, creosotes and other hydrocarbon mixtures in soil and promoted bioremediation of petroleum sludges (Franzetti et al. 2010a). Biodegradation of chlorinated hydrocarbons can be enhanced by addition of glycolipids to the medium containing poly-chlorinated bi-phenyls. Similarly, pesticide biodegradation was promoted by surfactin (Awasthi et al. 1999).

Direct Contact

The interactions between bacteria, contaminants and biosurfactant can be interpreted from a functional perspective, considering that the main natural role attributed to biosurfactants is their involvement in hydrocarbon uptake (Perfumo et al. 2010b). Microbial surfactants can promote the growth of bacteria on hydrocarbons by increasing the surface area between oil and water through emulsification and increasing pseudosolubility of hydrocarbons through partitioning into micelles (Volkering et al. 1998). In direct contact, the bacterial cells adhere to the surface of the hydrocarbon and were crucial to the bacterial degradation of hexadecane. Direct contact can facilitate biosurfactants and bioemulsifiers to enhance adhesion between the cell wall and the accessible hydrocarbon. For example, the Gram-negative bacterium, *Acinetobacter* spp. is widely reported to produce biosurfactants / bioemulsifiers; thus, it has a hydrophobic exterior to allow cellular contact with the hydrocarbon. Additionally, some bacteria naturally have hydrophobic cell surfaces enabling cellular adhesion to hydrocarbons. Further, it was observed that uptake of the biosurfactant-coated hydrocarbon droplets occurred, suggesting a pinocytosis mechanism, a process not previously reported in bacterial hydrocarbon uptake systems (Cameotra and Singh 2009).

Changing Cell-Surface Hydrophobicity

Hydrophobic interactions play a role in the adherence of micro-organisms to a wide variety of surfaces. In particular, the hydrophobic nature of the bacterial surface has been cited as a factor in the growth of cells on water insoluble hydrophobic substrates such as hydrocarbons. In this case, cell contact with hydrophobic compounds is a requirement because the first step in aromatic or aliphatic hydrocarbon degradation is the introduction of molecular oxygen into molecules by cell-associated oxygenases. Uptake and utilization of water-insoluble substrates require specific physiological adaptations. Various microorganisms have developed different strategies of interaction with hydrophobic compounds. Two general types of

hydrocarbon-cell interactions, depending on the state and size of oil droplets relative to the size of microbial cells, have been postulated: specific adhesion of cells to larger oil drops and pseudosolubilization involving the cellular assimilation of emulsified small hydrocarbon droplets. The proposed role of biosurfactants in hydrocarbon uptake is the regulation of cell attachment to hydrophobic and hydrophilic surfaces by exposing different parts of cell-bound biosurfactants, thus changing cell-surface hydrophobicity (Franzetti et al. 2010a). This natural role through exogeneous (bio) surfactants supplementation can increase the hydrophobicity of degrading microbial cells and can facilitate easier access to hydrophobic substrates.

28.5.5 Biosurfactant-Assisted Bioaugmentation: Laboratory Scale Studies

Feasibility studies are a prerequisite for any planned intervention that usually revolves around screening, followed by tailoring of a competent microbial formula for a particular site. The initial screening/selection step usually based on the metabolic potential of the microorganism and also on essential features to enable the cells to be functionally active and persistent under the desired environmental conditions. The best approach for selecting competent microbes is primarily based on the prior knowledge of the microbial communities inhabiting the target site (Thompson et al. 2005). In the case of co-contaminated sites, e.g. contaminated with both high metal concentrations and organic pollutants, the microbial population ability to degrade the organic compounds may be inhibited by the co-contaminants (Roane et al. 2001). The proposed strategies, in such cases, have involved the use of multicomponent systems such as a microbial consortium, which truly represent a real environment than model – based on single-component systems (Ledin 2000). From an applied perspective, application of microbial consortium rather than a pure culture for the bioremediation is more advantageous as it provides the metabolic diversity and robustness needed for field applications (Rahman et al. 2002; Nyer et al. 2002). Table 28.2 summarizes the use of biosurfactants to stimulate hydrophobic organic contaminant biodegradation.

Apparently, the combination of bioaugmentation, biostimulation, and biosurfactant addition, depending on the characteristics of the contaminated site, might be a promising strategy to speed up bioremediation (Baek et al. 2007). However, any such planned intervention must be followed by ecotoxicity and quality studies of the contaminated site to ascertain that it has regained its natural biological activity and integrity (Hamdi et al. 2007; Liu et al. 2010a).

28.5.5.1 Biosurfactant in Oil Spills

Conservative estimates revealed that approximately $0.8 \pm 0.4\%$ of the total worldwide production of petroleum eventually reaches the oceans. As per National

Table 28.2 Biosurfactant-aided bioremediation: laboratory studies[a]

Compound(s)	Surfactant	Medium	Organism	Biodegradation kinetics[b]	Reference
14–16 C alkanes, pristane phenyldecane, and naphthalene	Sophorose lipid	Liquid	Mixed population	+	Oberbremer et al. (1990)
Mixture of alkanes and aromatics	Biosurfactant	slurry reactor	mixed culture	Stimulation and emulsification	Oberbremer et al. (1990)
Crude oil	Emulsan	Liquid	*Acinetobacter calcoaceticus* RAG-1	0	Glaser (1991)
Octadecane	Rhamnolipid	Soil	*P. aeruginosa* ATCC 9027	+	Zhang and Miller (1992)
Hexachlorobiphenyl	Rhamnolipid Emulsan	Soil	*P. aeruginosa Acinetobacter calcoaceticus* RAG-1	+	Van Dyke et al. (1993)
Aliphatic and aromatic hydrocarbons	Rhamnolipid	Soil	*P. aeruginosa* UG12	+	Scheibenbogen et al. (1994)
Mixture of alkanes and naphthalene	Rhamnolipid and oleophilic fertilizer	Soil	*P. aeruginosa*	+	Churchill et al. (1995)
Metals, phenanthrene, and PCBs	Rhamnolipid	Soil	*P. aeruginosa* ATCC 9027	+	Miller (1995)
Naphthalene	Rhamnolipid	Soil	*P. aeruginosa* 19SJ	+	Providenti et al. (1995)
Phenanthrene, pyrene, and B[*a*]P	Sodium dodecyl sulfate and rhamnolipid	Soil	*P. aeruginosa* UG2	–	Deschenes et al. (1996)
4,4,9-dichlorobiphenyl	Rhamnolipid	Soil	*P. aeruginosa*	+	Robinson et al. (1996)
Crude oil	Biosurfactant	Liquid		0	Kanga et al. (1997)
Naphthalene and methyl naphthalene	Glycolipid and Tween 80	Liquid	*Rhodococcus* sp. H13A	+	Kanga et al. (1997)

(continued)

Table 28.2 (continued)

Compound(s)	Surfactant	Medium	Organism	Biodegradation kinetics[b]	Reference
Hexadecane and kerosene oil	Crude surfactin	Soil	*Bacillus subtilis* ATCC 2423	+	Makkar and Cameotra (1997)
Naphthalene and phenanthrene	Rhamnolipid	Soil	*P. aeruginosa* ATCC 9027	+	Zhang et al. (1997)
Phenanthrene, fluoranthene, pyrene, B[*a*]P, and pentachlorophenol	Rhamnolipid	Soil	*P. aeruginosa* #641	+	Awashti et al. (1999)
Endosulfan	Lipopeptide	Soil and liquid	*B. subtilis* MTCC 1427	30–45% enhanced degradation	Awasthi et al. (1999)
Phenanthrene, fluoranthene, and pyrene	Alasan	Liquid	*Acinetobacter radioresistens* KA53	+	Barkay et al. (1999)
Endosulfan	Crude surfactin	Soil	*Bacillus subtilis* ATCC 2423	+	Straube et al. (1999)
Phenanthrene and cadmium	Rhamnolipid	Soil	*P. aeruginosa* ATCC 9027	+	Maslin and Maier (2000)
Aliphatic and aromatic hydrocarbons	Crude surfactin	Seawater	*Bacillus subtilis* O9	+	Moran et al. (2000)
Phenanthrene and hexadecane	Rhamnolipid	Soil	*P. aeruginosa* UG2	+	Noordman et al. (2000)
Naphthalene and cadmium	Mono-rhamnolipid	Soil	*P. aeruginosa* ATCC 9027	+	Sandrin et al. (2000)
Phenanthrene	Sophorolipid	Soil	*Candida bombicola* ATCC 22214	+	Schippers et al. (2000)
Toluene, ethyl benzene, and butyl benzene	Di-rhamnolipid	Liquid	*Pseudomonas*	+	McCray et al. (2001)
Phenanthrene slurries	Rhamnolipid	Soil	*P. aeruginosa* UG12	+	Makkar and Rockne (2003)

Hexachlorobiphenyl	Rhamnolipid	Soil slurries	*P. aeruginosa* UG12	+	Makkar and Rockne (2003)
Phenanthrene and pyrene	Rhamnolipid	Soil-aqueous	*P. aeruginosa* ATCC 9027	Enhanced cell surface hydrophobicity, solubilization and transport	Jennings and Tanner (2004)
Hexadecane	Peptidyl-lipid	Liquid	*Bacillus* sp. JF2	High cmc micelles forms; increases dissolution	Jennings and Tanner (2004)
Naphthalene and Phenanthrene	Rhamnolipid	Silica-sand	*P. aeruginosa*	Sorption and transport in silica sand	Chen et al. (2005)
Phenanthrene	Rhamnolipid	Liquid	*P. putida*	Increased soil-sorbed PAHs desorption, solubilisation, and dissolution	Martinez-Toledo et al. (2006)
Petroleum hydrocarbons	Rhamnolipid	Liquid	*P. aeruginosa*	Rhamnolipid-induced cell surface hydrophobicity	Song et al. (2006)
Hexadecane	Biosurfactant	Liquid	*P. aeruginosa* GL1	Increased cell hydrophobicity, emulsification, pseudosolubilization	Bouchez-Naitali and Vandecasteele (2008)
Naphthalene, pyrene and benzo(a)pyrene	Emulsan	Liquid	*Acinetobacter calcoaceticus* BU03	Increased solubilization and uptake	Zhao and Wong (2009)
Pyrene	Rhamnolipid	Liquid	*Pseudomonas* sp. LP1	Increased solubilization and uptake	Obayori et al. (2009)
Phenanthrene	Lipopeptide	Liquid	*P. putida* PCL1445	Augment alkane bioavailability and thus degradation rate	Tecon and van der Meer (2009)
Tetrachloroethylene (PCE) and trichloroethylene (TCE)	Rhamnolipids and surfactin	Liquid	*P. aeruginosa* (MTCC 2297) *B. subtilis*	Increased bioavailability to enhance the biodegradation process	Albino and Nambi (2010)

(continued)

Table 28.2 (continued)

Compound(s)	Surfactant	Medium	Organism	Biodegradation kinetics[b]	Reference
Phenanthrene	Rhamnolipid	Batch soil slurry	*P. putida* ATCC 17484	Emulsification, solubilization and transport	Gottfried et al. (2010)
2-methylnaphthalene, hexadecane and pristane	Sophorolipid	Liquid	*C. bombicola* ATCC 22214	Surfactant solubilization and enhancement	Kang et al. (2010)
Phenol	Mono-rhamnolipid	Liquid	*C. tropicalis*	Surfactant solubilization and enhancement	Liu et al. (2010b)

[a]Studies found in the literature where biosurfactants were used to stimulate hydrophobic organics biodegradation by defined cultures

[b]+: beneficial effect defined as a significant increase in biodegradation rate and/or extent; −: detrimental effect; 0: no effect

Research Council (2003) about 1.3 million tonnes of crude oil is released into the marine environment each year and over 5.6 million tonnes of oil have been released in the environment since 1970 (Cho et al. 2006). Notable examples of massive oil spillage since Arabian Gulf War (1991–1992) include (i) Amoco Cadiz oil spill in Brittany coastal waters in 1978, (ii) Exxon-Valdez spill in the Prince William Sound in 1989, (iii) Haven spill on the coast of Italy in 1991, (iv) over 105 t of petroleum released in the Gulf waters, (v) Nakhodka tanker oil spill (1997) off the Oki Islands in the Sea of Japan, (vi) San Jorge tanker spill (1997) on the shores of Punta Del Este in Uruguay and (vii) Nissos Amorgos spill (1997) in the Maracaibo Channel in the Gulf of Venezuela (viii) release of about 11 million barrels of crude, (ix) pollution in more than 1,280 km coastline of Kuwait and Saudi Arabia and (x) British Petroleum's accidental spills off Gulf of Mexico and (xi) oil spills (2010) off Mumbai shore lines. Microbial system with biosurfactant activity have been recruited for removal of oil spillage at field scale levels.

Laboratory investigations have indicated that rhamnolipids were able to degrade (i) hexadecane, heptadecane, octadecane and nonadecane in seawater upto 47%, 58%, 73% and 60%, respectively (Shafeeq et al. 1989), (ii) n-paraffins, (iii) tetradecane, hexadecane and pristine, (iv) naphthalene, anthracene, phenanthrene, fluorine, 2,2′,5,5′-tetrachloro-biphenyl and 3,3′,4,4′,5,5′-hexachloro-biphenyl, (v) recovery of hydrocarbons to 25–70% and 40–80% in silt-loam soil and sandy-loam soil upon application of 5 gL^{-1} of rhamnolipid, (vi) recovery of aliphatic and aromatic hydrocarbons to 36% and 40%, respectively using a 0.08% mixture of rhamnolipids and (vii) 100% of C8-C11, 83–98% of C12-C21, 80–85% of C22-C31 and 57–73% of C32-C40 of petroleum sludge (Rahman et al. 2002). Further, *in situ* field experiments demonstrated that a 1.0% rhamnolipid solution yielded (a) twofold oil recovery at 10–80°C, (b) 3.5 times more recovery at 40^0 C, (c) 1 min contact time in the Prince William Bay after Alaskan Exxon-Valdez oil spills or oil-contaminated desert sand in Kuwait. Tang et al. (2007) enhanced crude oil biodegradability proportional to rhamnolipid production by *P. aeruginosa* ZJU after preservation in an oil-containing medium. Nitschke et al. (2009) removed 67% of 10% (w/w) of crude oil using 0.1% (w/v) rhamnolipid. Biosurfactant-producing microbes are currently being exploited in British Petroleum's spill off Gulf of Mexico and accidental spill off Mumbai shorelines in India in a joint effort by National Institute of Oceanography (N.I.O) and The Energy Research Institute (TERI), New Delhi.

28.5.5.2 Biosurfactant in Microbial Enhanced Oil Recovery (MEOR)

The concept of MEOR technology was poorly scaled up from laboratory-based studies (1980) to field applications (1990) due to failure of existing EORs to attend problems viz.: (i) low permeability of some reservoirs, (ii) high viscosity of oil leading to poor mobility, (iii) high IFT between the water and oil, (iv) high capillary forces retaining the oil in the reservoir rock, (vi) hazardous implications of chemical surfactants, (vii) high costs, (viii) difficult to dispose undesirable residues, (ix) only

30–50% oil recovery and (x) adsorption of surfactants on the surface of the reservoir by rock-oil-brine ternary interactions. As per National Institute of Petroleum and Energy Research (Dehradun, India), about (i) 27% of the oil reservoirs (600 reservoirs containing over 12 billion barrels of unrecoverable oil) and (ii) 40% of the oil-producing carbonate reservoirs in the US may be suitable for MEOR (Singh et al. 2007). At $100 per barrel, the entrapped but retrievable oil is valued at $32 trillion in the US alone and over $500 trillion worldwide. As of now, more than 400 MEOR tests have been conducted in the US alone.

A 3 year study (2004–2007) by the US Dept. of Energy (USDE) showed that just 250 mg L^{-1} rhamnolipids was sufficient to recover 42% of otherwise entrapped oil from sand-pack. The potential application of the biosurfactants produced by the thermo- and halo-tolerant species of *Bacillus licheniformis* JF-2 and *Bacillus subtilis* have been explored for enhanced oil recoveries in laboratory columns and reservoirs with oil recoveries from 9.3% to 62% (Perfumo et al. 2010b; Singh et al. 2007). Similarly, oil recovery was significantly elevated by 30% from underground sandstone using trehalolipids from *Nocardia rhodochrus* (Franzetti et al. 2010b). Flooding strata with suspensions of *Bacillus, Desulfovibrio, Clostridium, Micrococcus, Pseudomonas, Arthrobacter, Peptococcus, Microbacterium,* and other microorganisms of different taxonomic groups has been recommended. Injection of biosurfactants and bacteria such as *Pseudomonas aeruginosa, Xanthomonas campestris, B. licheniformis* and *Desulfovibrio desulfuricans* along with nutrients showed increase in oil recovery by 30–200%. Pornsunthorntawee et al. (2008) demonstrated that *P. aeruginosa* SP4 biosurfactants removed 57% oil effectively compared to three synthetic surfactants using sand-packed column. Further, a 70% bioremediation and bioreclamation rate of a slop-oil contaminated site has been achieved with emulsan®, a commercial biosurfactant. Moreover, it reduces the viscosity of Boscon heavy crude oil from 200,000 to 100 cP, and facilitated pumping of heavy oil to 26,000 miles through a commercial pipeline. Application of rhamnolipids and surfactin in this area is also visibly encouraging due to (i) possibility of 95% recovery of crude oil, (ii) retaining 100% hydrocarbon content, (iii) comparable American Petroleum Institute (API) values of extracted crude oil and the API range of standard crude, (iv) production of 5,550 barrels of saleable crude from about 750 m^3 of sludge and (v) recovery of cleaning costs by selling recovered crude oil at US $ 1,00,000–1,50,000/storage tank. Kuwait Oil Company has examined 90% oil recovery with biosurfactants for crude oil storage tank clean up.

Although off-site biosurfactant production is the most common practice in MEOR, its potential has not been fully analysed yet due to its high cost. The prospect for strategy like reducing the costs by genetically improved strains (Wang et al. 2007) are probably quite poor since the production of rhamnolipids in *Pseudomonas* is regulated through the quorum sensing system and hence, genetic intervention is difficult (Banat et al. 2010).

Several *in situ* applications of MEOR are reported at field scale level (Sen 2008), but none has clarified whether (i) introduced microorganisms can actually be effective in oil recovery or (ii) they are out-competed by indigenous bacteria (Wang et al. 2008; Banat et al. 2010), and (iii) predictability of biosurfactant-based MEOR

process performance (Banat et al. 2010). Thus, effective MEOR application requires substantial research on a case-by-case basis and the associated costs to minimize uncertainties for MEOR application.

28.5.5.3 Biosurfactant Enhanced Bioremediation: Organics

Interaction of bio-amphiphiles with contaminated soil (which contains at least six phases: bacteria, soil particles, water, air, immiscible liquid and solid contaminants) result into partioning of pollutants among different states: solubilised in the water phase, absorbed to soil particle, sorbed to cell surfaces and as a free/insoluble phase (Banat et al. 2010). Biosurfactants added to this system can interact with both the abiotic particles and the bacterial cells.

High-molecular-weight biosurfactants (bioemulsifiers) have great potential for stabilizing emulsions between liquid hydrocarbons and water, thus increasing the surface area available for bacterial biodegradation. However, they have been rarely tested as enhancers of hydrocarbon biodegradation in bioremediation systems, and contrasting results are reported in the literature (Banat et al. 2010; Franzetti et al. 2010a, b). Emulsan from *Acinetobacter* RAG-1 are known to degrade oil, aromatic and paraffinic hydrocarbons. Alasan, a bioemulsifier produced by *Acinetobacter radioresistens* KA53 effectively emulsifies a wide range of hydrophobic compounds, long chain alkanes, aromatics, PAHs, paraffins and crude oil. While polymeric biosurfactants like sphingans (from *Sphingomona*s strains) and biosurfactant from *Halomonas eurihalina* are able to adsorb to PAHs, but it is unclear about enhancement of apparent substrate solubility and therefore, the mass transfer to the cells.

For low-molecular-weight biosurfactants, above the CMC, a significant fraction of the hydrophobic contaminant partitions in the surfactant micelle cores. In some cases, it increases in the bioavailability of contaminants for degrading microorganisms. Rhamnolipids above CMC, enhanced the (i) apparent aqueous solubility of hexadecane, (ii) biodegradation of hexadecane, octadecane, n-paraffins, creosotes and other hydrocarbon mixtures in soil and (iii) bioremediation of petroleum sludges (Rahman et al. 2002), chlorinated hydrocarbons (PCBs) and pesticides (Singh et al. 2007). Almost double hydrocarbon recovery (from 25% to 70% and 40% to 80%) from contaminated soil using rhamnolipids from *P. aeruginosa* has also been demonstrated. Glycolipid biosurfactants have also been shown to enhance the hydrocarbon removal (from 80% to 90–95%) from soil; furthermore, the biosurfactant was reported to increase hydrocarbon mineralization by twofold and shorten the adaptation time of microbial populations to fewer hours. Recently, uptake, solubilization and biodegradation of 2-chlorobenzoic acid (75%), 3-chlorobenzoic acid and 1-methyl naphthalene (60%) by 0.5% rhamnolipids from *Pseudoxanthomonas* sp. PNK-04 has been examined by Nayak et al. (2009). External addition of a 0.1% commercial rhamnolipids increased (i) both growth and green fluorescent protein (GFP) expression of *Burkholderia sartisoli* RP037 and (ii) phenanthrene bioavailability compared to non-amended control (Tecon and van der Meer 2009). Further, Gottfried

et al. (2010) showed that rhamnolipid with salicylate or glucose in liquid solution increases the apparent aqueous solubility of phenanthrene, and overall degradation by 20% compared to solutions containing only salicylate or glucose. For solubilization of chlorinated solvents in surfactant enhanced aquifer remediation, Albino and Nambi (2010) reported 2.06 and 8.36 Weight Solubilization Ratio (WSR) with rhamnolipids for tetra-chloro-ethylene and tri-chloro-ethylene, respectively. Yuan et al. (2010) used rhamnolipids to accelerate aerobic degradation of tetrachlorobisphenol-A (TCBPA) in sediment samples. Besides being used in remediation of soil and water (Mulligan 2009), rhamnolipids are persistent enough to remain in soil for periods useful for phytoextraction (Wen et al. 2009). Average removal efficiency of PAHs by rhamnolipid-enhanced multi-technique phytoremediation reached to 60.5% and 251.8% vis-a-vis phytoremediation itself (17.19%) (Zhang et al. 2010). Henry and Abazinge (2009) used micro-encapsulated rhamnolipids in ε-polycaprolactone microparticles to optimize the formulation factors and achieved 100% release after 30 days in different release media. The presence of rhamnolipids (300 mgL^{-1}) increased the EC_{50} of phenol, 4-chlorophenol, 2,4-dichlorophenol and 2,4,5-trichlorophenol by about 12%, 19%, 32% and 40%, respectively (Chrzanowski et al. 2009). Kulkarni (2005) opened a new gateway towards rhamnolipid-assisted bioremediation of nitroaromatics by enhanced biodegradation of p-nitrophenol upto 300 ppm with 0.8 $ml{\cdot}L^{-1}$ of rhamnolipid. Similarly, Singh et al. (2009) found more than 98% degradation of chlorpyrifos (0.01 $g\ L^{-1}$) using rhamnolipid (0.1 $g\ L^{-1}$) as compared to 84% in control experiment after 120 h incubation.

Interestingly, the release of LPS by *Pseudomonas* spp. induced by sub-CMC levels of rhamnolipids allowed a more efficient uptake of hexadecane by rendering the cell surface more hydrophobic. It has been reported that rhamnolipid produced by *P. aeruginosa* UG2 facilitated the hydrocarbon uptake of the producer strain and increased the degradation of hexadecane but failed to stimulate the biodegradation of hexadecane by *Acinetobacter lwoffii* RAG1, *R. erythropolis* ATCC 19558, *R. erythropolis* DSM 43066 and BCG112 (Noordman and Janssen 2002). Zhong et al. (2008) showed that the adsorption of di-rhamnolipid biosurfactants on cells of *B. subtilis, P. aeruginosa* and *Candida lipolytica* depended on the physiological status of the cells and was species specific. Furthermore, the biosurfactant adsorption affected the cell-surface hydrophobicity and its physiological state by rhamnolipid concentration. The effect of exogenous rhamnolipids on cell-surface composition of *P. aeruginosa* NBIMCC 1390 studied by Sotirova et al. (2008) showed that (i) about 22% reduction of total cellular LPS content above the CMC, and (ii) changes in the bacterial outer membrane protein composition without affecting the LPS component below the CMC. But Chang et al. (2009) demonstrated that the cell-surface hydrophobicity was enhanced by the accumulation of different fatty acids at the cell surface during growth on hydrocarbon in *R. erythropolis* NTU-1. A significant correlation between the modification of the cell surface by saponins and the degree of hydrocarbon biodegradation was reported by Kaczorek et al. (2008). In addition, Wang and Mulligan (2009) observed the effect of ammonium ion concentration and pH on the potential application of rhamnolipid and surfactin for enhanced biodegradation of diesel. Similarly, a lipopeptide and protein-starch-lipid

produced by two strains of *P. aeruginosa* significantly favoured the solubilization and metabolism of phenanthrene, pyrene and fluorine concomitant with growth (Bordoloi and Konwar 2009).

Lipopeptides from hydrocarbon-degrading bacilli act to (i) solubilize and emulsify the substrate, (ii) modulate the bacterial hydrophobicity, and (iii) adsorb onto the cell surface alternately exposing the cyclic peptide (hydrophilic) or the fatty acid tail end (hydrophobic). The contribution of lipopeptides is not merely limited to hydrocarbon access but may confer an evolutionary advantage to the producing bacteria in response to prevailing environmental conditions and substrate availability.

Biosurfactant production was shown a key characteristic of alkane-degrading bacteria, for which it serves to augment alkane bioavailability and thus, degradation rate. A similar effect has been observed for the bacterial degradation of polycyclic aromatic hydrocarbons (PAHs), but it appears that biosurfactant production is not an essential trait of PAH degraders. The external addition of biosurfactants, however, is believed to increase the solubilization of PAHs from non-aqueous phase liquids (NAPLs) and solid particles. Yet, an augmentation of PAH solubilization is not necessarily associated with an equivalent increase of its bioavailability to microorganisms, and hence, the nature of biosurfactants effect on PAH degradation rate are complex (Banat et al. 2010). In fact, it might be possible that molecules dissolved in micelles are actually less available to certain bacteria than freely dissolved molecules, when these are incapable of releasing the molecules from the micelles. Also, biosurfactants themselves might be used as a preferential substrate by microorganisms, which would lead to a reduction of the degradation rate of the HOCs. While *in situ* biosurfactant production has been reported as easier and more cost efficient than external addition (Mulligan 2005). However, it may lead to numerous secondary effects that have no role in increasing availability of organics. On the other hand, augmentation of organic compounds and solubilization is not necessarily associated with an equivalent increase of its bioavailability to microbes. Reports on the efficacy of surfactants on bioremediation have, however, been mixed, inconclusive and remains inoscuous.

28.5.5.4 Biosurfactant Enhanced Bioremediation: Heavy Metals

Annual worldwide release of heavy metals reached (a) 939,000 t for copper, (b) 783,000 t for lead, (c) 1,372,000 t Zn and (d) 22,000 t for cadmium leading to agricultural land contamination to the tune of 10,000 ha in Germany and 100,000 ha in the US and Europe. Rhamnolipid and surfactin facilitate partitioning of metal-surfactant complexes in an aqueous phase and their subsequent removal from soil in the washing process, alleviate heavy metal toxicity and enhance degradation of organic pollutants through heavy metal complexation.

In batch scale, rhamnolipids were found to (i) possess high affinity for lanthanum, (ii) complex with Cd^{++} at binding capacity of 0.2 Cd/rhamnolipid molecule, (iii) have better stability constant ($\log K = 6.89$ and 8.58) for Cd^{++} and Pb^{++},

respectively as compared to oxalic acid, citric acid and SDS, (iv) preferentially complex with Cd^{++}, Pb^{++} and Hg^{++} by mono-rhamnolipid (RL-1), (v) remove 19.5 and 35.1% Zn^{++} and Cu^{++}, respectively, using a 12% rhamnolipid solution, and (vi) desorb Zn^{++} and Cu^{++} from contaminated soil with 12.6% oil and grease content. In column studies, 0.1% di-rhamnolipid solution facilitated 13-fold higher removal of Cr^{++} from the heavy metal-spiked soil, whereas removal of Pb^{++}, Cu^{++} and Cd^{++} was 10, 14 and 25 -fold higher, respectively (Juwarkar et al. 2008). Studies performed by Massara et al. (2007) showed that rhamnolipids (i) remove Cr (III) mainly from the carbonate and oxide/hydroxide portions of the kaolinite, and (ii) reduce close to 100% of the extracted Cr(VI) to Cr(III) over a period of 24 days. It was also found to improve the utilization of zinc (Zn) fertilizers by plant roots in Zn-deficient soils and in solution culture (Stacey et al. 2008). Jordan et al. (2002) examined the chelant-assisted phytoextraction of Cu, Pb and Zn by maize (*Zea mays*) and saltbush (*Atriplex numilaria*) from a soil contaminated by mine tailings using rhamnolipid application. Subsequently, Johnson et al. (2009) observed the effect of 43 and 347 μM of rhamnolipid (along with several other chelants) to improve Cu accumulation by Indian mustard (*Brassica juncea*) and ryegrass (*Lolium perenne*) in hydroponic culture and found (i) negligible toxicity of rhamnolipid to plant shoot growth, and (ii) enhancement of metal uptake. On the contrary, Wen et al. (2010) suggested that rhamnolipid in the soil contaminated by Cd and Zn remain long enough to promote metal phytoextraction, yet not long enough to raise concerns regarding metal transport in the long term.

Similarly, combinatorial amendments of EDDS, rhamnolipid and citric acid resulted in the highest shoot metal levels (Cu and Cd), but also caused severe phytotoxicity in perennial ryegrass (*Lolium perenne*) with negative synergism (Gunawardana et al. 2010). Detrimental effects of rhamnolipids on copper uptake, biomass yield, and the translocation of copper from roots to shoots in *Brassica juncea* and *Lolium perenne* plant species have raised concern about possible role in metal (Johnson et al. 2009).

28.5.6 Biosurfactant-Assisted Bioaugmentation: Field Case Studies

A successful process in the laboratory-controlled conditions does not imply similar success in an uncontrolled environ. Bioaugmentation and biostimulation studies at laboratory, simulated field and *in situ*, are very few so far. It can provide insight about the microbes and their growth requirements, before any on-site intervention for decontamination is carried out. Rosenberg et al. (1992) optimized conditions for bioremediation of crude oil using a combination of bioaugmentation and biostimulation technique in the laboratory, thereby successfully implementing the same for field and *in situ* beach remediation. While Gallego et al. (2007) performed laboratory, pilot, and full scale experiments to select nutrient sources, surfactants, and

other bioremediation amendments for *in situ* bioremediation of spilled oil. Some field remediation studies using bioaugmentation and biosurfactant are presented in Table 28.3. The published results of application of biosurfactants in field scale bioremediation processes are often limited to the statement that they were used to overcome bioavailability limitations without further evidence for beneficial effects of their addition.

28.5.7 Confined Systems and Real-Case Studies: Bridging the Gap

Despite its long-term use in bioremediation, bioaugmentation of contaminated sites with microbial cells continues to be a source of controversy within environmental microbiology. From an applied perspective, successful laboratory studies concerning bioremediation do not necessarily lead to reproducible *in situ* decontamination (El Fantroussi and Agathos 2005; Tyagi et al. 2010). This impending gap between laboratorial trials and on-field studies may be due to several factors influencing the remediation process: (i) strain selection, (ii) indigenous microbial ecology, (iii) type of contaminants, (iv) environmental constraints, and (v) the procedures used for the introduction of the remediation agents. Thus, contrasting effects of biosurfactant application are a result of the poorly understood complexity of interactions between soil/sediment, pollutant, surfactant and microorganisms in different environments. The recent observations that single biosurfactants can have contrasting effects on the degradation of organic pollutants and may further explain why applications of biosurfactants have yielded inconclusive results. There is certainly a need to design an optimal surfactant/biodegrader/target environment combination and to further unravel the underlying complex interactions. Thus, the current knowledge about the optimization of degradation by unknown metabolic communities on site through the addition of biosurfactants remains a futile approach. The combination of surfactant production with degradative capabilities in a single bacterial strain may offer advances for *in situ* bioremediation, but further insights into the genetic organization and regulation of surfactant production are essential.

28.6 Biosurfactant and Bioaugmentation: An Economical Perspective

Existing non-biological remediation technologies seem to be economically non-viable due to (i) impractical cost (US$ 750 billion) and time (about 30 years) of physico-chemical processes, (ii) escalating cost estimates (US$ 30 billion) and hundreds of years of work for organo-metallics, (iii) overburden project estimation of soil

Table 28.3 Biosurfactant-aided bioremediation: field trials

Technique/process	Contaminants removed	Groups and location	Reference
Biostimulation using oleophilic fertilizer and biosurfactant-producing organisms	Oil	Exxon Valdez oil spill, Alaska, USA	Pritchard et al. (1992)
Bioaugmentation and biostimulation using hydrophobic fertilizer as N and P source along with enriched microbial culture	Crude oil	Zvulon beach oil spill, Israel	Rosenberg et al. (1992)
Bioaugmentation and biostimulation with inorganic mineral nutrients and/ or inoculation of biosurfactant-producing microbes	Light crude oil contaminating a sandy beech	Delaware, USA	Venosa et al. (1996)
Low concentration of EPA and FDA approved biosurfactants	Residual wood treating oil (DNAPLs)	Surtek, Inc. Mobility controlled surfactant flushing	USEPA (1998)
Biostimulation using liquid inorganic and slow release mineral fertilizer	Crude oil and heavy fuel oil degradation	Sea Empress oil spill, UK	Swannell et al. (1999)
Biostimulation using forced aeration and nutrient supplementation	Spill simulation (light crude oil and fuel oil) at mature mangroves	Gladstone, Australia	Duke et al. (2000)
Oil spills and bioremediation strategy applied; Bioaugmentation using commercial Biosurfactant producing microbial culture	Heavy crude oil degradation	Nakhodka oil spill, Japan	Tsutsumi et al. (2000)
1% food grade sorbitan mono-oleate (3.8 $L.min^{-1}$ for 3.8 days)	TCE (DNAPL)	US DOE gaseous diffusion pint, Paducah, NY	Mulligan et al. (2001)
Biosurfactant: single phase microemulsion	DNAPL (PCE and perchloroethylene)	Dover AFB, Dover DE	Mulligan et al. (2001)
Biostimulation using oleophilic fertilizer	High molecular weight n-alkanes, alkylcyclohexanes, and benzenes, and alkylated PAHs	Prestige oil spill, Spain (2006)	Jimenez et al. (2006)
Surfactant enhanced pilot-scale *in situ* flushing of biosurfactant	Diesel, kerosene, and lubricating oil	Pusan, Korea	Paria (2008)
Biostimulation using fertilizers (urea and di-ammonium phosphate) and surfactant; moisture content and temperature amendments	Petroleum hydrocarbons degradation in polar desert	Tanquary fiord, high Arctic	Sanscartier et al. (2009)
Biostimulation of manure oily sludge-contaminated soil with biosurfactant	Total petroleum hydrocarbon (TPH) and PAHs	China	Liu et al. (2010b)

excavation in Europe (US$ 270–460 t^{-1}) and US (US$ 324–552 m^{-3}), and (iv) unrealistic demands of incineration (US$ 1.7 trillion; US $7,000/citizen). Comparatively, cost for application of bioremediation approach reduced to US$ 75 billion (US$ 14 billion $year^{-1}$) or 5–170 £ t^{-1} soil.

Due to these facts, biotensioactives-based bioaugmentation technologies as preferred *in situ* remediation techniques have attracted commercial interest. Emerging formulations and products are gaining attention because of successful application, thereby claims for rapid decontamination rates. However, these products are not panacea and need to be evaluated according to the requirements of the site before implementation. As of now, rhamnolipids are commercially available from Jeneil Biosurfactant Inc. (USA), Ecover (France) and Rhamnolipid holdings Inc., (USA), while sophorolipids are currently offered as sophoron™ from Saraya (Japan) and Soliance (France). The current production price of sophorolipids amounts to 2–5 € kg^{-1} whereas rhamnolipids cost US $ 5–20 kg^{-1}; at 20 m^3 US $20 kg^{-1}; when produced at 100 m^3 scale, it costs US $ 5 kg^{-1}, against ethoxylate or alkyl polyglycoside [US $1–3 kg^{-1}].

In bioremediation studies supported by Exxon company from 1993 to 1997 (spill of 41 million litres of petroleum from the Exxon Valdez in Alaska in 1989), has (i) spent > US$ 10 million dollars, (ii) generated seven patents, and (iii) made bioremediation second only to enhanced oil recovery during the first years of its implementation. The distribution of patents in specific areas of the biosurfactants oil industry includes 17 patents in soil and water bioremediation and 20 in enhanced oil recovery (Santos et al. 2011).

28.7 Future Directions and Concluding Remarks

A most sustainable alternative is an integrated approach more focused on (i) economical feasibility, (ii) ensuring protection to the environment and (iii) acceptable by stakeholders and the society in general. At present, biosurfactant-aided bioaugmentation hold the promise of epitomizing *in situ* bioremediation. The gap between R&D and the application of biotensioactives in bioremediation options is partly due to a lack of awareness by regulators and problem owners, a lack of expertise and knowledge by service providers. It would be naive to believe that by simply picking the ‘right’ biosurfactant-producing microbe(s) or manipulating the right field parameter, bioaugmentation will suddenly become as reliable and predictable as engineered systems. Development of a tailor-made additive mixture suitable for extreme reservoir conditions, consisting of a combination of suitable microbial strains, nutrients, biosurfactants and buffering agents in appropriate proportions, may foster a further productive line of research. Based on these considerations, a deeper understanding of microbial degradation abilities, together with their metabolic networks as well as their cellular resistance and adaptation mechanisms, will bring out a variety of appropriate microbial formula tailored for decontamination of a specific site.

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Chapter 29
Bioremediation of Metals Mediated by Marine Bacteria

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Abstract Metals are an intrinsic part of the earth's crust. With rapid industrialization and urbanization, enormous amounts of industrial wastes including metal wastes are accumulating, which require special treatment. Wastes from mining and metal refining industries, sewage sludge, power plant wastes and waste incineration plants often contain substantial amounts of toxic heavy metals viz. Hg, Cd, Pb, As, Sb, Zn, Cu which pose serious treat to the environment and need to be removed from the source of pollution.

Microorganisms from metal polluted habitats possess a variety of inherent mechanisms to tolerate high levels of toxic metals which include precipitation of metals as phosphate, sulphide, carbonate; volatilization via methylation/ethylation; physical exclusion in membranes and extracellular polymeric substances (EPS); energy driven metal efflux system and intracellular sequestration mediated by metallothionein like proteins. For the last several decades, metal resistant microorganisms including marine bacteria have been considered a potential alternative for heavy metal recovery and bioremediation resulting in the development and refinement of many bioremediation technologies for removal of toxic metals form contaminated soils and aqueous mining and industrial wastes/effluents. Interestingly, these bioremediation technologies are economically viable, environmental friendly and value added processes.

Keywords Bioremediation • Metallothioneins • Heavy metals • Marine environment • Biosurfactants • Biosorption

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29.1 Introduction

Heavy metals are intrinsic part of the earth crust. With rapid industrialization and urbanization enormous amount of industrial waste containing toxic heavy metals have been generated which need special treatment before release into the natural environment viz. terrestrial, aquatic and atmospheric environment. These toxic metals viz. Hg, Cd, Pb, Zn, Cr, Te, As, Sb and Cu released directly into the marine environment pose serious threat to the health and productivity of the marine biota including marine flora and fauna (Skei 1978; De et al. 2007, 2008; Jayaraju et al. 2010). Wastes from industries, sewage sludge, power plants and incineration plant often contain substantial amount of toxic heavy metals, which are of serious environmental concern and need to be removed from the source of pollution itself. It is interesting to mention that even Antarctic Ocean water, which is considered relatively more pristine than any other ocean water, is also contaminated with heavy metals due to anthropogenic activities (Bonner 1984). For instance, high level of several heavy metals in krills have been already reported (Yamamoto et al. 1987). Similarly level of Cd in caridean decapods, *Chorismus antarcticus* and *Notocrangon antarcticus* was highest among marine crustaceans (i.e.13 mg/kg dry weight). Some bacterial isolates from Antarctic Ocean have also shown high resistance to common antibiotics and heavy metals which clearly indicates that Antarctic Ocean is contaminated with heavy metals (De Souza et al. 2006).

For last several decades metal resistant microorganisms including bacteria have been considered a potential alternative for clean up and bioremediation of heavy metal contaminated environmental sites. Many bioremediation technologies have been developed for detoxification and removal of toxic heavy metals from metal contaminated aquatic sites and industrial wastes employing various indigenous metal resistant bacteria from heavy metal contaminated marine sites (Francis and Tebo 1999; Rathgeber et al. 2002; Iyer et al. 2004; De et al. 2007, 2008; Wang et al. 2009).

Microorganisms including bacteria possess a variety of mechanisms to tolerate and bioremediate high levels of toxic metals which include precipitation of metals as phosphates, carbonates and sulphides; volatilization via methylation/ethylation/reduction; intracellular bioaccumulation mediated by low molecular weight metallothionein proteins; ATP mediated efflux system; biosorption of metals at cell surface and sequestration in extracellular polymeric substances viz. EPS (Nakanura 1989; Blindauer et al. 2002; Nies 1999; Roane 1999; Rossbach et al. 2000; Borremans et al. 2001; Edgcomb et al. 2004; Mire et al. 2004; Naik and Dubey 2011). It is important to note that bioremediation technologies based on microbes are economically viable, cost effective, environment friendly.

29.2 Heavy Metal Pollutants in Marine Environment

Although essential metals viz. Zn, Cu, Mn, Mo, Ni, Co, Mg and Fe are required in trace amounts for various metabolic processes of organisms, they are toxic at higher concentrations (Gadd 1992; Choudhuri and Srivastava 2001).Toxic metals viz. Cd,

Pb, Hg, Cr and metalloids such as As and Te which are non-essential, non-biodegradable persist indefinitely in marine ecosystem as a result of natural and anthropogenic activities posing serious environmental and health problems to the marine biota. Consumption of seafood contaminated with mercury and cadmium has been reported to cause serious diseases such as Minamata and itai-itai in coastal population of Japan respectively (Lanford and Ferner 1999; Matsuda et al. 2003). Therefore, these heavy metals have been included as the most hazardous substances in the US-EPA list of priority (Cameron 1992).

29.3 Level of Heavy Metal Pollutants in Marine Environment

All heavy metals exist in surface water in colloidal, particulate and dissolved phases, although dissolved concentrations are generally low (Kennish 1992). The colloidal and particulate metals may be found as (i) hydroxides, oxides, silicates, sulphides; or (ii) adsorbed to clay, silica or organic matter. The soluble forms are generally ions or un-ionized organometallic chelators or complexes. Non-essential heavy metals and metalloids of particular concern to surface water systems are Cd, Cr, Hg, Pb, As and Sb (Kennish 1992).The level of heavy metals in trough sediments , Kara sea, arctic ocean have been determined which revealed presence of cadmium < 0.5 ppm; As 2 ppm; Co 23 ppm; Cr 112 ppm; Cu 30 ppm, Hg 310 ppb; Ni 51 ppm; Pb 17 ppm; Sb 1.8 ppm and Zn 96 ppm respectively (Siegel et al. 2001). Whereas in Minamata bay very high level of mercury was reported which caused a serious neurological disorder in humans referred as “Minamata disease”. The level of total mercury in sea water of Minamata bay ranged from 56 to 285 ng/L and 2.1–506 ng/L (Kumagai and Nishimura 1978). Interestingly, surface sediment sample from semi-enclosed bay, “Gunnekleivfjorden” from Southwest, Norway contained mercury ranging from 90 to 350 ppm (Skei 1978).

The behaviour of metals in natural waters is a function of substrate sediment composition, the suspended sediment composition, and the water chemistry. Sediment composed of fine sand and silt will generally have higher levels of adsorbed metals than quartz and detrital carbonate rich sediments. Metals also have a high affinity for humic acid, organo clays and oxides coated with organic matter (Connell and miller 1984). The water chemistry of the system controls the rate of adsorption and desorption of metals to and from sediment. Adsorption removes the metal from the water column and stores the metal in the substrate. Desorption returns the metal to the water column where recirculation and bioassimilation may take place. Metals may be desorbed from the sediment if the water experiences increase in salinity, decrease in redox potential or decrease in pH.

The contamination levels and distribution characteristics of heavy metals in coastal waters and sediments from Tianjin Bohai Bay, China revealed that Pb and Zn were the main heavy metal pollutants in the coastal waters of the bay. High levels of Pb and Zn appeared especially near the estuary, indicating that river discharge was the main pollution source. Analysis of data for the period 1987–2004 indicated that Pb pollution in coastal waters of Tianjin Bohai Bay originated primarily from

river discharge before 2001. Pb levels did not decrease after 2001 when annual runoff levels declined; indicating that Pb pollution by atmospheric deposition had increased due to the use of leaded petrol in motor cars. Pb, Zn, and Cd were the dominant polluting elements in superficial sediments from Tianjin Bohai Bay, with levels in excess of the corresponding upper limits of environmental background values. High concentrations of polluting elements were found in tidal sediments near water bodies such as Qihe and Dagu estuaries (Meng et al. 2008).

29.4 Heavy Metal Resistant Marine Bacteria

29.4.1 Heavy Metal Resistant Marine Bacteria from Coastal Waters

Since presence of metal resistant bacterial isolates in the environment directly suggests metal contamination, unusual rise in the number of mercury resistant bacteria along the Indian coast indicates significant mercury contamination. Several heavy metal resistant marine bacteria from coastal waters of India were isolated and evaluated for their ability to transform/efflux heavy metals viz. mercury, cadmium and lead. Interestingly, these marine bacteria were highly resistant to mercury and were capable of growing at 25 ppm or higher levels of mercury indicating their potential for detoxification not only of Hg, but also Cd and Pb along with polychlorinated biphenyls (Ramaiah and De 2003; De et al. 2006, 2007, 2008).These metal tolerant bacteria removed mercury by means of volatilization and were successful to detoxify mercury amended waters. Mercury resistant marine, *Pseudomonas aeruginosa* strain CH07 resists lead by EPS – entrapment mechanism which was clearly revealed by SEM-EDX analysis (De et al. 2007). Antarctic water samples were also examined for the presence of metal and antibiotic resistant bacteria (De Souza et al. 2006).The bacterial isolates from these waters showed varying degrees of resistance to antibiotics viz. chloramphenicol, ampicillin, streptomycin, tetracycline and kanamycin along with metals such as Cr, Cd, Zn and Hg which proved that even pristine environments are not devoid of metal pollutants and metal resistant bacteria. The metal resistant marine bacteria include *Enterobacter cloacae, Alcaligenes faecalis, Bacillus pumillus, Bacillus* sp., *Pseudomonas aeruginoasa, Alteromonas* sp., *Vibrio harveyi, Aeromonas* sp., *Corynebacterium* sp., *Brevibacterium iodinium, Streptomyces* sp., *Salinobacter* sp., *Micromonospora* sp., *Saccharomonospora* sp., *Flavobacterium* sp., *Moraxella* sp., *Micrococcus* sp. *and Aeromonas* sp. (Mire et al. 2004; Iyer et al. 2004; De Souza et al. 2006; De et al. 2008; Selvin et al. 2009; Matyar et al. 2010).

29.4.2 Heavy Metal Resistant Marine Bacteria from Marine Sediments

As a consequence of anthropogenic activities marine environment around the world is also contaminated by heavy metals. These toxic metal contaminants usually accumulate in the sediments and interestingly concentration of heavy metals is by several orders of magnitude higher in the sediment than their levels in surface water. It has long been recognised that aquatic microorganisms have a strong affinity for surfaces and that the majority of benthic bacteria are not suspended in waters but are attached to sediment particles. Adherent bacteria from Belgian continental plate showing metal contaminated marine sediment belongs to γ and δ proteobacteria (Gillan and Pernet 2007). Water and sediments of the Seine estuary are also contaminated by chemicals, especially Cadmium, which interestingly favours survival, growth and selective enrichment of Cadmium resistant bacteria. Among them 11 distinct isolates were found to carry *cadA* gene e.g. *Staphylococcus* sp., *Micrococcus* sp. and *Halobacillus* sp. (Oger et al. 2003).

Arsenite resistant bacteria were detected in deep sea sediments on the SouthWest Indian Ridge. Phylogenetic analysis based on 16S rRNA revealed that they mainly belonged to proteobacteria and actinobacteria. Denaturing gradient gel electrophoresis revealed that *Microbacterium esteraromaticum* was the dominant member in the arsenite enriched communities and this was reconfirmed by 16S rRNA gene library analyses. There is a significant diversity in arsenite resistant bacteria inhabiting the deep sea sediment which may play a role in the biogeocycling of arsenic in the marine environments (Chen and Shao 2009).

A deep sea sedimentary manganese-oxidizing bacterium, *Brachybacterium* sp. strain Mn 32 showed high Mn(II) resistance (MIC 55 mM) and Mn (II) – oxidizing/removing abilities. Strain Mn 32 removed Mn(II) employing two pathways : (i) oxidizing soluble Mn (II) to insoluble biogenic Mn oxides – birnessite (δ-MnO_2 group) and manganite (γ-MnOOH); (ii) the biogenic Mn oxides further adsorb more Mn(II) from the culture medium (Wang et al. 2009).

Few studies have focused on the mechanisms of adaptation to mercury contamination in marine sediment microbial communities. High frequency of Gram-negative bacterial isolates that are resistant to mercury were isolated from the aerobic culturable marine microbial community of marine sediment of Brunswick, GA, and Skidaway island near Savannah, GA, USA (Reyes et al. 1999).

29.4.3 Heavy Metal Resistant Marine Bacteria from Hydrothermal Vent, Sponges and Coral Reefs

Hydrothermal vents are highly rich sources of heavy metals which are considered to be toxic to living organisms. Since deep-sea hydrothermal vent fluids are enriched with toxic metals, it was hypothesized that (i) the biota in the vicinity of a vent is

adapted in the presence of toxic metals and (ii) metal toxicity is modulated by the steep physico-chemical gradients that occur when anoxic, hot fluids are mixed with cold oxygenated seawater. These metal enriched waters provide a constant selection pressure making the deep-sea hydrothermal vent environment a likely place to find heavy metal resistant microorganisms. For instance, heavy metal resistant heterotrophic bacteria were also isolated from the deep sea hydrothermal vent polychaete, *Alvinella pompejana* (Jeanthon and Prieur 1990). Mercury adaptation among bacteria from a deep-sea hydrothermal vent has also been reported (Vetriani et al. 2005). Deep-sea hydrothermal vents lie deep below the ocean and spew waters enriched with metals such as cadmium, zinc, lead, iron, and mercury. *Pseudomonas aeruginosa* CW961, an isolate from the vicinity of a deep-sea hydrothermal vent, grew in the presence of 5 mM Cd^{2+} and removed Cd^{2+} from solution. Sulphate was sufficient for growth when Cd $^{2+}$ was not present in the culture medium; however, thiosulfate was necessary for Cd^{2+} precipitation and cell survival in the presence of Cd^{2+} (Wang et al. 2002). Three hyperthermophilic vent archaea, the sulfur-reducing heterotrophs, *Thermococcus fumicolans* and *Pyrococcus* strain GB-D and the chemolithoautotrophic methanogen *Methanocaldococcus jannaschii* were tested for tolerance to heavy metals viz. Zn, Co, and Cu. The sulphide addition consistently ameliorated the high toxicity of free metal cations by the formation of dissolved metal-sulphide complexes as well as solid precipitates (Edgcomb et al. 2004).

Coral reef flora and fauna are at risk of exposure to high concentrations of heavy metals from contaminant inputs such as processing plant and mine effluent discharged into rivers and marine ecosystems (Gladstone 1996). Coral reef sponges often harbour communities of symbiotic microorganisms that fulfil necessary functions for the well-being of their hosts. Sponges invariably filter a large volume of seawater and potentially accumulate heavy metals and other contaminants from the environment. Sponges, being sessile marine invertebrates and modular in body organization, can live many years in the same location and therefore have the capability to accumulate anthropogenic pollutants such as toxic heavy metals over a long time period. Sponges and corals are often host to abundant and diverse communities of symbiotic bacteria and algae that contribute to their nutrition and health. Microbial communities associated with the sponge, *Rhopaloeides odorabile* were used as bioindicators for sublethal copper stress (Webster and Webb 2001). Not only do sponges have potential for monitoring elevated concentrations of heavy metals but also examining changes in their microbial symbionts which is a novel and sensitive bioindicator for the assessment of pollution on important microbial communities. The sponge associated bacteria including *Streptomyces* sp. MSI01, *Salinobacter* sp. MSI06, *Roseobacter* sp. MSI09, *Pseudomonas* sp. MSI016, *Vibrio* sp. MSI23, *Micromonospora* sp. MSI28, *Saccharomonospora* sp. MSI36 and *Alteromonas* sp. MSI42 showed resistance against Cd, Hg and Pb (Selvin et al. 2009).

29.5 Biochemical and Genetic Mechanisms of Heavy Metal Bioremediation by Marine Bacteria

29.5.1 Intracellular Sequestration by Metallothioneins and Other Metal Binding Proteins

Metallothioneins are small cysteine rich, low molecular weight proteins ranging from 3,500 to 14,000 Da and ubiquitous in eukaryotes but also present in bacteria and cyanobacteria (Hamer 1986).These proteins have potential to sequester multiple heavy metal ions viz. Hg^{+2}, Cd^{+2}, Pb^{+2}, Zn^{+2}, Ag^{+1} and Cu^{+2} through thiol groups of cysteine and also participate in metal homeostasis. Cysteine constitutes nearly 30% of the total amino acids present in the protein. Metallothioneins and other metal binding proteins present in diverse cyanobacterial and bacterial species regulate the intracellular toxic metal concentration through highly specific metal binding. Metallothioneins limit metal availability and is thereby believed to confer protection against deleterious effects of high concentration of toxic heavy metals in *Synecococcus* PCC 7942, *Anabaena* PCC 7120, *Pseudomonas putida* and *Pseudomonas aeruginosa* (Blindauer et al. 2002). Typically, the metals for which metallothioneins have the highest affinity are Cd, Pb, Hg, Cu and Zn but also bind to Ni though with lower affinity. The heterotrophic marine bacterium, *Vibrio alginolyticus* interestingly showed production of extracellular copper-binding compounds when exposed to copper in a sea water medium. Fractionation and analysis of copper and methionine incorporation in culture supernatant fractions showed that the copper-binding compound co-eluted with material which was radiolabeled with ^{35}S methionine. This suggested that the copper-binding compound is a protein (Schreiber et al. 1990).The data suggest a potential role for macromolecules excreted by heterotrophic bacteria in control of copper ion activity in seawater. Two copper-inducible supernatant proteins having molecular masses of 21 and 19 kDa (CuBPl and CuBP2) were identified in marine bacterium, *Vibrio alginolyticus* and these proteins were 25 and 46 times amplified in supernatants of copper-challenged bacterial cultures as compared with controls without copper (Harwood-Sears and Gordon 1990).

29.5.2 Exopolymers, Biosurfactants and Other Biomolecules

Prokaryotic microorganisms in nature produce diverse and chemically complex exopolymeric substances (EPS) comprising of a variety of high molecular weight organic molecules viz. polysaccharides, proteins, nucleic acids, phospholipids along with other non-polymeric constituents of low molecular weight. EPS localized on outer bacterial cell surface mediate exchange of nutrients and essential metal ions with surrounding environments and also play important role in cell adhesion, formation

of biofilms, flocks and protects cells from hostile environments (Vu et al. 2009). Electrostatic interaction of metal cations with negatively charged functional groups involved in metal immobilisation by EPS are COO^{-}, HPO_4^{-}, OH^{-} and SO_4^{-2} and possess both adsorptive and adhesive properties (Pal and Paul 2008). EPS is believed to play a substantial role in sequestration of toxic heavy metals with varying degree of specificity and affinity thus prevent their entry inside bacterial cells and protect bacterial cells from toxic heavy metals such as Pb, Cd, Hg and Zn. Enzymatic activities in bacterial EPS are also involved in detoxification of heavy metals by transformation and subsequent precipitation resulting in entrapment of metals in these polymeric substances (Loaec et al. 1998; Bhaskar and Bhosle 2006; Poli et al. 2010). Therefore use of microbial sorbents like EPS can be used to bioremediate heavy metals from marine waters since marine pollution control is a major challenge to the environmental biotechnologists. The use of isolated microbial biopolymer in metal bioremediation process is more economically viable and ecofriendly alternative to environmentally risky chemical methods such as precipitation, coagulation, ion exchange and electrochemical processes.

Bacterial extracellular polymeric substances work as a carrier of heavy metals in marine food chain since metal binding properties of bacterial EPS and its possible role in bioaccumulation of Cu and Pb in marine food chain was investigated using a partially purified and chemically characterized microbial EPS isolated from *Marinobacter* sp. (Bhaskar and Bhosle 2006). Marine isolates of sulphate reducing bacteria (SRBs) have been reported to release EPS in the liquid medium during growth which forms complex with Ni, Cr and Mo and may be employed for bioremediation of marine ecosystem (Beech and Cheung 1995). It has been demonstrated that heavy metal resistant, deep sea hydrothermal vent bacterial species also produce EPS with capacity to bind metals and other toxic substances (Loaec et al. 1998; Wuertz, et al. 2000). EPS produced by Antarctic bacterial isolate contained uronic acid and sulphate which may act as carrier of heavy metals in southern ocean environments (Mancuso, et al. 2005). In marine *Pseudomonas aeruginosa* CH07 Lead was entrapped in EPS indicating it as a possible resistance mechanism (De et al. 2007) (Fig. 29.1). Accumulation of hexavalent chromium by an exopolysaccharide producing marine *Enterobacter cloacae* which is not only resistant to chromium but also showed enhanced growth and exopolysaccharide production in the presence of 25, 50 and 100 ppm Cr (VI) (Iyer et al. 2004).

Surfactants and emulsifiers comprise a unique class of molecules that are distinguished by their capacity to interface between water-soluble and oil phases. This amphipathic quality is conferred by the presence of both polar and non-polar moieties, endowing these molecules with a hydrophilic-hydrophobic nature. Marine microorganisms produce low and high molecular weight biosurfactants. The low molecular weight biosurfactants include glycolipids viz. trehalose tetraesters, dicorynomycolates, fructose lipids, sophorolipids and rhamnolipids and lipopeptides which include surfactin and viscosin. Preferred use of biosurfactants for bioremediation of heavy metals over chemical surfactants and emulsifiers is due to their lower toxicity, better environmental compatibility and economic viability. Among the various bioactive compounds, biosurfactants (BS)/bioemulsifiers (BE) are

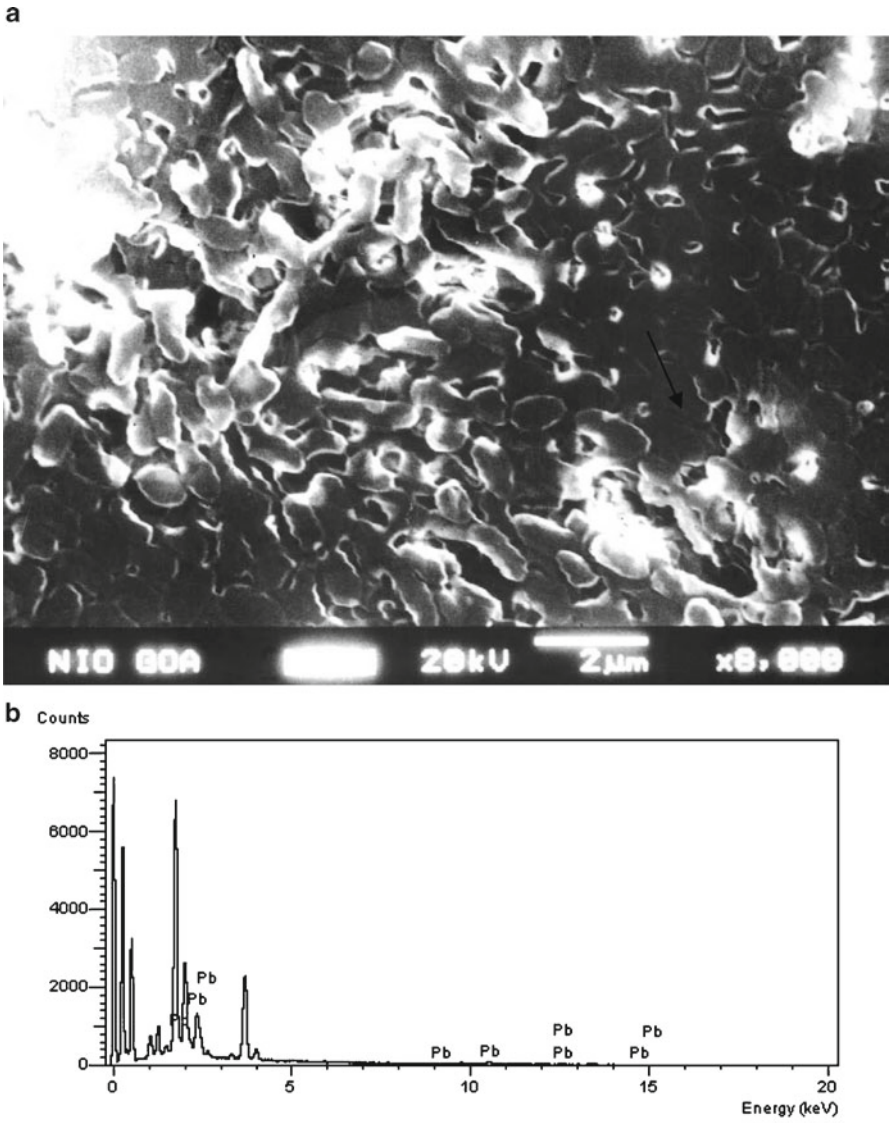

Fig. 29.1 Removal of Pb from Sea water nutrient broth (SWNB) amended with 50 mg/l Pb. **a**) SEM picture of the EPS-entrapped Pb (black arrow). **b**) The signal reflected from Pb as revealed by EDS. Results shown here are for the mercury-resistant marine Pseudomonas strain CH07. (Courtesy of De Jaysankar, in Microbes and Environment 22(4):336–345, 2007)

attracting major interest and attention due to their structural and functional diversity. Marine microorganisms such as *Acinetobacter* sp., *Arthrobacter* sp., *Pseudomonas* sp., *Halomonas* sp., *Myroides* sp., *Corynebacteria* sp., *Bacillus* sp., *Alteromonas* sp. have been studied for production of BS/BE and exopolysaccharides (Satpute et al. 2010). Hexavalent chromium reduction and trivalent chromium tolerance of

marine *Bacillus* sp. MTCC 5514 is known to be mediated by its extracellular enzyme, chromate reductase and its biosurfactant (Gnanamani et al. 2010). An excellent alternative for enhanced metal bioremediation is the use of microbial biomolecules such as microbial surfactants and extracellular polymers which would increase the efficiency of metal reduction/sequestration by microorganisms to achieve field bioremediation of metal contaminated sites (Singh and Cameotra 2004). It is interesting to note that these biomolecules exhibit dual advantage in bioremediation of sites contaminated with metals and organic compounds since microorganisms have capability to utilize organic compounds such as hydrocarbons as carbon source and simultaneous sequestration of heavy metals.

29.5.3 ATPase Mediated Efflux

In order to maintain heavy metal homeostasis, intracellular concentration of toxic heavy metal ions has to be tightly controlled. Soft-metal-transporting P-type ATPases are group of proteins involved in transport of heavy metals across the biological membranes and thus responsible for bacterial heavy metal resistance. ATPase mediated efflux pump prevents over accumulation of metals inside the bacterial cells by effluxing excessive heavy metals outside the cells. The best examples of Zn(II)/Cd(II)/Pb(II)-translocating ATPases are encoded by gene, *zntA* from *E. coli* and *cadA* gene from *S. aureus* plasmid pI258 (Silver 1999; Rensing et al. 1999). Complete operon *pbrUTRABCD* confering efflux mediated lead resistance has been well studied in *Ralstonia metallidurans* CH34 (Borremans et al. 2001; Taghavi et al. 2009).The mechanism of Zn resistance in multiple metal-resistant *Pseudomonas putida* strain S4 is based on inducible efflux. An ATPase in this strain mediates active extrusion of Zn^{+2}, which occurred during the exponential phase of growth (Choudhuri and Srivastava 2001).

29.5.4 Biosorption

Biosorption of metals is mediated by several mechanisms such as ion exchange, chelation, adsorption and diffusion through cell walls and membranes. Biosorption encompasses those physico-chemical mechanisms by which metal ions are removed from an aqueous solution. This phenomenon is often attributed to the binding of metals on to the bacterial cell surface and EPS (Fig. 29.2) (Van Hullebusch et al. 2003). For example bacterial biomass was produced by culturing polysaccharide-producing marine *Bacillus circulans* in liquid medium containing glucose as carbon source and biomass was used to remove copper and cadmium ions from aqueous solutions through biosorption. A biomass of 1.48–1.52 g dry weight/l was found to remove 80% of copper and 44% of cadmium from solutions containing 495 ppm copper and 492 ppm cadmium respectively (Sahoo et al. 1992). Heavy metal chelation property of exopolysaccharide produced by *Enterobacter cloacae*, a marine

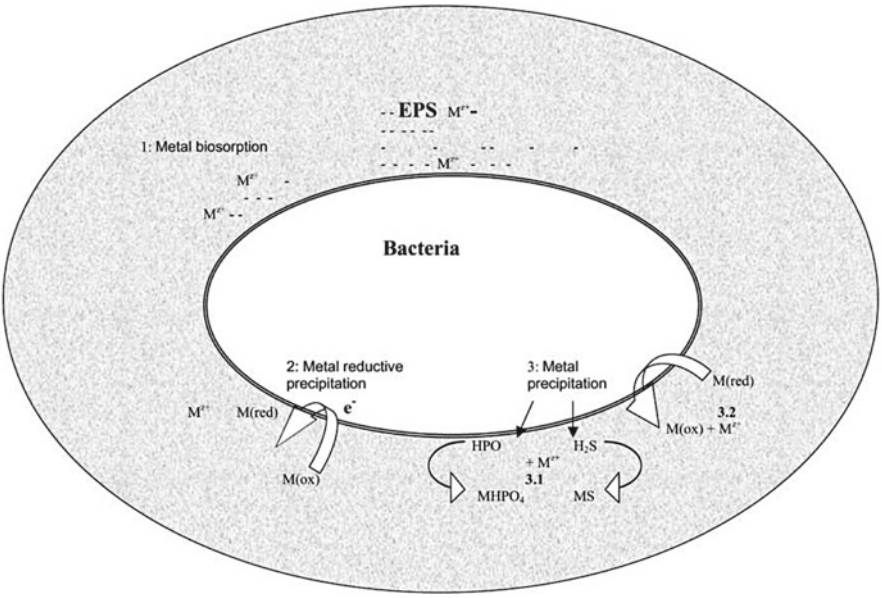

Fig. 29.2 Schematic representation of the interaction between metals and biofilm. 1: Metal biosorption by EPS; 2: Reductive precipitation of metal by enzymatic transformation; 3: 3-1: Metal precipitation as sulfide and phosphate; 3-2 Metal precipitation as biogenic oxide (iron and manganese) and metal adsorption on biogenic oxides. (Courtesy of Lens et al. 2003 ; Environ. Sci. Bio/Technol. 2: 9–33)

bacterium, isolated from the West Coast of India has already been reported. The exopolysaccharide demonstrated excellent chelating properties with respect to cadmium (i.e. 65%) followed by copper (20%) and cobalt (8%) at 100 mg/l level of respective heavy metal (Iyer et al. 2004). Mn(II)-oxidizing spores of marine *Bacillus* sp. strain SG-1 may be used successfully for bioremediation of Mn due to their inherent physically tough nature, unique metal binding capacity and oxidative precipitation (Francis and Tebo 1999). Photosynthetic bacterium, *Rhodobacter sphaeroides* S and another marine photosynthetic bacterium *Rhodovulum* sp. PS88 in a batch culture were capable of significant cadmium removal in the presence of 30 g/l Sodium chloride and divalent cations Mg^{2+} and Ca^{2+} (Watanabe et al. 2003).

29.5.5 Bioprecipitation as Phosphates and Sulphides

Marine bacteria immobilise toxic metals through bioprecipitation as phosphates and sulphides and protect bacterial cells from toxic effects of metals. Three pleiotropic, quorum sensing-defective *Vibrio harveyi* mutants were observed to precipitate soluble Pb^{+2} as an insoluble compound. The compound was purified and subjected to X-ray diffraction and elemental analyses. These assays identified the precipitated compound as $Pb_9(PO4)_6$, an unusual and complex lead phosphate salt that is produced synthetically

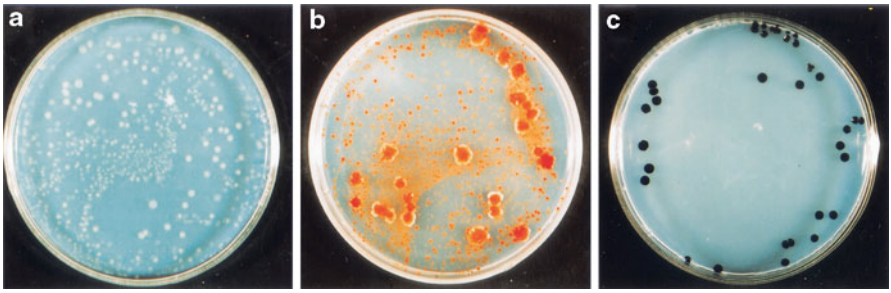

Fig. 29.3 Plates used for the enumeration of metalloid-resistant or - reducing bacteria in bacterial-film-like formations taken from the Melarie Summit site, Main Endeavor Field. (**A**) Metalloid-free control plate; (**B** and **C**) Plates containing 100 μg Na2SeO3 per ml and 100 μg K2TeO3 per ml, respectively. The red-orange color of colonies (**B**) is due to the accumulation of elemental Se, and the black color (**C**) is due to the accumulation of elemental Te. (Courtesy of Vladimir Yurkov; Appl. Environ. Microbiol. 68(9):4612–4622, 2002)

at 200°C (Mire et al. 2004). Sulphate reducing bacteria (SRBs) are anaerobic heterotrophic bacteria in marine environment which also precipitate heavy metals as insoluble metal sulphides e.g. ZnS, PbS, CdS and CuS (Chamberlain et al. 1988). A fluorescent *Pseudomonas* strain CW-96-1 isolated from a deep-sea vent sample under aerobic conditions tolerated cadmium up to 5 mM and Cadmium was removed by precipitation on the cell wall as cadmium sulphide (Wang et al. 1997, 2002).

29.5.6 Redox Reactions and Volatilization

Certain heavy metals viz. Cr, Mo, Se, U, Tc and Au can be immobilised by bacteria following enzymatic reduction of these metals into an insoluble lower redox state. Under oxidizing conditions, they occur as highly soluble and mobile ions, however, under reducing conditions they usually form insoluble phases (Van Hullebusch et al. 2003).Various marine mercury resistant bacterial isolates detoxify toxic mercury through reductive volatalization of mercury. Hg^{+2} gets reduced to elemental mercury (Hg^0) through catalytic activity of mercuric reductase enzyme encoded by *merA* gene (De et al. 2007, 2008). Various species of bacteria reduce selenite to elemental selenium which is insoluble and nontoxic thus eliminate its toxic characteristics. Tellurite- and selenite-reducing bacterial strains were isolated in high numbers from ocean water near hydrothermal vents, bacterial films, and sulfide-rich rocks. Growth of these isolates in media containing K_2TeO_3 or Na_2SeO_3 resulted in the accumulation of metallic tellurium or selenium (Figs. 29.3 and 29.4) (Rathgeber et al. 2002). Copper and nickel tolerant marine sulphate reducing bacteria (SRBs) were isolated from 90/10 copper-nickel alloy and were shown to attach and grow on this alloy. Using energy dispersive X-ray analysis, X-ray photoelectron spectroscopy and electrochemical polarization analyses it was confirmed that intracellularly immobilized metal sulphides produced resemble with synthetic sulphides

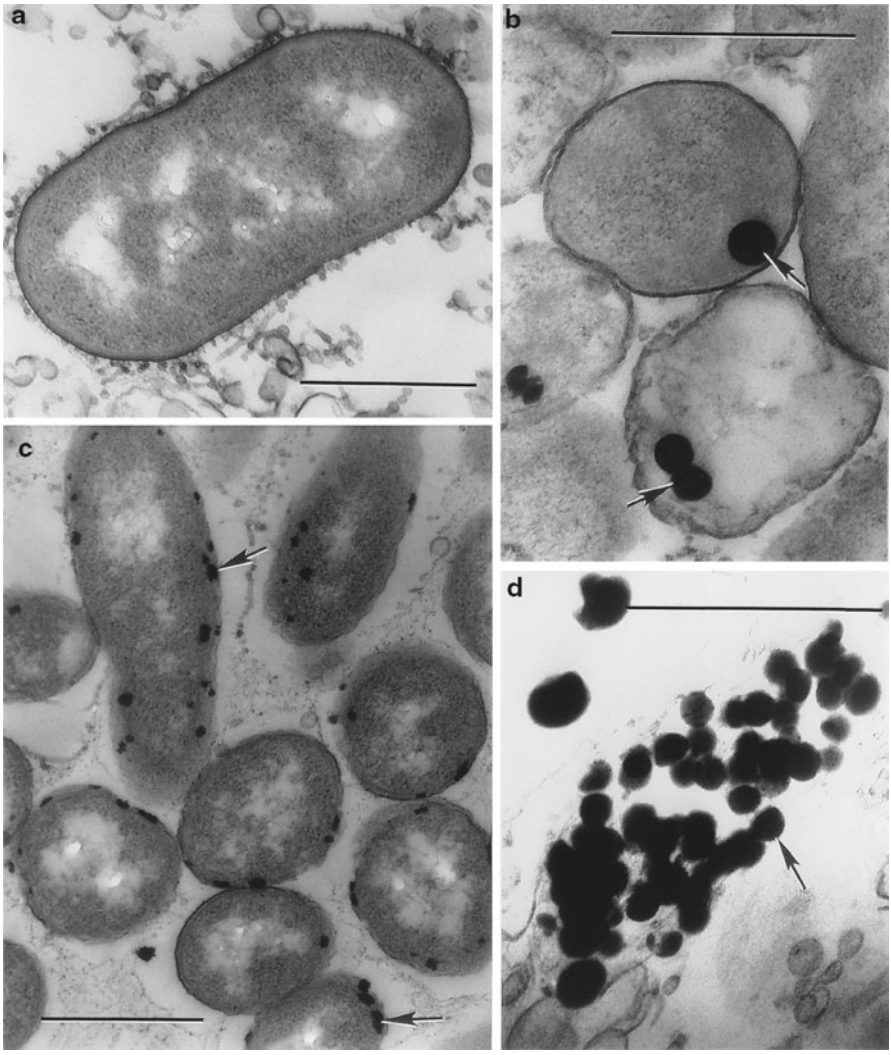

Fig. 29.4 Electron microscopy of ultrathin sections (**A**) Strain Se-1-2-red grown in metalloid-free medium; (**B** and **C**) intracellular localizations of Se (**B**) and Te (**C**), as reduction products of selenite and tellurite, in strains Se-1-2-red (48-h-old culture) and Te-1-1 (72-h-old culture), respectively; (**D**) granules of Se released from cells of Se-1-2-red (48-h-old culture). Bars: 0.5 μm. (Courtesy of Vladimir Yurkov; Appl. Environ. Microbiol. 68(9):4612–4622, 2002)

(Chamberlain et al. 1988). A deep sea sedimentary manganese-oxidizing bacterium, *Brachybacterium* sp. strain Mn 32, showed high Mn(II) resistance (MIC 55 mM) and Mn(II)-oxidizing/removing abilities. This bacterial strain removed Mn (II) employing a simple pathway involving oxidation of soluble Mn (II) to insoluble biogenic Mn oxides (Fig. 29.5) (Wang et al. 2009).

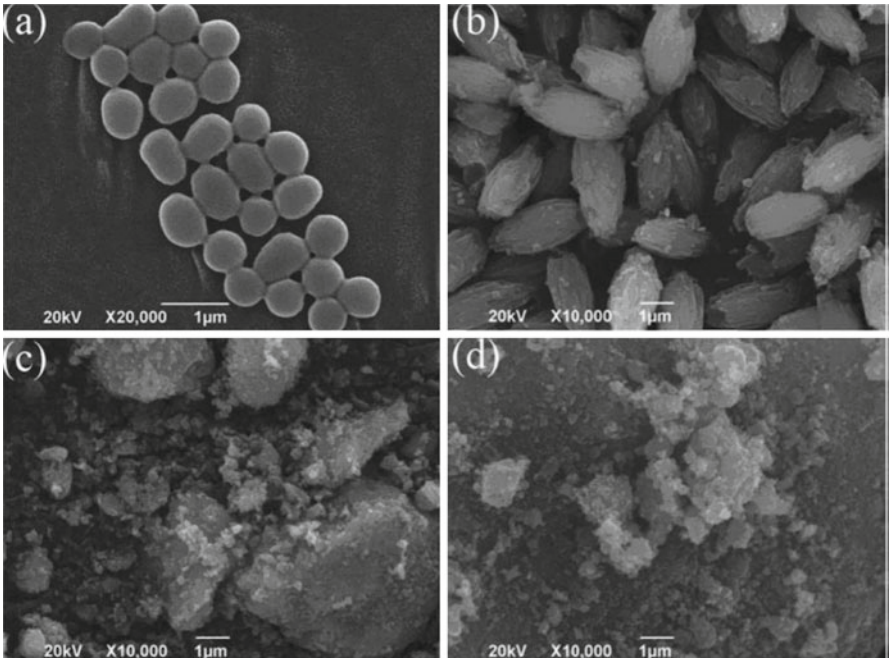

Fig. 29.5 SEM images of (**a**) cells of Brachybacterium sp. strain Mn32 (after 9 days of cultivation in liquid A medium without MnCl2); (**b**) biogenic Mn oxides produced on the cell surface of strain Mn32 (9 days of cultivation with 0.2 M MnCl2 in liquid A medium); (**c**) commercial MnO_2; (**d**) fresh synthetic MnO2 (Courtesy of Wang et al.; Microbiology 155:1989–1996, 2009)

29.5.7 Metal Resistance Conferring Bacterial Genes and Their Significance in Metal Bioremediation

Bacterial metallothionein genes *bmtA* from *Anabaena* PCC 7120, *Pseudomonas aeruginosa* and *Pseudomonas putida* encoding metallothionein that bind multiple zinc and copper ions with high affinity have already been cloned. Thiol modification demonstrates that cystein coordinates zinc in all these proteins and participates primarily in metal homeostasis. Bacterial isolates with this specific protein accumulate very high intracellular levels of metals without any deleterious effects on cell physiology and metabolism. This unique property of hyper-metal accumulating bacterial isolates can be employed in heavy metal bioremediation (Blindauer et al. 2002). Two copper-inducible supernatant proteins having molecular masses of 21 and 19 kDa (CuBPl and CuBP2) were identified in marine bacterium, *Vibrio alginolyticus* and these proteins were 25–46 times amplified in supernatants of copper-challenged cultures as compared with controls, which help in metal accumulation and therefore homeostasis of metals (Harwood-Sears and Gordon 1990).

Volatilisation of mercury by reducing Hg^{+2} to highly volatile elemental mercury, Hg^0 is mediated by the enzyme mercuric reductase encoded by the gene *merA* which

is a Hg detoxification mechanism employed by marine bacteria viz. *Alcaligenes faecalis*, *Bacillus pumilus, Pseudomonas aeruginosa* and *Bacillus iodinium* (De et al. 2008). Similarly *mer B* gene in marine bacteria encodes organomercurial lyase which is responsible for break down of organomercurial compounds causing cancer. Marine bacteria possessing this gene can be used to detoxify marine waters contaminated with organomercurials by using bioremediation technology.

Bacterial plasmids possess genetic determinants encoding resistance to several toxic metal ions viz. Ag^{+2}, AsO_2^{-}, AsO_4^{-3}, Cd^{+2}, Co^{+2}, CrO_4^{-2}, Cu^{+2}, Hg^{+2}, Ni^{+2}, Pb^{+2}, Sb^{+3}, TeO_3^{-2}, Tl^{+} and Zn^{+2} (Silver 1999). The function of most resistance systems is based on the energy dependent efflux of toxic ions. Efflux systems are ATPases and others are chemiosmotic cation/proton pumps e.g. *czc* system conferring resistance to Cd^{+2}, Zn^{+2} and Co^{+2}. Some arsenate resistant bacterial isolates possess *ars* operon including *arsC* gene encoding arsenate reductase enzyme which reduce arsenate (As^{+5}) to arsenite (As^{+3}) and *arsA* gene encoding ATPase enzyme which efflux toxic arsenite to the cell exterior in order to maintain metal homeostasis (Silver 1999). ATPase based efflux pumps prevent over accumulation of toxic metals inside the bacterial cells by effluxing excessive heavy metals outside the cells. For instance Zn(II)/Cd(II)/Pb(II)-translocating ATPases are encoded by *zntA* in *E. coli* and *cadA* of *S. aureus* plasmid pI258 confers cadmium efflux mediated by cadmium ATPase (Silver 1999; Rensing et al. 1999).

29.5.8 Genetic Engineering of Marine Bacteria for Bioremediation of Metals

Significant developments in the field of molecular biology, microbiology, microbial biochemistry, biotechnology and genetics resulted in successful development of genetically engineered microbes for bioremediation of toxic metals. A number of genetic engineering approaches have been developed recently which proved useful in introducing the desired traits in bioremediating marine bacteria for designing microbes with novel remedial properties (Chen et al. 1999; Pieper and Reneke 2000).

Insertion of metallothionein encoding *bmtA* gene into a suitable expression vector and its transformation in a marine bacteria is already accomplished. These genetically modified marine bacteria expressing extremely high amount of metallothionein can be employed to bioremediate marine sites highly contaminated with heavy metals. These hyper-metal accumulating bacteria maintain metal homeostasis by reducing metal bioavailability. Expression of metallothioneins on the cell surface by fusion with cell surface proteins improves the bioaccumulation capacity of bacteria (Chen et al. 1999).

Marine bacterial isolates with natural plasmids have been well characterized which possess *merA* gene responsible for reduction of Hg^{+2} to elemental Hg^{0} which is volatile and non toxic. This plasmid can also be transformed in marine bacteria or *merA* gene can be cloned into marine bacteria using standard genetic engineering techniques. These genetically engineered bacteria can be employed to

detoxify mercury contaminated marine sites. Introducing genes responsible for resistance to multiple heavy metals into a marine bacterium makes it resistant to multiple heavy metals and may be used to bioremediate sites contaminated with multiple heavy metals.

Marine bacteria can be genetically engineered to express high amount of metal binding groups viz. carboxyl, hydroxyl, sulphate, phosphoric, amine for biosorption of high amount of toxic metals either on cell surface or microbial products such as EPS and biosurfactants. Modification of bacterial isolates which can over produce EPS and biosurfactants may be a good strategy for bioremediation of significant amount of heavy metals. Microbial enzymes can be modified to increase their kinetics to reductively precipitate heavy metal ions or increase efficiency of enzyme to effectively detoxify metals such as mercury.

29.6 Metal Bioremediation Technologies Based on Marine Bacteria and Future Prospects

Natural marine bacterial isolates have got surprisingly tremendous potential to tolerate, sequester and remove toxic metal pollutants from ambient environment. Therefore, it is imperative and highly desirable to characterize such marine bacterial isolates with reference to their biochemical and genetic mechanism of resistance against heavy metals. Highlighting the fundamental mechanism of resistance in marine bacteria would prove useful in designing bioremediation sites polluted with toxic metals. The combination of genetic engineering of bacterial catalysts with judicious eco-engineering of polluted sites will be of paramount importance in future bioremediation strategies. Valuable properties already present in certain strains can be combined or improved through state-of-the-art genetic engineering tools. In future, expression of metallothioneins (MTs) on the surface of environmentally robust organisms such as marine *Pseudomonas aeruginosa* could prove to be a very promising strategy. Application of genetically engineered microorganisms (GEMs) in bioremediation has received a great deal of attention but has largely been confined to laboratory environment. Their practical impact and delivery under field conditions need to be studied. GEMs can also be employed to develop bioreporter sensors for biomonitoring sudden changes in the level of marine metal pollutants released due to several anthropogenic activities and natural geochemical cycling. However, ecological and environmental concerns and regulatory constrains are major obstacles for testing GEMs in the field. Legislations and biosafety norms should be strictly adhered to in this regard before employing these bacteria in metal bioremediation under field conditions. Since release of GEMs is banned worldwide their release to the environment will not be possible. Therefore, only alternative for metal remediation remains to be under controlled laboratory conditions.

An excellent option for enhanced bioremediation of metal contaminated sites is use of microbial products viz. surfactants, emulsifiers and extracellular polymers which would increase the efficiency of metal reducing/sequestering organisms for

field bioremediation. Sustainable and ecofriendly biotechnologies should be used in future for bioremediation and environmental impact assessment (EIA) should be performed prior to introducing metal resistant, marine GEMs in fields for bioremediation of toxic metals.

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Chapter 30
Microbial Denitrification and Its Ecological Implications in the Marine System

Trelita de Sousa and Saroj Bhosle

Abstract Microbial denitrification is an essential component of the nitrogen cycle and occurs extensively in the estuarine, coastal and marine ecosystems. Denitrifying organisms are unique because they are facultative and can switch between aerobic and anaerobic modes of respiration by utilizing nitrogen oxides as electron acceptors via a series of reductases under conditions of oxygen limitation and nitrate availability. Oxygen plays a regulatory role in aerobic denitrification and controls the electron transport to oxygen or nitrate. Denitrifiers are ubiquitously distributed encompassing a wide array of microorganisms ranging from bacteria and archaebacteria to fungi and foraminifers. Techniques like terminal restriction fragment length polymorphism analysis, functional single-cell isolation method and fluorescent in situ hybridization have provided new insights into the community structure and functioning of these organisms. *Pseudomonas, Paracoccus and Alcaligenes* are among the most frequently isolated and studied denitrifying bacterial genera. Denitrification acts as an important feedback mechanism and on a global scale has critical impacts on the Earth's climate. The process operates as a nitrogen sink in estuaries and controls marine biological productivity. The recently discovered anoxic ammonia oxidation process or anammox, which is greatly responsible for the loss of fixed nitrogen in the oxygen minimum zones in the marine system, is also dependent on denitrification for its nitrite. Denitrification also contributes to significant consequences in global warming and hydrocarbon bioremediation.

Keywords Denitrification • Denitrifying bacteria • Anammox • Nitrate • Marine • Hydrocarbon

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30.1 Introduction

Denitrification has been recognized as a major process in the global nitrogen cycle, sustained by microorganisms (Gamble et al. 1997; Zumft 1997) and has stimulated considerable interest over the years. However, in the past two decades, studies on denitrification have gained momentum on account of its essential ecological and geochemical implications in both terrestrial and aquatic systems.

This intricate process occurs ubiquitously in nature and is instrumental in balancing the highly interwoven and complex nitrogen cycle, a simple schematic representation of which is given in Fig. 30.1 (Prescott et al. 2003; Ward et al. 2007). Denitrification plays a critical role in regulating the amount of primary production, in counteracting eutrophication as well as in sustaining the global nitrogen budget particularly in aquatic systems (Zehr and Ward 2002; Seitzinger et al. 2006; Fulweiler 2007; Gruber and Galloway 2008; Fennel et al. 2009; Schlesinger 2009).

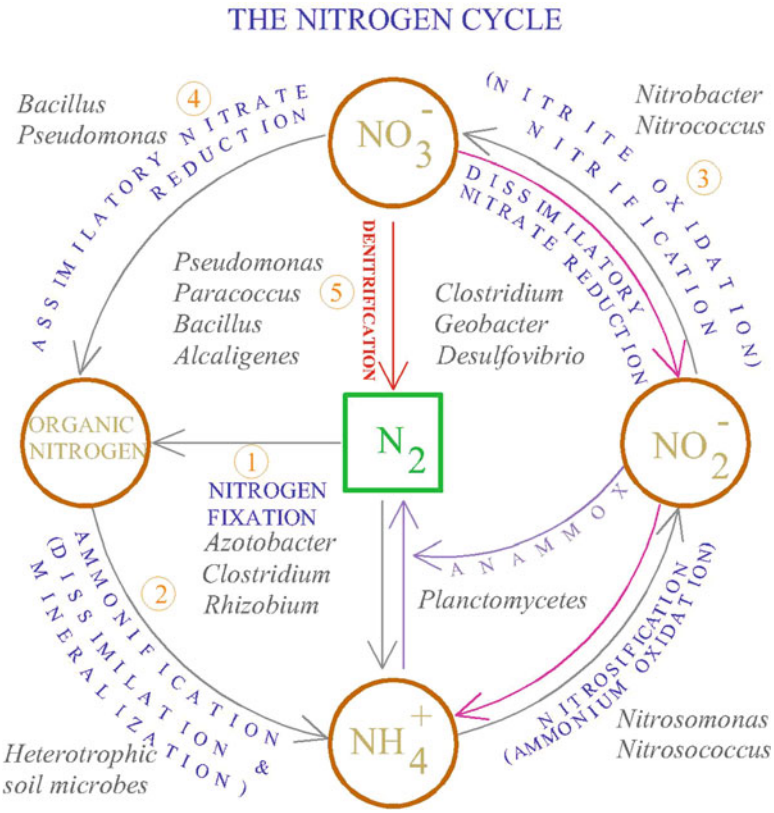

Fig. 30.1 The various transformations of the intricate nitrogen cycle sustained by microorganisms. Abbreviations: Anammox, anoxic ammonia oxidation (Prescott et al. 2003; Ward et al. 2007)

Its contribution of nitrous oxide to the atmosphere has made it an important element in global warming investigations (Takaya et al. 2003; Sandbach 2007). So also, the prevalence of denitrifying bacteria in hydrocarbon contaminated sites has provided a new dimension to bioremediation studies (Wilson and Bouwer 1997; Chawala and Lu-Kwang 2000; Chénier et al. 2003; Shinoda et al. 2004; Gallagher et al. 2005; Song and Ward 2005; Quan et al. 2007; Callaghan et al. 2009; Cao et al. 2009).

This chapter compiles data on the denitrification mechanism as a whole, its microbial diversity, and its ecological implications in the estuarine, coastal and marine ecosystems with a perspective on pollution abatement and environment management.

30.2 Critical Review

30.2.1 Occurrence of Denitrification

Denitrification is fundamentally an anaerobic process occurring in oxygen limited environments with NO_3^- availability (Baumann et al. 1996; Zumft 1997; Flores-Mireles et al. 2007). It occurs in essentially all river, lake, ground water, estuarine and coastal marine ecosystems but in general, the range of denitrification rates measured in coastal, marine and estuarine sediments is greater than that measured in lake or river sediments (Seitzinger 1988; Gamble et al. 1997; Bachman and Krantz 2008). This is so because these systems receive ample amounts of anthropogenic nutrient inputs and is rich in nutrient supply that comes from mineralization of organic matter (Seitzinger 1988). Studies comparing the rates of denitrification across a range of ecosystems suggest that the coastal and marine systems account for the largest proportion of total global denitrification (58%), followed by the terrestrial systems (22%) and freshwater systems (20%). The oxygen minimum zone in the ocean and the continental shelf individually make up 14% and 44% respectively in the marine system fraction while the estuarine system comprises 1% in the freshwater system (Seitzinger et al. 2006).

30.2.2 Physiology and Biochemistry

Denitrification is the sequential exergonic dissimilatory reduction of one or both of the ionic nitrogen oxides; nitrate (NO_3^-) and nitrite (NO_2^-), brought about by aerobic to facultatively anaerobic heterotrophs, to the gaseous nitrogen oxides; nitric oxide (NO) and nitrous oxide (N_2O). These are finally reduced to form the ultimate product, dinitrogen (N_2) (Knowles 1982; Seitzinger 1988; Zumft 1997). During denitrification, a nitrogen oxide, instead of oxygen, serves as the terminal electron acceptor thus generating an electrochemical gradient across the cytoplasmic membrane. The concerted action of a series of terminal oxidoreductases that transfer

electrons through several nitrogen oxides results in the sequential transformation of NO_3^- to N_2. Thus, denitrification is a multi-step process involving the participation of four enzymes: nitrate reductase (NaR), nitrite reductase (NiR), nitric oxide reductase (NOR) and nitrous oxide reductase (N_2OR) to form the ultimate product, dinitrogen as given below (Zumft 1997; Prescott et al. 2003; Sipkoska-Gastarova et al. 2008).

$$NO_3^- \xrightarrow{NaR} NO_2^- \xrightarrow{NiR} NO \xrightarrow{NOR} N_2O \xrightarrow{N_2OR} N_2$$

Under anaerobic conditions, in the presence or absence of NH_4^+, the dissimilatory nitrogen oxide reductases in denitrifying bacteria undergo derepression within a period of 40 min to 3 h (Knowles 1982). The initial step in denitrification is catalyzed by NaR and the presence of this membrane-bound enzyme represents indirect evidence of denitrification (Knowles 1982; Wilson and Bouwer 1997; Zumft 1997). Assays for studying the NaR activity have been demonstrated in a number of reports (Wang et al. 2007; De Sousa et al. 2009). NaR is a terminal component in the electron transport chain and its synthesis is induced by the presence of NO_3^- and the absence of O_2. NiR subsequently, is the key enzyme of denitrification in catalyzing the first committed step that proceeds towards the formation of a gaseous intermediate. For the denitrification process to lead to N_2 formation, the NiR reaction is complemented by the activity of the two distinct metalloenzymes; NOR and N_2OR, which use NO or N_2O as substrates respectively (Knowles 1982; Wilson and Bouwer 1997; Zumft 1997; Dang et al. 2009). However, the obligatory participation of NOR in the sequence of reductions is still to be understood.

Most denitrifiers possess all of the reductases necessary to reduce NO_3^- to N_2 and thus bring about complete denitrification of NO_3^- to N_2 via NO_2^-, NO and N_2O. These are termed 'true denitrifiers'. Some lack NaR and are termed 'nitrite dependent', and others lack N_2OR and thus yield N_2O as the terminal product. Still other organisms possess N_2OR but cannot produce N_2O from NO_3^- or NO_2^-. Many denitrifying bacteria only have the enzymatic ability to reduce NO_3^- to NO_2^- with no further reduction of the NO_2^- produced, hence capable of 'incomplete denitrification' (Knowles 1982). It is important to note that all these reactions are in reality part of the denitrification pathway.

Denitrification is frequently referred to as a modular organization. Even in a complete denitrifier, the respiratory complexes maintain a certain degree of independence since they respond to a mixture of different external and internal signals. Bioenergetic studies suggest that when bacteria shift to denitrification, the initial reactions in the aerobic electron transport chain involving the pyridine nucleotides, flavins and quinones remain unchanged. However, from the point of the newly produced cytochrome *b* onwards, separate and specific enzymes function in the energetically profitable reductions of the nitrogen oxides (Payne 1983; Zumft 1997). A model of the probable pathways of electron transport in *Paracoccus denitrificans* is illustrated by Knowles (1982). ATP is generated at the expense of succinate oxidation and NO_3^- reduction. In the whole cells and extracts of *Pseudomonas denitrificans*, phosphorylation is also associated with reduction of NO_2^- to N_2 at the expense

of lactate (Payne 1983). It has been suggested that redox conditions regulate the synthesis of NO_3^- reduction, thus, increase in the redox potential represses the synthesis of NaR in some microbial species. The redox state of ubiquinone, the last common component of both NO_3^- and O_2 electron transport systems, plays a role in the regulation of denitrification. The presence of O_2 may serve to affect the redox state of the components of the respiratory chain, thereby playing a major role in controlling the electron transport to O_2 or NO_3^- (Wilson and Bouwer 1997).

In many bacteria, the synthesis and activity of denitrifying enzymes is inhibited by O_2. O_2 inhibits NO_3^- reduction in bacteria by probably competing for electrons, preventing synthesis of denitrifying enzymes, and inhibiting NO_3^- transport to the active site of NaR (Knowles 1982; Wilson and Bouwer 1997). Because of the inhibitory effect of O_2, some investigators believe that denitrification is strictly an anaerobic process. However, quite a few reports have shown that organisms do denitrify in microaerophilic conditions or in the presence of O_2 despite the ability of the latter to inhibit denitrification (Wilson and Bouwer 1997; Ozeki et al. 2001; Takaya et al. 2003; Okada et al. 2005; Wang et al. 2007). Several studies demonstrated that nitrogen oxide reducing enzymes were in fact induced in the presence of O_2 thus contradicting the conventional concepts and indicating that denitrification is not strictly an anaerobic process (Wilson and Bouwer 1997). These observations have led investigators to reconsider several aspects of the process. The phenomenon of denitrification in the presence of O_2 in otherwise aerobic aquifers is called 'aerobic denitrification' (Wilson and Bouwer 1997; Zumft 1997; Okada et al. 2005).

Under aerobic conditions, denitrifiers are able to transfer electrons from a reduced compound to O_2 via the electron transport chain and gain energy in the process. In the absence of O_2, NO_3^- can serve as the terminal electron acceptor with NaR, cyt *b* and ubiquinone acting as electron donors during NO_3^- reduction. Thus, the presence of O_2 results in the oxidation of reduced components in the electron transport chain such as cyt *b*. This prevents the transfer of electrons to cyt *c* and NaR. Once the concentration of O_2 is lowered or removed, reduced cyt *b* resumes its transfer of electrons to cyt *c* resulting ultimately in the reduction of NO_3^- (Wilson and Bouwer 1997).

In aerobic denitrification, there occurs a co-respiration of O_2 and NO_3^- which means that both O_2 and NO_3^- (or NO_2^-) are used as electron acceptors concurrently (Okada et al. 2005). Nitrogen oxide respiration is found in a broad range of O_2 concentrations, besides the onset of nitrogen oxide utilization differs from one denitrifier to another. Aerobic denitrification is stimulated when the denitrification genes are activated at a high O_2 level. Regulatory control usually operates in favor of channeling electrons towards aerobic respiration. Phase shifts between O_2 respiration and denitrification establish themselves as independent oscillations at low O_2 concentrations. Thus, if O_2 does not repress nitrogen oxide utilization, the outcome is a co-respiration of the two substrates ensuing aerobic denitrification (Zumft 1997). Considerable research has been devoted towards aerobic denitrification and several aerobic denitrifiers have been isolated (Baumann et al. 1996; Wilson and Bouwer 1997; Zumft 1997; Ozeki et al. 2001; Takaya et al. 2003; Okada et al. 2005; Wang et al. 2007). Further, studies have also proved that tolerance to O_2 can be induced by

familiarizing samples with nitrate as the sole substrate under long-term high dissolved oxygen concentration conditions (Wang et al. 2007).

30.2.3 Microbial Denitrifiers

Denitrifiers are widespread and randomly distributed throughout the world. According to various survey estimates, denitrifiers represent about 10% of the total recoverable bacterial population of various soils, sediments and waters including marine systems. No particular relationship or association appears to connect these organisms and they are solely defined on the basis of their ability to reduce NO_3^- to gaseous products. Although, denitrifiers are mostly heterotrophs, the mechanism is also found in chemolithotrophic organisms (Knowles 1982; Payne 1983; Fries et al. 1994; Gamble et al. 1997; Zumft 1997; Rich et al. 2003; Okada et al. 2005; Heylen et al. 2006).

Denitrifiers are facultative microorganisms and their facultative quality allows them to proliferate in an extensive range of habitats with different O_2 concentrations as compared to other microbial groups. Of the various electron acceptors other than O_2 available to sub-surface microorganisms, NO_3^- is the preferable alternative to O_2 and is normally the first electron acceptor used by bacteria for electron transport phosphorylation via denitrification. In fact, in marine ecosystems, sedimentary respiration occurs consuming oxidants in the order, O_2, NO_3^- and Mn^{+2}, Fe^{+3} and SO_4^{2-}. This preference of NO_3^- over other electron acceptors is because of the higher energy yield of the denitrification process as compared to other anaerobic mechanisms (Knowles 1982; Fries et al. 1994; Wilson and Bouwer 1997; Zumft 1997; Braker et al. 2001; Chénier et al. 2003).

Bacteria capable of denitrification belong to a variety of groups and include a wide range of physiological traits. There is no recognizable pattern of distribution as denitrifiers are found within the alpha, beta and gamma classes of the Proteobacteria. *Pseudomonas, Alcaligenes and Paracoccus* are the most dominant and significant genera and have stimulated wide research directed at understanding this intriguing group of microorganisms (Knowles 1982; Payne 1983; Baumann et al. 1996; Gamble et al. 1997; Zumft 1997; Chawala and Lu-Kwang 2000; Ozeki et al. 2001; Joo et al. 2005).

The most commonly isolated and studied denitrifying bacterial genera include *Pseudomonas* (represents the most active denitrifiers in natural environments), *Paracoccus, Alcaligenes*, *Bacillus, Achromobacter, Thiobacillus* and *Hyphobacterium*. Other denitrifying bacterial genera include *Agrobacterium*, *Spirillum*, *Corynebacterium, Flavobacterium, Moraxella, Neisseria, Propionibacterium, Vibrio, Zavarzinia* and *Xanthomonas*. Some rhizobial strains are also known to reduce NO_3^- and NO_2^- to N_2 or to nitrogen oxides or to both (Knowles 1982; Gamble et al. 1997; Zumft 1997). Adav et al. (2010) reported denitrifying cultures belonging to less frequently observed genera such as *Comamonas,*

Alicycliphilus, Diaphorobacter, Delftia and *Acidovorax* capable of this remarkable pathway using the Fluorescent In Situ Hybridization (FISH) technique. Also, two novel denitrifying bacteria belonging to the genera *Stenotrophomonas* and *Oceanimonas* were isolated from industrial wastewater and soil samples (Yu et al. 2009).

This pathway is also known to occur in certain archaebacteria. The existence of denitrification in the hyperthermophilic branches of *Archaea* is of evolutionary significance since it indicates that there must have been an early origin and occurrence of the process before the branching of the archaeal and bacterial domains (Zumft 1997). The existence of similar enzymes in both kingdoms supports this view. *Halobacterium marismortui* (redescribed as *Haloarcula marismortui*) was the first archaeon shown to produce gaseous nitrogen oxides from NO_3^-. Other denitrifying halobacterial genera are *Haloarcula*, *Pyrobaculum, Halovibrio, Halospina* and *Ferroglobus* (Zumft 1997; Sorokin et al. 2006).

Formerly, denitrification was always thought to be exclusively a prokaryotic trait until it was discovered to occur in certain fungi (Koyabashi et al. 1996; Zumft 1997). N_2O formation was found in the genera, *Fusarium, Giberella, Trichoderma, Cylindrocarpon, Chaetomium, Penicillium, Aspergillus* and *Hansenula* and in some members of Fungi Imperfecti, filamentous fungi and yeasts (Zumft 1997) of which the denitrifying systems of *Fusarium oxysporium* and *Cylindrocarpon tonkinense* have been best characterized (Koyabashi et al. 1996). Fungal systems usually lack N_2O reductase and thus evolve N_2O as the final denitrification product. Many systems lack *nar.* Fungal *nor* involves cyt P_{450} while bacterial *nor* is of the cyt *bc* type. Fungal *nir* however, is very similar to the bacterial counterparts. Furthermore, in certain fungi like *C. tonkinense*, the denitrification systems have shown to support anaerobic cell growth. The mitochondrion is normally regarded as an aerobic eukaryotic organelle. The occurrence of anaerobic respiration in the mitochondria of denitrifying fungi undoubtedly demonstrates the presence of a novel respiratory metabolism evoking interesting questions. During evolution, the mitochondria of eukaryotic cells may have lost adaptive systems such as denitrification. In any case, fungal denitrification is clearly an energy yielding process just like in the case of bacteria (Koyabashi et al. 1996).

Recent studies show that two marine benthic foraminiferal species *Globobulimina turgida* and *Nonionella* cf. *stella* are capable of complete denitrification of nitrate to nitrogen gas (Risgaard-Petersen et al. 2006; Høgslund et al. 2008; Piña-Ochoa et al. 2010). Benthic foraminifers are amoeboid protists and inhabit a wide range of aquatic environments including open marine, brackish and freshwater environments. Piña-Ochoa et al. (2010) suggest that the contribution of these eukaryotes to the removal of fixed nitrogen by respiration may equal the importance of bacterial denitrification in ocean sediments.

Certain microbial species are capable of denitrification concomitant with other phenomena such as diazotrophy, phototrophy and nitrification (Table 30.1) (Knowles 1982; Zumft 1997; Flores-Mireles et al. 2007).

Table 30.1 Denitrifying diazotrophs, phototrophs and nitrifiers (Knowles 1982; Zumft 1997; Flores-Mireles et al. 2007)

Denitrifying diazotrophs	*Azospirillum* (formerly *Spirillum*) *lipoferum*
	A. brasilense
	Magnetospirillum magnetotaticum Bradyrhizobium japonicum
	Azoarcus tolulyticus
	A. evasii
Denitrifying phototrophs	*Rhodobacter sphaeroides*
	Rhodobacter azotoformans
	Rhodoplanes elegans
	Rhodoplanes roseus
	Rhodopseudomonas palustris
	Roseobacter denitrificans
Denitrifying nitrifiers	*Nitrosomonas europaea*

30.2.4 Cultivation of Denitrifying Bacteria

The isolation and cultivation of a specific physiological group of bacteria, such as the denitrifiers, requires the comprehension of the various interactions between a large number of medium components and growth conditions (Heylen et al. 2006). Several studies have been directed towards the isolation and characterization of denitrifying bacteria and have been accomplished successfully (Fries et al. 1994; Chawala and Lu-Kwang 2000; Rich et al. 2003; Takaya et al. 2003; Heylen et al. 2006; De Sousa and Bhosle 2009). Denitrifying bacteria are characteristically rod-shaped, coccoid, coccal and spiral-shaped and a few have more complex cell types. They grow on a variety of carbon and energy sources; some utilize one-carbon compounds, while other autotrophic denitrifiers grow on H_2, CO_2, reduced inorganic sulphur compounds, ammonia or NO_2^- (Knowles 1982; Payne 1983; Zumft 1997). A denitrification medium should ideally consist of a salt mixture and a carbon and NO_3^- source. Succinate is known to enhance denitrification activity (Kariminiaae-Hamedaani et al. 2004). Denitrification medium containing sodium succinate, tryptone, ammonium chloride, and potassium nitrate among other salts and minor nutrients is most suitable for the isolation and maintenance of denitrifying bacteria (De Sousa et al. 2009). The pH should be preferentially adjusted to 7.0–7.2 and the most suitable temperature ranges from 28°C to 30°C (Fries et al. 1994; Ramesh 2005). However, since denitrifiers encompass a wide range of organisms, these conditions vary with different denitrifiers. So also, the oxygen requirement will differ with each organism. Nevertheless, denitrifiers have been isolated and cultivated under aerobic, microaerophilic and anaerobic conditions (Baumann et al. 1996; Chawala and Lu-Kwang 2000; Takaya et al. 2003; Shinoda et al. 2004; De Sousa and Bhosle 2009).

Various methods are used to measure denitrification, most of them being indirect, partly due to the large background concentration of N_2. Several detailed reviews

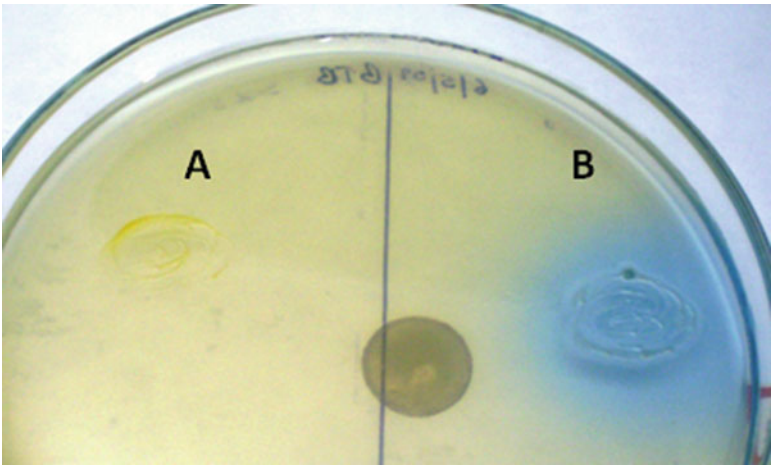

Fig. 30.2 Blue halos obtained on bromothymol blue (BTB) medium. On incubation, a change in pH caused by the depletion of nitrate on account of denitrification induces the formation of a blue halo around the colony. '*A*' is a nitrate reducer and '*B*' is a denitrifier (De Sousa et al. 2009)

of denitrification methodology are available (Knowles 1982; Seitzinger 1988; Kana 1998). The screening for denitrifying bacteria is checked by the formation of blue halos (Fig. 30.2) on bromothymol blue medium (Takaya et al. 2003) and the denitrification activity is assessed by the nitrate reduction test using α-naphthylamine, sulphanilic acid and zinc dust (Norris and Ribbons 1971; Wang et al. 2007). Denitrification is also evaluated by observing gas bubble formation in nitrate broth containing inverted Durham tubes, followed by N_2O analysis using gas chromatography (Fries et al. 1994). NO_3^- and NO_2^- concentrations in culture supernatant may be determined spectrophotometrically (Nagaraja and Kumar 2002; Wang et al. 2007) and confirmed by High Performance Liquid Chromatography (Fries et al. 1994; Rich et al. 2003; Heylen et al. 2006). Most direct assays of denitrification involve the addition of NO_3^- followed by mass spectrometric detection of the N_2O and N_2 released (Seitzinger 1988; Gamble et al. 1997; Kana 1998).

30.2.5 Community Composition and Functioning

Despite the crucial and fundamental role of denitrification in the global nitrogen cycle, little is known about the community composition and functioning of this essential group of microbes in nature. The relation between the denitrifying community structure and the denitrification rates in the natural environment still needs to be clearly understood. Investigating these aspects and examining the spatial variations in denitrification communities and nitrogen cycling processes may improve the understanding of the process as a whole and provide insights into the functional

role of the microbial diversity (Braker et al. 2001; Rich et al. 2003; Yoshie et al. 2004; Khan et al. 2007).

The classical technique using terminal restriction fragment length polymorphism analysis of 16S rRNA gene sequences employed successfully in exploring various communities of eubacteria and archaebacteria is not suitable for community analysis of the phylogenetically diverse denitrifying bacteria (Braker et al. 2001). A more systematic and accurate approach involves the use of terminal restriction fragment length polymorphism analysis of PCR-amplified *nir*S genes and other such functional genes (Braker et al. 2001; Flores-Mireles et al. 2007; Khan et al. 2007). The genetic diversity of denitrifiers in marine sediments was explored in a number of habitats including marine and estuarine systems by cloning nitrite reductase genes, *nir*K (encoding copper containing NiR) and *nir*S genes (encoding cytochrome cd_1-containing NiR). Denitrifying communities in marine sediments exhibit a higher diversity for *nir*S rather than *nir*K (Braker et al. 2001; Flores-Mireles et al. 2007). This approach not only indicates similarities in denitrifying communities but can also be used to establish the influence of environmental parameters on the denitrifying community structure and distribution (Dang et al. 2009).

In addition, Functional Single-Cell (FSC) isolation method and microscopic analysis techniques such as FISH combined with immunofluorescence, microautoradiography and stable isotope probing and rRNA slot-blot hybridizations with 16S rRNA-targeted oligonucleotide probes are being developed to quantify and thereby study the diversity of denitrifiers (Van de Pas-Schoonen 2005; Adav et al. 2010; Ashida et al. 2010).

Most studies in this line of research has been restricted to a few denitrifying species; *Paracoccus denitrificans, Pseudomonas denitrificans* and *Pseudomonas perfectomarinus* or the organisms isolated from the particular site under study. The numerically dominant organisms isolated in any environment are also generally assumed to be the functionally dominant ones. However, a study of the numerically dominant denitrifying bacteria from world soils showed that *Pseudomonas fluorescens* and *Alcaligenes faecalis* were the most frequently encountered organisms and that *Pseudomonas denitrificans,* previously assumed to be the most dominant denitrifier, interestingly, did not feature at all among the isolates (Gamble et al. 1997). Our studies showed that the most dominant of the denitrifying bacteria isolated from the estuarine (mangroves in Fig. 30.3) and coastal ecosystems off the coast of the Arabian Sea belonged to the *Pseudomonas* genera (De Sousa and Bhosle 2009).

Bacterial denitrification in coastal, estuarine and marine systems is under complex environmental control owing to diverse physical, chemical and biological factors that affect it both directly and indirectly. Direct effects at the microbe level on the process include substrate, that is, NO_3^- availability, O_2 inhibition, organic matter supply, temperature and pH (Knowles 1982; Payne 1983; Seitzinger 1988; Kana 1998; Chawala and Lu-Kwang 2000; Takaya et al. 2003; Tomaszek and Czerwieniec 2003; Ramesh 2005; Fennel et al. 2009). At the ecosystem scale in aquatic environments, denitrification is influenced by the

Fig. 30.3 Mangrove ecosystems at Dr. Salim Ali bird sanctuary at Chorao, part of the estuarine ecosystems of River Mandovi, Goa – India (De Sousa and Bhosle 2009)

seasonality of physical and chemical factors, hydrodynamics of the system, organic cycling and anthropogenic effects on nutrient loading (Kana 1998). Denitrification in the bottom sediments are also greatly affected by the depth of the sediments, redox potential and nitrogen concentrations in marine, lake and river systems (Tomaszek and Czerwieniec 2003). Studies also showed the effect of phosphate content in water on the number of denitrifying bacteria. Increase in phosphorous inhibited denitrifying activity of bacteria. The reductases involved in denitrification are also susceptible to inhibition by a variety of compounds such as sulfide and acetylene (Knowles 1982; Payne 1983; Kumar et al. 2000; Sipkoska-Gastarova et al. 2008).

However, denitrifying community composition may be related to potential denitrification and nitrification activities and environmental factors, including soil C/N ratios and exhibit only slight shifts with respect to parameters such as pH (Rich et al. 2003; Deiglmayr et al. 2006; Bachman and Krantz 2008). Molecular studies using *nir*S and *nos*Z genes show that the structure of denitrifying communities are also influenced by geographic distance and carbon resources and not so much by redox gradients as previously thought (Braker et al. 2001). Further research in this area would help in developing a more comprehensive study of the relationships among qualitative and quantitative community compositions, functions and process stabilities.

30.2.6 Ecological Significance of Denitrification

As discussed before, denitrification is predominant in nature and occurs ubiquitously in a number of habitats. Its not surprising therefore, that the process has several essential and significant ecological and geochemical consequences. Some of the major implications of the process in the coastal, estuarine and marine ecosystems are discussed below.

30.2.6.1 Regulation of Primary Production

The nitrogen cycle controls the availability of nitrogenous nutrients and biological productivity in marine systems and thus is linked to the fixation of atmospheric CO_2 and export of carbon from the ocean's surface via various feed-back loops (Thamdrup and Dalsgaard 2002; Zehr and Ward 2002; Ramaiah 2005; Bachman and Krantz 2008; Beman et al. 2008; Lam et al. 2009). Phytoplankton production in numerous coastal marine areas has been identified as being limited by N or by both N and P (Seitzinger 1988). The various forms of nitrogen are required by marine organisms in order to synthesize structural components or to gain energy for growth. These nutrients are lost to the deep ocean and recycled by physical upwelling or various remineralization processes. While most chemical forms of nitrogen in the ocean are bioavailable (fixed nitrogen), the most abundant chemical form, dissolved nitrogen gas (N_2) is usually not and this unavailability of N_2 for marine organisms makes the removal of fixed nitrogen by denitrification an important process in regulating the amount of primary production in such system (Seitzinger 1988; Gruber and Galloway 2008; Lam et al. 2009; Zehr 2009). The removal of nitrogen by denitrification limits biological growth leading to a selection of nitrogen fixing microorganisms. A balance between nitrogen fixation and denitrification determines the amount of bio-available nitrogen in the ocean and consequently reflects marine productivity. These two processes must be therefore coupled with each other in order to prevent massive swings and perturbations in the availability of fixed nitrogen content in the ocean. With the rapid increase in atmospheric anthropogenic nitrogen input, an increase in marine productivity and subsequent CO_2 sequestration is inevitable (Diaz and Rosenberg 2008; Gruber and Galloway 2008; Lam et al. 2009; Zehr 2009). But the exact outcome of these global changes and their ensuing feedbacks needs to be precisely understood.

30.2.6.2 Control of Eutrophication

Nitrogen fertilizer producing industries and most wastewater treatment plants release N_2 as nitrates (Bachman and Krantz 2008). This presents no major problems to receiving waters such as the ocean and rivers; since either there is sufficient dilution of the nitrates or sufficient flow to prevent accumulation of

nitrates respectively. However, increased concentration of nitrates in estuaries can have major negative ecological implications since nitrates cause the proliferation of algal growth creating an ecological imbalance (Ramesh 2005; Burgin and Hamilton 2007; Fulweiler 2007; Bachman and Krantz 2008; Diaz and Rosenberg 2008; Sipkoska-Gastarova et al. 2008; Schlesinger 2009). NO_3^- is the limiting nutrient in the growth cycle of algae and excessive NO_3^- may lead to increased algal growth causing algal blooms. This subsequently prevents the penetration of sunlight required for photosynthesis that is carried out by the aquatic life in the water body and also prevents O_2 production otherwise released during photosynthesis thereby completely destroying the productivity of the water body (Ramesh 2005). Also, algal blooms cause the development of a secondary BOD (biological oxygen demand) associated with decomposition of the dying algae leading to anaerobic conditions and H_2S production. Excessive algal blooms in estuaries lead to eutrophication in which the water body prematurely slits up (Ramesh 2005; Burgin and Hamilton 2007; Fulweiler 2007; Schlesinger 2009). Aggravated eutrophication results in increased primary productivity encouraging the formation of dead zone that have serious ecological repercussions (Diaz and Rosenberg 2008). The removal of nitrogen from the system as dinitrogen gas can counteract the negative effects of eutrophication. Most of the reactive nitrogen in estuaries is denitrified in the coastal zone returning N_2 to the atmosphere. Denitrification therefore plays a critical role in maintaining the ecological integrity of estuaries (Fulweiler 2007; Bachman and Krantz 2008; Schlesinger 2009).

30.2.6.3 Nitrogen 'Sink' in Estuaries and the Continental Shelf

Denitrification not only decreases the amount of fixed nitrogen within a system, but it can also decrease the amount of nitrogen transported downstream. It appears to be a significant process in removing nitrogen inputs in estuaries (Seitzinger 1988; Burgin and Hamilton 2007; Fennel et al. 2009; Gruber and Galloway 2008). As mentioned in Sect. 30.2.6.2, in estuaries receiving substantial amounts of anthropogenic nutrients the removal of nitrogen via denitrification decreases the degree of eutrophication of coastal marine waters (Seitzinger 1988; Sipkoska-Gastarova et al. 2008). Denitrification thus, acts as a cleanser for estuaries and because the process is so important in the global marine nitrogen budget, estuaries have been described as 'filters' or 'sinks' (Thamdrup and Dalsgaard 2002; Naqvi et al. 2006; Fulweiler 2007). Denitrification decreases by about 40% the amount of continentally derived, river borne nitrogen transported to the oceans (Seitzinger 1988). However, new research also attributes some importance to processes that remove nitrate in freshwater ecosystems, including dissimilatory nitrate reduction to ammonium, denitrification coupled with sulfide oxidation, reduction of nitrate coupled with abiotic or biotically mediated oxidation of iron. But an entirely new outlook has been revealed with the unearthing of the anaerobic ammonium oxidation (anammox) (Burgin and Hamilton 2007).

Anammox, discovered only recently in 1995, couples the anaerobic oxidation of NH_4^+ with the reduction of NO_2^- to produce N_2 by the following reaction:

$$NO_2^- + NH_4^+ \rightarrow 2N_2 + 2H_2O$$

It has been suggested that certain chemolithoautotrophic members of the phylum *Planctomycetes* (photosynthetic bacteria) play a role in the process. It is considered to be the primary process responsible for the loss of fixed nitrogen in the oxygen minimum zone (OMZ) of the ocean rather than the previously accepted denitrification process. However, it must be considered that anammox is dependent on denitrification for its NO_2^- and the anammox organisms therefore compete with denitrifying bacteria for nitrite under anaerobic conditions (Prescott et al. 2003; Burgin and Hamilton 2007; Ward et al. 2007; Beman et al. 2008; Lam et al. 2009; Schlesinger 2009; Zehr 2009). Further, from a biogeochemical perspective, heterotrophic denitrification and anammox are both denitrification reactions in the sense that they both lead to the loss of fixed nitrogen from the ecosystems to the atmosphere. What is intriguing is that the simultaneous loss of nitrogen by these two processes has different and important implications. Anammox results in the removal of nitrogen with CO_2 fixation while denitrification leads to the loss of nitrogen with release of CO_2 (organic carbon metabolism). Also there is an interesting difference in the loss of substrate (nitrogen source) due to both these competing pathways (Lam et al. 2009; Schlesinger 2009; Zehr 2009).

30.2.6.4 Global Warming

Recognition of the role of N_2O in the stratospheric ozone depletion and consequently in global warming has raised quite a few alarms leading to numerous studies devoted to the sources, sinks and biogeochemical cycling of this trace gas. N_2O is a gaseous nitrogen oxide and is produced by three microbial processes of the nitrogen cycle: nitrification, dissimilatory NO_3^- reduction to NH_3 and denitrification (Seitzinger 1988; Kumar et al. 2000; Lam et al. 2009; Zehr 2009). In addition to these, other sources of N_2O include enteric bacteria, lightning, coal-utilizing and other power stations, automobile exhausts, NO production by catalytic oxidation of NH_3 and the burning of biomass (Knowles 1982). Although, the concentration of N_2O in the atmosphere is considerably lower than that of CO_2 (the major greenhouse gas), N_2O has a 200–300 fold stronger greenhouse effect than CO_2 and a potential to completely destroy the ozone layer. Therefore, the N_2O balance is critical to the natural environment (Takaya et al. 2003; Sandbach 2007).

The role of denitrification becomes even more evident since it is not only involved in the production of N_2O but also is the most important means of consumption of the greenhouse gas in freshwater, estuarine and coastal marine systems. Consumption of N_2O by denitrification also appears to be responsible for the low N_2O concentrations in various O_2-depleted coastal and marine waters. The ratio of net N_2O:N_2 production is very small and similar in these systems and is greatly affected by the

O_2 concentration in the coastal and marine sediments. Studies show that N_2O production by denitrifying bacteria decreased with decreasing O_2 concentrations (Seitzinger 1988; Lam et al. 2009; Zehr 2009). Based on a study, novel aerobic denitrifiers that produce low levels of N_2O under aerobic conditions have been isolated and characterized (Takaya et al. 2003). These strains identified as *Pseudomonas stutzeri* TR2 and *Pseudomonas* sp strain K50 should be useful for future investigations of the mechanisms of denitrifying bacteria that regulate N_2O emissions. Quantification and assessment of denitrifying gene abundances are also being used for determining nitrous oxide emissions from soils (Morales et al. 2010).

30.2.7 Role of Denitrifiers in the Biodegradation of Hydrocarbons

Research on microaerophilic and anaerobic degradation of hydrocarbons especially by denitrifying bacteria has been stimulated by the fact that dispersal of oil and fuel in the environment may lead to the pollution of deep aquifers that are frequently anoxic (Fries et al. 1994; Wilson and Bouwer 1997; Chawala and Lu-Kwang 2000; Chénier et al. 2003; Shinoda et al. 2004; Gallagher et al. 2005; Song and Ward 2005; Quan et al. 2007; Callaghan et al. 2009; Cao et al. 2009). Knowledge of bacterial capacities for degradation of oil hydrocarbons in the absence of molecular O_2 is important to predict the fate of hydrocarbons and the effectiveness of bioremediation efforts under such conditions. The addition of NO_3^- to oil-contaminated sites has been regarded as a potential means of enhancing bioremediation efforts on site (Fries et al. 1994; Wilson and Bouwer 1997; Song and Ward 2005; Al-Turki 2009; Callaghan et al. 2009).

The facultative trait of denitrifying bacteria makes them especially important in the biodegradation of hydrocarbons under mixed electron acceptor conditions. Because of their ability to use both O_2 and NO_3^-, denitrifying bacteria typically occur in transition redox zones where microaerophilic conditions may exist. Being facultative, they are capable of using O_2 as the electron acceptor. They may possess enzymes that enable the bacteria to use aerobic degradation pathways to metabolize organic compounds in the presence of molecular O_2. When O_2 levels drop below a certain concentration, the bacteria will switch to NO_3^- respiration (Wilson and Bouwer 1997).

Denitrifying bacteria that degrade hydrocarbons are widely distributed in nature and include several genera such as *Pseudomonas, Azoarcus, Thiosphaera, Thauera*, *Rhodopseudomonas* and *Acidovorax* (Fries et al. 1994; Wilson and Bouwer 1997; Chawala and Lu-Kwang 2000; Shinoda et al. 2004; Gallagher et al. 2005; Song and Ward 2005; Nestler et al. 2007). Several new isolates of denitrifiers capable of mineralizing a wide spectrum of aromatic compounds under anaerobic conditions have been discovered and the number is steadily growing. Research on *Thauera aromatica* and *Azoarcus evansii* have led to substantial mechanistic advances in understanding the anaerobic degradation of benzoate. The range of substrates utilized

under denitrifying conditions includes alkanes, toluene, xylenes, phenols, cresols, phthalate, cyclohexanol, benzoate and other aromatic acids, alcohols and aldehydes (Zumft 1997; Song and Ward 2005; Nestler et al. 2007; Quan et al. 2007; Callaghan et al. 2009; Martínez-Hernández et al. 2009; Mehboob et al. 2009).

30.3 Future Perspectives

There exists a strong impact of denitrification in the estuarine, coastal and marine habitats but the lack of information particularly concerning the microbial groups responsible for the denitrifying activities hampers the scientific and ecological exploitation of the process. Many aspects of the process are inadequately understood and require more investigation such as the actual role of each of the reductases in denitrification and the feedback mechanisms involved in their activity. Further information concerning the aerobic branch of the pathway, the shuffling of electrons between O_2 and NO_3^- still requires clarifications and research. A lot desires to be understood about the relatively new anammox especially concerning its interaction with denitrification. Methodological inadequacies continue to impede studies on denitrification and novel techniques have to be devised and constantly refined to aid further research and subsequently improve our understanding of the biochemical, physiological, genetic and molecular facets of the process.

30.4 Concluding Remarks

Undoubtedly, denitrification is a fascinating process and the wide selection of microorganisms responsible for this important phenomenon is equally intriguing. These facultative microbes capitalize on their unique ability to grow on a broad substrate spectrum over an extensive range of habitats with varying oxygen concentrations. Their distinctive style of switching from aerobic to anaerobic respiration has received tremendous attention and opened new avenues of research.

The major implication of denitrification is emphasized in the aquatic ecosystems. Its essential function in balancing the nitrogen cycle and indirectly affecting the marine productivity and global climate makes it a crucial pathway in the coastal, estuarine and marine systems. The role of denitrifying bacteria in hydrocarbon mineralization is evidently most significant. Biodegradation of oil and petroleum contaminants under mixed electron acceptor conditions offers a promising solution to the present scenario of recalcitrant hydrocarbon contamination in the coastal marine environment. Exploiting the characteristics of denitrifying bacteria in this field may prove to be technologically profitable in a bioremediation design. From their frequency of isolation, bacteria belonging to the *Pseudomonas, Paracoccus* and *Alcaligenes* groups are clearly of greatest significance as denitrifiers. However, new discoveries are paving the way for new theories!

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Chapter 31
Genomic Technologies in Environmental Bioremediation

Thangamani Rajesh, Jeyaprakash Rajendhran, and Paramasamy Gunasekaran

Abstract Environmental pollution is a serious global problem. In recent years, microorganisms have been successfully employed in bioremediation of polluted environments. Studies on microorganisms have gained momentum due to the availability of complete genome sequences for microbes in well studied ecosystems. Due to limitations in understanding the culture conditions of environmental microbes, culture independent approaches are gaining much attention in the field of bioremediation. High-throughput technologies coupled with metagenomic analyses of soil microbes have led to the identification of catabolic pathways involved in degradation of organic and inorganic contaminants in soil. The novel properties of identified gene(s) clusters suggested metagenomics studies to have a real impact in bioremediation. Identified organisms have not only proved important in development of bioremediation processes but also in the design of new biosensors to detect the presence of pollutants in the environment. With the advent of environmental arrays, it is highly possible to understand the global analysis of gene expression of soil microbes in response to environmental changes. The application of genomics in understanding soil microorganisms involved in bioremediation has been presented in this review.

Keywords Environmental genomics • Bioremediation • Pathway analysis • Microarray • Proteomics • Metagenomics • Metabolic engineering

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31.1 Introduction

Bioremediation refers to the biological process used for complete removal or transformation of polluted compounds in an environment. Environmental adaptation plays a vital role that renders an innate species of an environment to participate in the degradation of pollutants (Alexander 1995). Every inherent organism including microbes, plants and animals participate in biodegradation with the aid of catabolic enzymes. Microorganisms are shown to be promising candidates for environmental bioremediation due to their flexibility to resist/degrade the newly introduced compounds within a limited time span (Gao et al. 2010). Due to our limited understanding on the role of degradative enzymes produced by microbial communities, bioremediation still seems to be a challenging area (Gerhardt et al. 2009). Genomics and proteomics approaches allow better understanding of environmental adaptation of both closely and distantly related species (Allen and Banfield 2005; Feder and Mitchell-Olds 2003). In addition, preliminary studies on culture independent metagenome analysis of mixed microbial populations involved in the degradation process has led to the construction of environmental models related to bioremediation (Connon et al. 2005; Hugenholtz et al. 1998). The results from such studies helps in understanding the specific pathways related to biodegradation. As inferred by most studies, the mixed analysis of microbial diversity involved in biodegradation has been shown to throw insights into the gene expression profiles and protein expression patterns under various environmental conditions (Covert et al. 2001; Liu et al. 2003; Singh and Nagathihalli 2006). Based on these global gene expression patterns, distinct enzymes involved in degradative pathways specific to bioremediation have been identified. Thus it is evident that the genomic approaches are valuable in providing information to understand the key molecules that govern the interaction between species, their metabolic pattern and the role of specific enzymes involved in a degradative pathway.

31.2 Microbial Dynamics During Bioremediation

Microbial diversity analysis and the study of microbial dynamics under various environmental conditions provide information on key species that dominate a particular environment (MacNaughton et al. 1999). Advanced methodologies have been developed to understand the abundance of various phylogroups and their role in a given ecological niche. Classical techniques used to study microbial diversity and population dynamics are the measurement of lipid biomarkers, in specific phospholipid fatty acids (PLFA). By this method, the lipid profiles of microbial communities in environment are quantified to determine the characteristics of microbial communities, the variability in biomass, the order of individual microbes in a given community and their response to environmental pressure (MacNaughton et al. 1999; White et al. 1979, 1998). Recently, microbial fingerprinting with 16S ribosomal DNA (rDNA) is performed in combination with lipid-based biomarker techniques.

Combined techniques provide information on both culturable and uncultivable microorganisms in an environment (Stephen et al. 1999). As 16S rDNA and culture independent analysis requires enormous sequencing efforts, electrophoresis based analysis were shown to be feasible to monitor microbial community structure. Denaturing gradient gel electrophoresis (DGGE), Temporal Temperature Gradient Gel electrophoresis (TTGE) and single-strand-conformation polymorphism (SSCP) (Muyzer et al. 1993; Watanabe 2001) analysis are sensitive and less expensive techniques usually adopted in microbial diversity analysis. In these methods, molecular markers such as 16S rDNA, *rpoB*, *gyrA* gene sequences that are highly species specific are being used to profile microbial populations. Employing these methods, profiling microbial populations from oil spills has led to the identification of a significant proportion of bacteria involved in the alkane and aromatic hydrocarbon degradation. Based on these data, it was reported that the microbial dynamics drastically varies at the final stage of degradation. Microbial communities that could significantly degrade the organic compounds were higher at the final stage and this indicates that by selecting this particular population efficient treatment of oil spills could be performed (Kasai et al. 2001; Maruyama et al. 2003). Although PCR-SSCP method was initially described for the evaluation of various DNA extraction and purification methods from soil samples, in the field of microbial ecology it is also extensively applied to monitor microbial population dynamics. Phylogenetic assessment and statistical inference on microbial community involved in biodegradation under varying aerobic and anaerobic environment, pH conditions has been successfully performed (Dabert et al. 2001).

One of the potential tools to monitor efficient microbes involved in biodegradation is the analysis of microbial community structure *in situ* (Fig. 31.1). This provides information on remediation potential of microbial community within a limited time frame (Lee et al. 1999). Available methods for microbial community analysis such as phospholipid, fatty acid analysis or PCR based methods to monitor microbial dynamics *in situ* offers only limited coverage and basic knowledge on metabolic activities and efficiency of mixed microbial populations. To study the cost effectiveness and to offer long-term efforts, microarray based *in situ* monitoring of microbial communities is performed in recent years. At present, custom arrays are available for the analysis of functional genes, mRNA and direct detection of 16S rRNA genes in the environment (Stenuit et al. 2008; Ye et al. 2001). Specific arrays containing probes based on the entire Ribosomal Database Project, a system has been successfully applied in the analysis of microbial communities involved in reduction and reoxidation of aromatic wastes (Brodie et al. 2006). A major drawback considered with the microarray based analysis is the high cost, lack of reproducibility and the data obtained needs to be validated using other quantitative methods such as Q-PCR. This limits the practical application of this technology in industries. As a promising effort, simple, field portable microarray system to monitor microbial community succession has been developed. These arrays termed low density arrays allow one to diagnose the microbial community structure and the identification of key indicator species. This reduces the financial constraint and provides closer monitoring in the field site (Chandler et al. 2010).

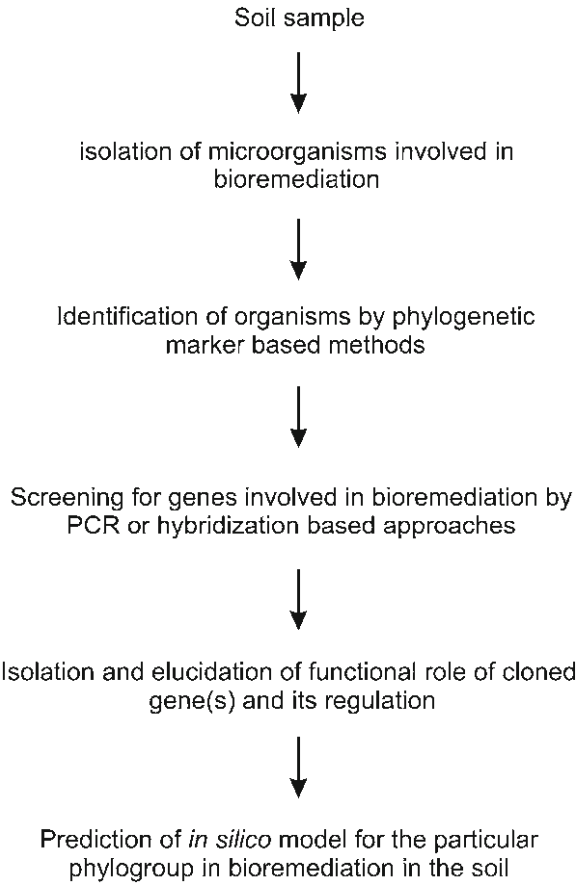

Fig. 31.1 *In situ* analysis of microbial community structure

The microbial composition, particularly the uncultivable microorganisms that resist environmental stress could be studied by culture independent techniques (Lovley 2003). These studies provided information on the complete structural composition and synergistic action of microorganisms that participate in bioremediation. For instance, in the microbial communities involved in hydrocarbon biodegradation, 90–99% of the species that are proficient degrading communities could not be cultivated under standard laboratory techniques (Leahy and Colwell 1990). This phenomenon is due to the fact that, in the contaminated site species that are capable of utilizing and tolerating the toxic contamination alone survive. The degradation of hydrocarbon could be achieved by a single microbial community or by the coordinated actions of mixed population depending on the nature of toxic compound and the time of exposure (Whiteley and Bailey 2000). For example, remediation sites contaminated with BTEX complex (benzene, toluene, ethylbenzene, xylene), were a major threat to ground water sources.

As these compounds are common effluents from industries, and as they are relatively soluble in water, remediation measures were intensified in this area. Successful treatment of such phenol containing water is limited due the lack in knowledge on microbial population involved degradation of phenol contamination. Further, relatively little was known about the structure of a microbial community during aerobic phenol degradation. In this direction, with the use of metagenomic approaches, a particular group of uncultivable bacteria was identified. This particular group of bacteria was consistently present at all stages of treatment, which explained that this particular microbial community contained essential genes involved in catabolic pathways for phenolic compounds. Due to their structural evolution and long-term adaptation in the environment, they became the key members involved in degradation of BTEX complex (Bowlen and Kosson 1995; Margesin et al. 2003).

31.3 Technical Hurdles in Bioremediation

Technologies available for microbial community analysis including the methods of environmental DNA extraction indicate that *in situ* analysis of microbial communities requires further optimization. Although a wide range of protocols are currently available for extraction of community DNA from the environments, each method has its own limitation (Blackburn and Hafker 1993; Griffiths et al. 2000; Krsek and Wellington 1999; Rajendhran and Gunasekaran 2008). A primary concern to these methods is the complexity of the sample, contaminating substances such as humic matter, organic contaminants, and heavy metals. Therefore, the methods that are currently available are not suitable for all types of environment (Dua et al. 2002; Stapleton et al. 1998). In the community DNA isolation from environment, the complete lysis of entire microbial community and the removal of interfering substances are major obstacles (Dua et al. 2002). Lack of knowledge for the comparison of local and global microbial diversity in degradation of similar compounds also remains a main problem. Inter-laboratory data comparison is a routine practice in microbiological research, but has been done to limited extent in environmental microbial ecology.

Environmental microarrays and culture independent community analysis are considered promising approaches as an alternative to pure culture dependent methods. Although the speed, effectiveness and low cost of sequencing methodologies are constantly improving, reconstruction of a complete bacterial genome from a metagenome is still at the stage of infancy (Tyson et al. 2004). Functional analysis of environmental library mainly target on identifying resistance genes rather than the catabolic genes that are involved in biodegradation of genotoxic compounds (Mirete et al. 2007). Even though, this could be studied using environmental arrays, the nature of microbial diversity and limited availability of universal probes, poses problem in our understanding signal intensity and its correlation with species

diversity (Bourgeois et al. 2003). Environmental contaminants often affect DNA hybridization process leading to artifacts in microarray analysis. Additionally, array based microbial diversity analysis are always qualitative as they only detect only the presence or absence of particular groups but fail to provide quantitative information on the population that are efficiently involved in biodegradation (Rhee et al. 2004). Fluorescent label free methodologies employing gold nanoparticles, quantum dots and magnetic particles are introduced in recent years and they offer promising strategies for *in situ* community analysis. All these methods follow simple operating protocols and are devoid of demerits such as instability of labels and sophisticated signal detection mechanisms. The wealth of information generated from these studies emphasizes the importance of investigating relative differences in biological processes among qualitatively different environments (Gerion et al. 2003; Taton et al. 2000). However, to date, none of them can provide directly incorporated specific measurements of the environment. As environment parameters are a set of complex, continuous features, it seems highly cumbersome to build models to determine a diverse array of biochemical activities, and in particular metabolic flexibility based on environmental differences.

31.4 Functional Genomics Approaches for Bioremediation

Field application of bioremediation technology is the need of the hour, which could be greatly facilitated by functional genomics approaches. To understand the natural microbes involved in bioremediation of specific areas that needs to be addressed are: the interaction of microbes with environment, the basis for their tolerance under stress conditions and the resulting genotype or phenotype due to the presence of pollutants. Genomics route provide a multidisciplinary approach to contemplate these issues. Genomics approaches applied in the field of bioremediation includes protein engineering, transcriptomics, metabolic engineering, proteomics and metagenomics (Fig. 31.2).

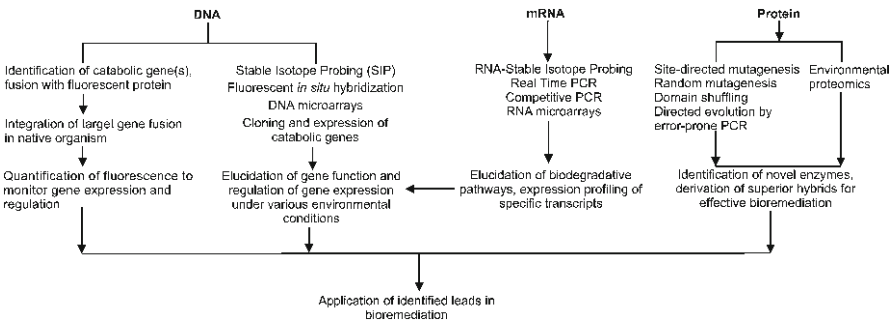

Fig. 31.2 Genomics approaches in bioremediation

31.4.1 Protein Engineering

Traditionally, protein engineering is considered to be the selection and alteration of a particular amino acid sequence of target protein for desired activity. In recent years, parameters such as basic knowledge on protein structure, its primary function and its reaction mechanisms are taken into considered for rational design (Steipe 1999). Often, active site residues that are in contact with substrates are considered for mutational analysis. Amino acid substitutions are then made by site-directed mutagenesis, site-saturation mutagenesis, gene shuffling, and generation of insertions or deletions for derivation of a hybrid protein (Costa et al. 1996; Farinas et al. 2001; Lutz and Patrick 2004; O'Donohue and Kneale 1996). Alternatively, error-prone PCR is also performed to introduce random mutations within the desired gene (Lutz and Patrick 2004). Initial attempts on protein engineering were introduced by rational designing of engineered Cytochrome P450 monooxygeneses (P450) for bioremediation (England et al. 1996; Jones et al. 1996). P450 are enzymes involved in the transformation of xenobiotics with broader substrate specificity and they have been studied in a number of organisms. The availability of resolved structures for P450 provided significant information on the conserved catalytic site that was structurally similar in all the cases. The centered heme-binding core and the distribution of surface charge were also found to be similar in all reported P450. By substituting Y96A in $P450_{cam}$, it was shown that the mutant protein oxidized diphenyl-methane which was not oxidized by the wild-type enzyme. Additionally, another mutant Y96F exhibited the ability to hydroxylate the cyclohexane ring in phenylcyclohexane which resulted in aliphatic oxidation, a significant reaction required for efficient bioremediation (Kellner et al. 1997). With the known three-dimensional structure of microbial P450s-$P450_{cam}$, $P450_{terp}$ and $P450_{BM-3}$, the replacement of $P450_{aldo}$ residues with similar amino acids in P45011β was shown to result in suppressed aldosterone synthesis and an increased 11β hydroxylase activity. Results from these studies suggested that by systematically altering specific amino acids, it is possible to alter substrate specificity for enhanced bioremediation (Li and Wackett 1993). Apart from site-directed mutagenesis, error-prone PCR based mutagenesis has been performed with toluene *ortho*-monooxygenase (TOM) coding genes from *Burkholderia cepacia* G4 species. The TOM operon of 3.5 kb was entirely subjected to mutagenesis to identify a critical residue Val 106. With a mutant enzyme, oxidation of indole was shown to be achieved in an enhanced manner (Canada et al. 2002). DNA shuffling and site-saturation mutagenesis has been applied to engineer monooxygenase, toluene-oxylene monooxygenase from *P. stutzeri* OX1 for chlorinated ethene degradation and enhanced degradation of p-nitrophenol (Vardar and Wood 2004). These studies clearly suggested the importance of protein engineering in biocatalysis, green chemistry and bioremediation. Successful protein engineering attempts in bioremediation is presented in Table 31.1.

Table 31.1 Successful protein engineering attempts in bioremediation

Protein engineering approach	Target enzyme	Application	Reference
DNA shuffling	Toluene *ortho*-monooxygenase (TOM)	Oxidation of indole, increased activity for chloroform	Canada et al. (2002)
DNA shuffling	Organophosphorus hydrolase	Degradation of the pesticide methyl parathion	Cho et al. (2002)
DNA shuffling	Arsenic resistance operon	Increased resistance to arsenic	Crameri et al. (1997)
DNA shuffling in combination with Site-saturation mutagenesis	Dioxygenases and monooxygenases	Bioremediation of nitroaromatics	Leungsakul et al. (2005)
Domain shuffling	Biphenyl dioxygenase	Degradation of biphenyl compounds, toluene, and benzene	Kumamaru et al. (1998)
Domain shuffling	Dioxygenase	Hydroxylation of double ortho- and double para-substituted polychlorinated biphenyls	Kimura et al. (1997)
Site-saturation mutagenesis	Toluene-o-xylene monooxygenase	Enhanced chlorinated ethane degradation, *p*-nitrophenol degradation	Rui et al. (2004a, b)
Site-saturation mutagenesis	Epoxide hydrolase	Degradation of dichloroethylene	Rui et al. (2004a, b)
Site-directed mutagenesis	Cytochrome P_{450}	Degradation of styrene and ethyl benzene	Stevenson et al. (1998)
Directed evolution	Haloalkane dehalogenase	Haloalkane dehalogenation	Bosma et al. (2002)

31.4.2 Transcriptomics

Genome-wide analysis of gene expression involves the organized analysis of genes involved in regulation of a physiological state with respect to a particular environmental stress. Employing high-throughput analysis, candidate gene targets involved in pathway regulation are then identified (Singh and Nagathihalli 2006). Transcriptome profile of a individual organism refers to a catalog of transcripts produced due to differential gene expression in response to varying growth conditions.

As vital metabolic processes are clearly depicted at the transcript level, this provides a snapshot of controlled biosynthesis of regulatory elements produced to degrade external pollutants (Gomase and Tagore 2008). In the field of bioremediation, the understanding of functional regulation is limited to distinct signaling pathways and the unregulated metabolic pathways (Singh and Nagathihalli 2006). A major drawback is the non-availability of commercial microarrays with suitable probes that provides sufficient coverage for unregulated transcripts in response to the presence of xenobiotics (Eyers et al. 2004). Extensive transcriptomics studies have been made with *Polaromonas* sp. strain JS666, an ideal candidate for bioremediation of chlorinated solvents such as tetrachloroethene and trichloroethene under aerobic conditions (Jennings et al. 2009). This organism was proposed to be evolved recently due to horizontal gene transfer of numerous transposable elements and degradative gene duplications. As the presence of *cis*-dichloroethene (cDCE), a potential carcinogen limits chloroethene degradation, investigations on the mechanisms of cDCE degradation by *Polaromonas* sp. has been carried out. Genomics studies on this system suggested that the operon involved in degradation of cDCE were surrounded by genes that are not involved in biodegradation. As monooxygenase transcript was in a higher amount, it was confirmed by transcriptional studies that this gene could be involved in cDCE degradation. Additionally, transcriptome profiling of this strain also led to the identification of new genes and enzymes involved in cDCE which are HADs (Bpro0530 and Bpro5186), GST (Bpro0645), CMO (Bpro5565), Hlase (Bpro5566), and CO DHase (Bpro0577) (Jennings et al. 2009). Studies of this kind opens gateway to identify lead candidates for degradation of specific compounds such as cDCE.

31.4.3 Metabolic Engineering

Metabolic engineering refers to engineering the metabolic pathway of a cell/strain in order to achieve desirable cellular traits for required function. By minimizing the stability of undesirable products, extended productivity of the desired product is strengthened for ideal applications (Camacho et al. 2005; Fiehn 2002). For bioremediation applications, cells are engineered to resist environmental stress and in certain cases, to degrade the introduced compounds (Villas-Boas and Bruheim 2007). Metabolic engineering seems feasible provided if the end product could be achieved by engineering a single gene. But in the case of bioremediation, the catabolic genes

involved in degradation are often organized as clusters or as complex regulatory circuits that require clear understanding of the response of bacterial cells to toxic organic molecules (Granato et al. 2007; Parisi et al. 2009; Stenuit et al. 2008). For instance, in *Clostridium acet*obutylicum ATCC 824 the overexpression of heat-shock or stress proteins (HSP), chaperones assist protein folding and refolding that leads to increased tolerance to organic solvents. As HSPs stabilize the proteins encoded by solvent-resistance genes it results in solvent tolerance. By incorporating such solvent tolerant genes and assisting HSP coding genes in suitable organism, effective bioremediation could be achieved (Tomas et al. 2003). In another study, an evolved haloalkane dehalogenase from *Rhodococcus* sp. m15-3 resulted in productive binding of 1,2,3-trichloropropane (TCP) in the active site, leading to its utility as a carbon source. This engineered enzyme was introduced in *Agrobacterium radiobacter* AD1 which led to utilization of the product of the haloalkane dehalogenase reaction. Both these strains are now employed in bioremediation (Bosma et al. 2002). Yet another study of metabolic engineering involves the surface display of organophosphorus hydrolase for organophosphorus pesticide degradation in *E. coli*. By creating a organophosphorus hydrolase-truncated ice nucleated protein hybrid, the enzyme was surface displayed. By selecting engineered cells based on the formation of p-nitrophenol from methyl parathion, variants that could degrade the pesticide 25-fold higher were identified (Shimazu et al. 2001).

cis-1,2-Dichloroethylene (*cis*-DCE) is primarily degraded by aerobic pathway, which results in the formation of a human carcinogen, vinyl chloride. Initially metabolic engineering was attempted with *E. coli* to degrage cis-DCE by introducing toluene *ortho*-monooxygenase from *Burkholderia cepacia* G4. Glutathione *S*-transferase (GST), and a variant of *ç*-glutamylcysteine synthetase (GSHI*) that could allow the overexpression of GST in *E. coli*. This engineered strain of *E. coli* was found to efficiently mineralize *cis*-DCE fourfold higher in comparison to other strains (Rui et al. 2004b).

31.4.4 Microarray Technologies

Studies on application of microarrays technology to analyze microbial communities have rapidly increased in recent years (Wu et al. 2006; Zhou 2003). An overview of available microarray technologies is presented in Table 31.2. For community analysis in any environment, array systems termed PhyloChips are employed which allows the simultaneous detection of bacterial and archaeal taxa (Hamady et al. 2010; Sagaram et al. 2009). PhyloChip analysis offers a range of advantages over conventional techniques like DGGE, SSCP, RFLP and RADP etc. As in recent years, a wide range of environmental arrays are being developed based on environments, tremendous information are expected to be submitted in databases within a limited time. Promising results have been obtained with environmental array analysis on Fossil Bluff and Coal Nunatak environments. Although only a limited number of bacterial and archaeal taxa were detected in these samples, it was much more

Table 31.2 Microarray technologies for bioremediation

Microarray platform	Target	Reference
PhyloChip	16S of 8, 741 operational taxonomic units (OTUs)	DeSantis et al. (2007)
GeoChip	30 K probes targeting 10, 337 functional genes	He et al. (2007)
Psychrochip	16S of 159 polar bacteria involved in biodegradation	Yergeau et al. (2007)
Catabolic chip	130 genes involved in organic pollutant degradation	Yergeau et al. (2007)
Combination of Geo Chip and PhyloChip	Detection of co-occurrence of OTUs and functional genes	Yergeau et al. (2009)

than expected in comparison to results from traditional techniques. Remarkably the phylogenetic pattern was also found to correlate well with 16S rRNA gene libraries analysis (Brodie et al. 2006; DeSantis et al. 2007).

For functional gene analysis, another set of arrays termed GeoChips that targets key genes involved in the geochemical cycling of N, C, and P, sulfate reduction, metal resistance and reduction, and contaminant degradation have been reported (Gentry et al. 2006). These GeoChips are sensitive, specific, and enables high-throughput analysis of metabolic processes linked with geochemical cycles. Currently, functional gene arrays (FGA) that contains probes for genes encoding enzymes involved in specific degradative pathways are getting attention (Gentry et al. 2006; He et al. 2007; Wu et al. 2001). As observed with PhyloChips, FGAs allow the simultaneous examination of several functional gene groups in a single experiment (Wagner et al. 2007; Wu et al. 2006; Zhou et al. 2008; Zhou and Thompson 2002). In combination with PhyloChips, FGAs provide information regarding the potential microbial communities and their genes involved in geochemical processes. GeoChip 2.0 is a broad range FGA currently available for studying various environmental metabolic processes (He et al. 2007). Successful application of GeoChip 2.0 includes bioremediation of UV(I), diesel contamination, identification of genes involved in the degradation of cellulose, phthalate, biphenyl, cyclohexanol, benzoate, and naphthalene, microbial N and C cycling in Antarctic sediments, denitrification, metal resistance genes, stable isotope probing experiments for microbial profiling etc. (Gao et al. 2007; Leigh et al. 2007; Yergeau et al. 2007; van Nostrand et al. 2007; Liang et al. 2009).

31.4.5 Proteomics

Proteomics studies provide a basic pipeline to examine the sequential participation of biomolecules, their association and involvement in living cells (Pandey and Mann 2000; Yeates 2000). Proteomics methods are adopted to identify functional properties of individual proteins under *in vivo* conditions, to define experimental objectives and

to design analytical strategies. Recently, several studies using proteomics approaches have been performed to identify biologically important proteins with superior bioremediation capabilities. All these studies employ high-resolution 2-D gels and electrospray MS of protein complexes to explicitly assign protein(s) its biological function in bioremediation. Initial studies to understand degradation pathway for benzoate was performed with *Acinetobacter* sp. KS-1. Proteome analysis of benzoate treated cultures revealed the differential expression of 18 proteins of which catechol 1,2-dioxygenase and β-ketoadipate succinyl-CoA transferase were identified to be involved in benzoate degradation (Kim et al. 2003). Similar studies on *Delftia acidovorans* MC1 treated with 2,4-dichlorophenoxypropionic acid and its by products 2,4-dichlorophenol and 3,5-dichlorocatechol revealed the involvement of two chlorocatechol 1,2-dioxygenases (Tomas-Gallardo et al. 2006). Difference in metabolic pathways involved during the degradation of phthalate by *Rhodococcus* sp strain TFB was also identified by proteomics approaches. The proteome profiles of phthalate-grown cells were compared with proteome profiles of induced cells with tetralin or naphthalene. Since, phthalate, tetralin and naphthalene were previously believed to be degraded by a single pathway, the observed difference in proteome profiles led to the reconstruction of specific metabolic pathways involved in degradation of individual aromatic hydrocarbons (Tomas-Gallardo et al. 2006). Community proteome analysis is an advancement in the field of proteomics that facilitates the coordinated study of cellular activities by microbial communities during environmental adaptations. For instance, proteome profile of different *Pseudomonas* sp grown with phenolic compounds has led to the identification of proteins coordinately expressed under oxidative stress response, general stress response, energetic metabolism, fatty acid biosynthesis, transcription regulation and transport of small molecules (Kim et al. 2006; Lupi et al. 1995; Zhao et al. 2004). By proteome profiling of different strains of *Corynebacterium glutamicum*, several putative proteins involved gentisate/3-hydroxybenzoate degradative pathway has been reported. By cloning and expression analysis of the identified putative protein coding genes, a novel GSH-independent gentisate pathway was also proposed (Feng et al. 2006). As a combinatorial approach for metabolite identification, genome and proteome analysis of aromatic hydrocarbon degradation pathway has led to the construction of a complete and integrated pyrene degradation pathway for *Mycobacterium vanbaalenii* PYR-1. In this study, complete genome sequencing and proteome profiling was performed with *M. vanbaalenii* PYR-1 cells exposed to pyrene. The results obtained in genomic and proteomic data analysis revealed the involvement of 27 enzymes required for pyrene degradation through o-phthalate and β-ketoadipate pathway (Kim et al. 2004). Similar study on *Herminiimonas arsenicoxydans* has led to the identification of key proteins involved in determining the survival of this microorganism in arsenic-rich environment based on oxidoreduction reactions. The identified key degradative enzymes upregulated in response to aromatic hydrocarbons can be utilized to genetically engineer microorganisms with superior bioremediation capabilities. Recombinant *E. coli* that could express toluene monooxygenase involved in degradation of trichloroethylene (TCE) and phenol has been shown successful followed by identification of these proteins by 2D-E analysis and complete sequencing of the respective proteins (Muller et al. 2007).

31.5 Culture Independent Approaches – Metagenomics

Each environment has its own microbial community composed of different phylogenetic groups. It is well known that environmental microbial communities are complex and are recalcitrant to culture under laboratory conditions (Pace 1997). By analyzing the 16S RNA gene sequences from direct environmental DNA, it is clearly understood that only a small fraction of microorganisms could be cultivated. This kind of cultivable small proportion taxonomic group is a characteristic feature for non-organic environments. In contrast, several thousands of taxonomic groups are reported in soil and ocean environments due to the abundance in carbon sources (Venter et al. 2004). Culture independent community genome analysis for the presence of functional genes is greatly facilitated by metagenomics studies (Voget et al. 2003; Handelsman 2004). In environmental bioremediation, metagenomics studies are centered on reconstruction of the microbial genomes for organisms efficient in bioremediation but remains uncultivable (Foerstner et al. 2006). An example for genome reconstruction is the metagenomics study of an acid mine drainage biofilm. The results obtained revealed the complete genomic description of two chemolithotrophic microorganisms namely, *Leptospirillum* sp. and *Ferroplasma acidarmanus* (Tyson et al. 2004). Similarly, reconstruction of the genome of uncultured bacterium *Kuenenia stuttgartiensis*, *Olavius algarvensis* involved in ammonium oxidation and sulphur-metabolism has also been achieved by metagenomics (Strous et al. 2006; Woyke et al. 2006). The techniques related to examination of genome sequence data have led to the identification of new molecules involved in degradation of recalcitrant products, including many pollutants such as organohalogenated compounds. The design and development of biosensors to detect the presence of toxic pollutants in the environment are also attempted using metagenomic approaches (Parales and Ditty 2005; Zylstra and Kukor 2005).

Sequence based catabolic gene identification in contaminated environments by metagenomics rely on conserved nucleotide sequence for the designing primers to amplify catabolic genes. However, as most of the catabolic genes involved in bioremediation are organized as clusters, they narrow down the identification of conversed regions. As an alternative, environmental genomic libraries can be subjected to functional screening to retrieve genes catalyzing a desired function. For environmental microbes, the presence of a number of metals is vital for catalysis and they aid co-factors requirements, redox activity processes and stabilizing of protein structures (Bruins et al. 2000). Most of the environmental microbes survive despite the presence of heavy metals via efflux of the toxic metal out of the cell or its enzymatic conversion, intracellular or extracellular restoration, exclusion or reduction in sensitivity signals. Thus environmental libraries of these microbial communities will essentially carry the genes involved in these processes. By functional metagenomics genes such as *nirS, nirK, dsrAB, amoA*, and *pmoA* involved in denitrification, sulfate reduction, nitrification and methane oxidation have been identified (Fields et al. 2006; Hwang et al. 2009; Palumbo et al. 2004; Yan et al. 2003). Certain novel genes involved in bioremediation identified by culture-dependent studies

include oxygenases for benzene/toluene subfamily, isopropylbenzene dioxygenases, ester- and glycosyl-hydrolases, polyphenol oxidase, extradiol dioxygenases etc. (Beloqui et al. 2006; Ferrer et al. 2005; Suenaga et al. 2007; Witzig et al. 2006).

Metagenomic profiling is an emerging field in environmental genomics which employs hybridization of novel insert DNA coding for a degradative enzyme to the genomes of bacterial isolates, reference strains, and environmental DNA. This kind of studies is being developed to specifically target the unculturable majority in the environment. Recently, a microarray platform was used to screen a metagenomic library with community genomes. The microarray termed COSMO was fabricated with 1 kb PCR products amplified from of 672 cosmid clones. In the next step, bacterial isolates were obtained from the environment and genomic DNA was isolated. Additionally, metagenomic DNA was also extracted from the mixed population. The isolated genomes were considered as test and were labeled with Cy5-dCTP and probed with COSMO. To eliminate false positives due to nonspecific hybridization, two-color based hybridization protocol was used. In this methodology, each test genome was compared to a reference common bacterial DNA and was labeled with Cy3. This approach was termed as comparative genomic hybridization (CGH). CGH performed with all environmental isolates and metagenomic DNA revealed clones that were specific to a test strain or community (Sebat et al. 2003).

31.6 Conclusions and Future Perspectives

Genomics technologies have potential applications in environmental bioremediation. Site-specific assessment of individual microbes guarantees the design for appropriate technologies to be applied for efficient bioremediation. For instance, if multiple contaminants are observed in an environment, it would be appropriate to design a combination of these techniques to reduce the concentrations of pollutants. Basic ideas of designing such combinatorial approaches are facilitated by genomics approaches. Despite their high cost, genomics route represent a driving factor in the development of new bioremediation technologies. For effective elimination of contaminants and to identify new enzymes, genomics technologies seem to be appropriate. Although some of the described technologies are still primarily in research and development phases, the field application of the identified leads in a limited scale have shown potential success. This in turn has led to an increase in public interest to further develop genomics techniques not only for identification of key molecules, but also for their efficient field application. Future concern on genomics advancements needed in the field of bioremediation includes development of methodologies for accessing co-metabolic capability of distant enzymes involved in degradation, identification of favorable conditions that facilitate the upregulation of degradative enzymes, approaches for sequential treatment of mixed wastes, improvement of high bioavailability of mixed microbial populations.

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Chapter 32
Coal Mine Drainage Pollution and Its Remediation

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Abstract Contaminated mine water is a ubiquitous persistent and detrimental environmental problem. Coal mine water is often acidic and if acid consuming minerals are present it is having neutral pH. The terms acid mine drainage or acid rock drainage (AMD/ARD) are not universally applicable to all mine drainages, however these terms are used even for non acidic mine water. Several chemical and biological factors are responsible for coal mine drainage generation. The environmental issues related to coal mine water pollutants are attributable to the exposure of reduced iron, sulphides and coal materials to oxygen and water. In the generation of mine drainage pollution microorganisms play both beneficial and destructive roles. *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* accelerate the chemical rate of pyrite oxidation about 500,000–1,000,000 fold, which is responsible for high concentration of sulphate, acid and turbidity of surface and underground waters. Control of sulphide oxidation may reduce or even eliminate the possibility of AMD/ARD generation, which is the main cause of most of the mine water pollution problems. On the other hand biochemical processes involving sulphate reducing bacteria and wetland or controlled reactor systems are used for removal of sulphate, metals and neutralisation of acid mine drainage. Other established treatment procedure for prevention of sulphide oxidation and AMD generation includes isolation, selective handling, co-disposal, blending and surface treatment of waste materials produced at mine site. Microbial activity may be controlled by addition of organic waste and use of bactericides.

Keywords AMD • ARD • Coal mine • Remediation • Iron and sulphur oxidizers

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32.1 Introduction

The origin of mining industry goes back with beginning of civilisation. It has been carried out since at least Neolithic time. With this historic mining activity there is a legacy of contamination of both land and water ecosystems. The modern mining industry is of significant importance to the world economy as it caters great diversity of mineral products for industrial as well as household consumers. The nature of coal and metal deposits makes contaminated mine water an ubiquitous problem. The mining industry is responsible for huge volume of wastes produced thus even lower concentration of pollutants play significant impact on environment. The mine water problems have more significance if the mining operations have ceased and mines are abandoned. Since the end of the nineteenth century, it is a legal requirement to inform a Mine's inspector of an abandonment of a mine within 2 months of ceasing operations. Mining and processing of minerals involve many complex steps; some of these are producing solid and aqueous wastes that must be properly treated to prevent environmental pollution (Hutchison and Ellison 1992). Microorganisms play both beneficial and devastating roles at the mining sites. Certain bacteria are playing significant role for one of the most persistent, destructive and ubiquitous environmental problems, acid rock drainage (ARD) or acid mine drainage (AMD). However, the same bacteria are commercially exploited for efficient, economically viable and environmental friendly metal extraction technology (Brierley and Brierley 2002). Mine water treatment involves physical, chemical and biological process and there appropriate combinations for prevention and control of ARD. The aim of the chapter is to take an over view of coal mining industries, their source of pollution, various treatment schemes to assess their applicability for the prevention and control of pollution due to coal mining or coal processing. It is also tried to include AMD management strategies to reduce the AMD liability.

32.2 World Coal Reserves and Sources of Pollution

32.2.1 Coal Reserves

Archaeologists have discovered in 2005 a 120,000 year old Stone Age coal stone in an opencast coal stone mine in Germany. China Coal Information Institute reports the use of Chinese mined coal stone for fuel since 10,000 years ago at the time of the 'New Stone Age', or 'Neolithic Era'. China remained the world's largest producer and consumer of coal until the eighteenth century. The earliest use of coal in the America was by the Aztecs. They used coal not only for heat but for ornaments also. Coal deposits were discovered by colonists in Eastern North America in the eighteenth century. The first practical coal fired electric generating station, developed by Thomas Edison, went into operation in New York in 1882 (The coal resource

Table 32.1 Some of the major reserves of coal worldwide

Country	Reserves of coal (billion tonnes)
USA	250
Russia	160
China	120
India	85
Australia	80
Germany	70
South Africa	50
Ukraine	30

2005). Coal has been used worldwide for electricity generation, steel production, cement manufacturing, as a liquid fuel and for other industrial processes. Coal is a fossil fuel. It is a combustible sedimentary organic rock, which is composed mainly of carbon, hydrogen and oxygen. It is formed from vegetation, which has been consolidated between other rock strata and altered by combined effects of pressure and heat over millions of years to form coal seams.

Coal formation was begun during the carboniferrous period- known as the first coal age, which spanned 360–290 million years ago. The quality of each coal deposit is determined by temperature, pressure and by the length of time formation, which is referred to as its organic maturity. The degree of change undergone by a coal as it matures from peat to anthracite – known as 'colification'. Initially the peat is converted into lignite or brown coal. Over the millions of years, the continuing effects of temperature and pressure produces further changes in the lignite, progressively increasing its organic maturity and transforming it into the range known as sub-bituminous coals. Further chemical and physical changes occur until these coals become harder and blacker, forming the bituminous or hard coals and finally these conditions lead to the formation of anthracite (The coal resource 2005).

Lignite is the lowest rank of coal formed during the Miocene period 16 million years ago (Rumpel et al. 2002; Rumpel and Kögel-Knabner 2003). High moisture content and susceptibility to spontaneous combustion can cause problems in transportation and storage of lignite. It has been estimated that there are over 984 billion tonnes of proven coal reserves worldwide. It can found on every continent in over 70 countries with the biggest reserves in the USA, Russia, China and India (Table 32.1). There is enough coal to last us over 190 years if it is mined properly. If we look to the Indian scenario, coal is the most important and abundant fossil fuel in the country and accounts for 55% of India's energy need. About 75% of the coal in India is consumed in the power sector. In addition, other industries like steel, cement, fertilizers, chemicals, paper and thousands of medium and small-scale industries are also dependent on coal and lignite for their process and energy requirements. The lignite reserves in India are estimated at around 36 billion tonnes, of which 90% occur in the southern state of Tamilnadu. A 4,150 million tonnes spread over 480 km^2 is in the Neyveli Lignite fields in Cuddalore district, of which around 2,360 million tonnes have been proved. Geological reserves of about 1,168 million tonnes of lignite have been identified in Jayamkondacholapuram of Trichy district

of Tamilnadu. In Mannargudi and east of Veeranam, geological reserves of around 22661.62 million tonnes and 1342.45 million tonnes of lignite have been estimated respectively. Other states where lignite deposits have been located are Rajasthan, Gujarat, Kerala, Jammu and Kashmir and Union Territory of Pondicherry (http://www.nlcindia.com/investor/dreport0809.pdf). Neyveli lignite mine is the biggest open-cast mechanized lignite mine in India. It mines 24 million tonnes of lignite annually and power generating with installed capacity of 2,490 MW of power.

Deposits of 200 million tonnes of lignite have been identified at different locations in Gujarat. Panandhro, the largest of these pockets, has been developed as major mining centre of lignite, which is used as a fuel in a large number of process boilers in the state as well as to produce power to meet the demand. Other than Panandhro mine, Rajpardi and Tadkeshwar are major mines in Gujarat. Both are located near Bharuch, South Gujarat region.

32.2.2 Source of Pollution

Coal is mined by surface or open cast and underground or deep mining. The choice of mining method is largely determined by the geology of the coal deposit. Surface mining of coal accounts for around 80% of production in Australia, while in USA it is used for about 67% of production. In India 75% coal reserves are amenable to surface mining. Underground mining currently accounts for about 60% of world coal production (Singh 2006). Opencast or open cut mining is only economic when the coal seam is near the surface.

This method of mining recovers a higher proportion of the coal deposit than underground mining as all coal seams are exploited 90% or more of the coal can be recovered. Large opencast mines can cover an area of many square kilo meters. Opencast mining for coal seriously affects the environment as mining operations changes topography and disturbs both soil structure and hydrologic regime (Duis 2001). Devastated unfertile land is prone to erosion, acid mine drainage and release of combustion products of lignite and lignite dust (Hüttl 1999; Rumpel and Kögel-Knabner 2003). In extensively mined areas these impacts can be identified even at the landscape level (Hüttl and Bradshaw 2001; Schaaf et al. 2006). In terrestrial ecosystems a severe disturbance or even destruction of soil and their functions, habitats, biocoenoses and species composition is observed. Surface and ground water is affected both qualitatively and quantitatively (Grünewald 2001; Schaaf et al. 2006).

Large number of coal mines are abandoned although the exact number is not know, but according to the Coal Authority data base the number exceed 10,000 (Younger 1994; Connelly et al. 1995).Where ever mining occurs, there is likely to be an associated mine water pollution problem which is of most environmental significance for abandoned mines. As per the US Bureau of mines estimates the abandoned coal and metal mines adversely affect more than 19,000 km of rivers and streams and over 73,000 ha of lakes and water reservoirs.

Some geological environments, in particular, metallic ore deposits, phosphate ores, coal seams, oil shale and mineral sands may contain abundant amount of sulphides. These sulphides are stable only under strongly reducing conditions. Mining of these resources can expose the sulphides to an oxygenated environment. During mining activities large amounts of sulphides can be exposed to air and water in the form of: tailing, waste rock dumps, coal spoil heaps; lignite spoil heaps; heap leach piles; run-off mine and low grade ore stock piles; open pit floors and faces; underground workings; haul roads; road cuts; quarries; and other rock excavations. When mining activity exposes sulphidic materials it becomes chemically unstable. The weathering of these minerals is a natural process, and thus generation of mining drainage pollutions can also be considered to be a natural process. A series of complex chemical weathering reactions are spontaneously initiated. Weathering of the minerals proceeds with the help of atmospheric gases, meteoric water and microorganisms. The sources of contaminated mine water from mining operations includes

- Drainage from underground workings,
- Runoff from open pit workings,
- Waste rock dumps from mining activities,
- Mill tailings,
- Ore stockpiles and
- Spent ore piles from heap leach operations.

32.3 Mechanism of ARD Generation and Its Neutralization

32.3.1 ARD Generation

The generation of polluted mine water is a combined chemical and microbial process. Major four pollutants of mine water are: acidity, ferric precipitates, turbidity and trace metals (Table 32.2). Oxidation reduction potential and pH observed at different coal mine of India is depicted in Table 32.3. Physicochemical diversity and extent of pollution recorded at various site of Rajpardi lignite mine is shown in Table 32.4. These data will give an idea that even at one mine site the pollution is varied in terms of several parameters. The variation is due to types and quantity of minerals and gangue material present at the site. Moreover, it also depend on physical, chemical and biological factors prevailed at the site. All these factors and minerals are different at different notch within the mine itself, which control the type and magnitude of the pollutant generated. Thus, it is not always possible to illustrate all the mechanisms of pollution generation even at one mine ecosystems. However, not all of these pollutants will be prominent in all mine water discharges. The significance of each factor varies within and between affected ecosystems, thus it is not always possible to predict their individual effect on the ecosystem (Gray 1997).

Table 32.2 Major characteristics of coal drainage

	Concentrations (ppm)				
Pollutants	Typical coal mine drainage	Bowden close colliery	Gwynfi discharge	Benbar colliery	Panandhro
Aluminum	5–50	5.29	–	67.4	7.2
Total iron	50–300	15.9	13.9	226	660
Manganese	20–300	130	–	66.4	–
Sulphate	20–2,000	–	1,370	2,416	1,833
pH	3.0–5.5	6.4	6.8	2.7	1.7

Table 32.3 Redox potential and pH characteristics of selected lignite and coal mine drainage of India

No.	Source	pH	Redox potential (mV)
1.	Neyveli lignite mine	5.0–6.0	+150 to +196
2.	Panadhro lignite mine	1.7	+640
3.	Rajpardi lignite mine	2.8–6.0	+240 to +530
4.	Tadkeshwar lignite mine	2.86–7.67	+60 to +535
5.	Moonidih coal mine	6.9–7.3	+125
6.	Chirimiri colliery	2.46	+485

Table 32.4 Physico-chemical diversity of Rajpardi Lignite Mine samples

Sample	pH	Redox potential (mV)	Conductivity (mS)	TDS (g/L)	Salinity (ppt)	Fe^{2+} (g/L)	SO_4 (g/L)
RJLig-1	2.8	440	2.30	1.39	1.30	1.68	2.55
RJLig-2	2.4	500	3.60	2.02	1.80	2.23	4.45
RJLig-3	5.1	305	1.02	0.653	0.50	0.39	0.77
RJLig-4	5.4	280	4.05	0.292	0.20	0.50	0.16
RJLig-5	5.6	240	0.23	0.154	0.10	0.36	0.13
RJLig-6	5.8	240	0.28	0.181	0.10	0.47	0.29
RJLig-7	2.8	530	3.10	1.66	1.60	0.36	0.28
RJLig-8	6.0	305	2.0	1.07	1.10	0.02	0.13

The most extensively studied mechanism for the formation of contaminated mine water is the oxidation of pyrite, which is the most abundant sulphide minerals, commonly associated with coal and metal ore deposits. Biotic and abiotic degradation can be caused by oxygen i.e. direct oxidation or by oxygen and iron i.e. indirect oxidation (Evangelou and Zhang 1995; Lottermoser 2007a). Different pyrite oxidation mechanisms are summarized in Fig. 32.1.

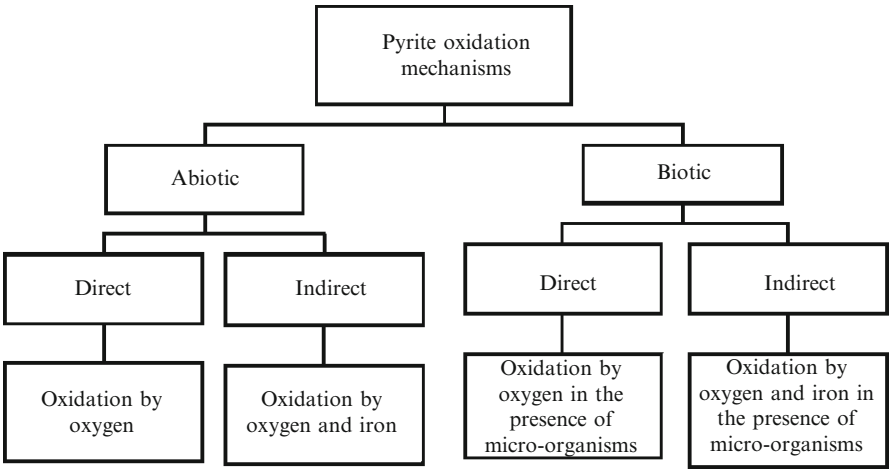

Fig. 32.1 Various mechanisms of pyrite oxidation

All these reactions are acid producing reactions. In the abiotic and biotic direct oxidation processes, oxygen directly oxidizes pyrite as shown in Eq. 32.1.

$$FeS_{2(s)} + 7/2O_{2(g)} + H_2O_{(l)} \rightarrow Fe^{2+}{}_{(aq)} + 2SO_4{}^{2-}{}_{(aq)} + 2H^+{}_{(g)} + \text{energy} \quad (32.1)$$

The pyrite chemically oxidizes, creating a slightly acidic environment conducive for the development of *Acidithiobacillus ferrooxidans* formerly *Thiobacillus* ferrooxidans (Kelly and Wood 2000) and *Leptospirillum ferrooxidans*. These naturally occurring and ubiquitous, chemolithotrophic microorganisms colonize the exposed mineral surfaces. *Acidithiobacillus ferrooxidans* accelerates the chemical rate of pyrite oxidation some 500,000–1,000,000 fold (Kelly and Tuovinen 1988; Schrenk et al. 1998; Brierley and Brierley 2002). Bacterially catalysed oxidation of this mineral is the single most important reaction contributing to acid rock drainage or acid mine drainage. Such biological process can increase the overall rate of acid generation by a factor of 20. Acid generation in last 10 years at Rajpardi lignite mine is sown in Table 32.5. The amount of acid mine drainage accumulated at the site (Fig. 32.2) is more than 1,000,000 m^3 having pH in the range of 2.0–3.5, this indicate the magnitude of AMD generation and its significance.

The indirect oxidation of pyrite involves the chemical oxidation of pyrite by oxygen and ferric iron (Fe^{3+}), which occurs in three interconnected steps that is illustrated in Eqs. 32.2–32.4.

$$4FeS_{2(s)} + 14O_{2(g)} + 4H_2O_{(l)} \rightarrow 4FeSO_{4(aq)} + 4H_2SO_{4(aq)} + \text{energy}$$

OR

$$Fe^{2+}{}_{(aq)} + 1/4O_{2(g)} + H^+{}_{(g)} \rightarrow Fe^{3+}{}_{(aq)} + 1/2H_2O_{(l)} + \text{energy} \quad (32.2)$$

Table 32.5 Ten years profile of ARD generation at Rajpardi Lignite Mine Gujarat, India

Year of sampling	pH of mine drainage
21.08.2000	2.6
2.11.2000	2.0
5.02.2001	2.5
5.11.2001	2.0
28.01.2002	2.6
23.07.2002	3.5
19.09.2003	2.6
27.09.2005	2.8
3.05.2007	2.9
23.10.2007	3.4
15.2.2008	2.8
20.2. 2009	2.9
5.2.2010	3.0

Fig. 32.2 Accumulated acidic water sump at Rajpardi lignite mine

$$FeS_{2(s)} + Fe_2\left(SO_4\right)_{3(aq)} + 2H_2O_{(l)} + 3O_{2(g)} \rightarrow 3FeSO_{4(aq)}$$

OR

$$Fe^{2+}{}_{(aq)} + ¼O_{2(g)} + H^{+}{}_{(g)} \rightarrow Fe^{3+}{}_{(aq)} + ½H_2O_{(l)} + \text{ energy} \qquad (32.3)$$

$$FeS_{2(s)} + Fe_2(SO_4)_{3(aq)} + 2H_2O_{(l)} + 3O_{2(g)} \rightarrow 3FeSO_{4(aq)} + 2H_2SO_{4(aq)} + \text{energy}$$

OR

$$FeS_{2(s)} + 14Fe^{3+}{}_{(aq)} + 8H_2O_{(l)} \rightarrow 15Fe^{2+}{}_{(aq)} + 2SO_4{}^{2-}{}_{(aq)} + 16H^{+}{}_{(aq)} + \text{energy} \quad (32.4)$$

In ferric iron mediated pyrite oxidation, no molecular oxygen is required; thus even in flooded mine working, where air pyrite contact is blocked, pyrite weathering can still occur, of course at much reduced rate as compared to in the presence of oxygen, since there will be no aerobic microbial actions. Moreover, natural water contain up to 10–15 ppm dissolved oxygen depending on the temperature, thus flooding could not guarantee of anaerobic condition.

The bacterial oxidation of ferrous iron reaction (32.3) produces ferric iron, a strong oxidant that chemically oxidizes pyrite reaction (32.4). The ferrous iron resulting from this reaction is regenerated to ferric iron by *At. ferrooxidans* and *L. ferrooxidans*

$$4Fe^{2+} + O_2 + 4H^+ \rightarrow 4Fe^{3+} + 2H_2O \quad (32.5)$$

The ferric iron is then available to oxidize more pyrite, and the cycle continues.

As acidic, sulphuric, iron-bearing solutions seeps from the sulphide rich environment and encounters rocks, soils and/or water of higher pH (pH >2.5), the soluble ferric iron produced in reaction (32.5) will hydrolyse and precipitate as ferric hydroxide, this reaction generate additional acid;

$$4Fe^{3+} + 2H_2O \rightarrow FeOH^{2+} + H^+ \quad (32.6)$$

$$Fe^{3+} + 2H_2O \rightarrow Fe(OH)_2^{+} + 2H^+ \quad (32.7)$$

$$Fe^{3+}{}_{(aq)} + 3H_2O_{(l)} \rightarrow Fe(OH)_{3(s)} + 3H^{+}{}_{(aq)} \quad (32.8)$$

$$Fe^{3+}{}_{(aq)} + 2H_2O_{(l)} \rightarrow FeOOH_{(s)} + 3H^{+}{}_{(aq)} \quad (32.9)$$

As a result of reactions 32.6–32.9 more and more H^+ ions are release, which lowers pH that leads to more soluble ferric iron in solution. This ferric iron is then stagger rapid oxidation of pyrite, which results in further fall in pH. The generated acidic pH accelerates microbial activity and these cyclic processes will continue. AMD waters typically precipitate iron hydroxides, oxyhydroxides or oxyhydroxysulphates, which are collectively termed as 'ochres', 'boulder coats', or 'yellow boy'. The precipitation of iron hydroxides in reactions 32.8 and 32.9 is termed as hydrolysis. In this chemical process, water molecules react with dissolved cations; the cations become bonded to the hydroxyl group and hydrogen ions are released and cause the

pH to fall. Hydrolysis reactions of iron are pH dependent (Brierley and Brierley 2002). Under acidic conditions of less than about pH 3, ferric iron remains in solution. At higher pH values, precipitation of ferric hydroxides occurs. Such a precipitate is commonly observed as the familiar reddish-yellow to yellowish-brown stain, coating, slimy sludge, gelatinous flocculent and precipitate in AMD affected streams and seepage areas (Zänker et al. 2002; Kim and Kim 2004; Lee and Chon 2006).

Depending on the pH and ionic composition of the bulk solution, 'jarosite' (basic ferric sulphate compounds) may form. Jarosite is the result of the chemical reaction between ferric iron, sulphate and soluble potassium and other monovalent ions such as NH_4^+ and Ag^+ which can induce jarosite formation:

$$K^+ + 3Fe^{3+} + 2SO_4^{2-} + 6H_2O \rightarrow KFe_3(SO_4)_2(OH)_6 + 6H^+ \quad (32.10)$$

$$K^+ + 3Fe^{3+} + 2HSO_4^- + 6H_2O \rightarrow KFe_3(SO_4)_2(OH)_6 + 8H^+ \quad (32.11)$$

Jarosite formation is an acid producing reactions. Under low pH conditions, iron oxides such as goethite [αFeO(OH)] and lepidocrosite [γFeO(OH)] can also form (Brierley and Brierley 2002). Iron minerals such as jarosite [$KFe_3(SO_4)_2(OH)_6$], ferrihydrite [$Fe_5HO_8 \cdot 4H_2O$], schwertmannite [$Fe_8O_8(SO_4)(OH)_6$] and the FeOOH polymorphs goethite, ferroxyhyte, akaganeite and lepidocrocite are very common. Different iron minerals appear to occur in different AMD environments (Bigham et al. 1996; Lottermoser 2007b). Acidity, metal content and turbidity of contaminated mine water are mainly influenced by: sulphide grain size and surface area; porosity and permeability of the deposit; nature of the gangue materials; composition of sulphide ore; nature and amount of acid consuming minerals; and different physico-chemical factors influencing the activity of the microorganisms. Generation of mine water drainage is thus highly site specific, and can greatly vary even within a single mine site. Sulphide minerals weathering will continue as long as there are exposed sulphides present, and is thus likely to occur for hundreds of years after mining activity have ceased. Predicting the amount of acid generation is likely to be uncertain due to complex geology and hydrology prevails at mining ecosystem. Continuous monitoring is highly essential for reliability of prediction of acid producing potential of the mining site (Fytas et al. 1992).

32.3.2 ARD Neutralization

The oxidation of pyrite, the precipitation of iron and aluminium hydroxides and the dissolution of some secondary minerals release hydrogen to solution. These processes increase the solution's acidity unless the hydrogen is consumed through buffering reactions. Much of the buffering of the generated acidity is achieved through rock forming gangue minerals, which is having the capacity to buffer acid. The minerals will react with and consumes the hydrogen ions. Acid buffering is largely caused by the weathering of silicates, carbonates and hydroxides. The individual gangue minerals dissolve at different pH values and buffering of the solution pH by

individual minerals occurs within certain pH regions. Carbonate minerals such as calcite ($CaCO_3$), dolomite [$CaMg(CO_3)_2$], magnesite ($MgCO_3$) neutralize acid generated from sulphide oxidation. Calcite neutralizes acid by dissolving and forming complex with hydrogen ion to form bicarbonate (HCO_3^-) and carbonic acid (H_2CO_3) (Lottermoser 2007b).

Depending on the pH of the weathering solution, acidity is consumed either by the production of bicarbonate in weakly acidic to alkaline environments reaction (32.12) or by the production of carbonic acid in strongly acidic environments reaction (32.13),

$$CaCO_{3(s)} + H^+_{(aq)} \leftrightarrow Ca^{2+}_{(aq)} + HCO_3^-{}_{(aq)} \tag{32.12}$$

$$CaCO_{3(s)} + 2H^+_{(aq)} \leftrightarrow Ca^{2+}_{(aq)} + CO_2 + H_2O \tag{32.13}$$

A reversal of the reactions (32.12) and (32.13) is possible when there is a change in temperature, loss of water or loss of carbon dioxide. Re-precipitation of carbonates will occur, which in turn releases hydrogen ions, causing the pH to fall.

Neutralization of acid by dolomite has shown in reaction (32.14),

$$4H^+ + CaMg(CO_3)_2 \rightarrow Ca^{2+} + Mg^{2+} + CO_2 + 2H_2O \tag{32.14}$$

The reaction of acid with aluminosilicates (potassium and calcium feldspars) forming kaolinite is presented by reactions (32.15) and (32.16).

$$2KAlSi_3O_8 + 2H^+ + H_2O \rightarrow Al_2Si_2O_5(OH)_4 + 4SiO_2 + 2K^+ \tag{32.15}$$

$$CaAl_2Si_2O_8 + 2H^+ + H_2O \rightarrow Al_2Si_2O_5(OH)_4 + Ca^{2+} \tag{32.16}$$

The formation of gypsum is shown in equation 32.17

$$CaCO_3 + SO_4^{2-} + 2H^+ + H_2O \rightarrow CaSO_4.2H_2O + CO_2 \tag{32.17}$$

Bacteria play significant role in oxidation of some divalent base metal sulphides, is illustrated in reaction 32.18

$$MS + 2.5O_2 + 2H^+ \rightarrow M^{2+} + SO_4^{2-} + H_2O \tag{32.18}$$

where MS is metal sulphide and M^{2+} is a divalent metal such as Cu^{2+}, Zn^{2+} or Ni^{2+} (Brierley and Brierley 2002).

Carbonate minerals such as siderite ($FeCO_3$) and ankerite [$Ca(Mg, Fe)(CO_3)_2$] are common gangue minerals of coal bearing strata and these carbonates contain Fe^{2+}. The weathering of siderite consumes hydrogen ions as long as the released Fe^{2+} does not undergo oxidation and hydrolysis because the hydrolysis of Fe^{3+} releases hydrogen protons. Thus, siderite dissolution in an oxidizing environment has no neutralizing effect on acidic waters. In contrast, the dissolution of ankerite consumes more hydrogen protons than the subsequent oxidation and hydrolysis of the release iron. Consequently, ankerite possesses a net neutralization potential for acidic waters (Lottermoser 2007b).

32.4 Microbiology of Coal and Lignite Mine

Coal and lignite mine ecosystem are normally having presence of some sulphidic materials. Highly acidic environments formed by the oxidation of pyrite and other sulphidic minerals are known to be populated by a range of acidophilic and acid tolerant prokaryotic and eukaryotic life forms (Johnson et al. 1992). Acidophiles are categorized based on phenotypic traits, such as temperature (as mesophiles, moderate thermophiles and thermophiles), pH optima for growth and the basis of cellular carbon acquisition viz. autotrophs assimilate CO_2, heterotrophs assimilate organic carbon and mixotrophs use both (Bhattacharya et al. 2006; Yamanaka 2008). Some of the acidophilic microorganisms associated with mine water are shown in Table 32.6.

Microbes can dissolve minerals by direct (enzymatic) or indirect action under aerobic and anaerobic conditions. When oxidized metal compounds such as Fe^{3+}, Mn^{4+} or As^{5+} act as terminal electron acceptors (dissimilatory reduction), anaerobic respiration becomes an example of direct dissolving action under anaerobic condition.

Indirect dissolution of minerals can be the result of microbial activity connected with production of organic and inorganic acids, and oxidizing agents, which can influence soil conditions including changes in pH and redox potential. As a result of microbial metabolism, insoluble metal deposits or metals contained in wastes are converted into soluble metal sulphates. Acidic sulphate soils as well as acid mine drainage, enriched with heavy metals, can be formed causing serious environmental problems. Pyrite isolated from lignite coal consisted of porous and non-porous irregular grains. Acid mine drainage is hostile to most forms of aquatic life. The majority of bacterial species found in unpolluted streams are killed on exposure to AMD, yet microorganisms are dominant forms of life in AMD. Most forms of life inhabiting AMD, in which temperatures generally range between 10°C and 35°C, are either acidophilic or acid tolerant. Besides acidophilic iron oxidizing chemolithotrophs, varieties of heterotrophic iron oxidizers are also found in AMD (Table 32.6). Heterotrophic microbes play an important role in metabolizing organic materials potentially toxic to autotrophic iron oxidizing bacteria (Kurek 2002).

The oxidation of pyrite is an exothermic reaction with standard reaction enthalpy $\Delta_f H° = -1{,}546$ kJ/mol (Schippers et al. 1995). At temperatures about 10–40°C *Acidithiobacillus* and *Leptospirillum* species predominate. But beyond 40°C, mesophilic chemoautotrophs begin to die (Niemela et al. 1994) and moderately thermophilic, acidophilic, chemoautotrophic bacteria appear. At 30–40°C temperature, mesophilic and moderately thermophilic bacteria coexist. An archaeon, *Ferroplasma acidarmanus* was isolated from ARD at pH ~ 0.5 (Brierley and Brierley 2002). This microbe can grow at 40°C and is believed to be a major factor in formation of ARD from a mine. At a temperature, of approximately 55°C, the moderate thermophilic acidophilic archaea such as *Sulfolobus*, *Acidianus*, *Metallosphaera*, *Sulfobacillus*, *Sulfurococcus* and *Sulfolobus metallicus* has been

Table 32.6 Some important iron and sulphur bacteria identified from ARD

Group/classification	Name of the organism
Iron oxidisers	*Acidithiobacillus ferrooxidans*
	Leptospirillum ferrooxidans
Sulphur oxidisers	*Thiobacillus thioparus*
	Thiobacillus versutus
	Thiobacillus intermedius
	Thiobacillus delicatus
	Thiobacillus kabobis
	Acidithiobacillus thiooxidans
	Thiobacillus neopolitanus
Heterotrophs grown on sulphur media	*Hyphomicrobium facilis*
	Pseudomonas aeruginosa
	Alcaligens spp.
	Pseudomonas stutzeri
	Brevundimonas diminuta
	Stenotrophomonas maltophilia
Ferric precipitator	*Sphaerotilus* spp.

isolated from an acid generating coal spoil heap and the other archaea are found in geothermal sites. These microorganisms oxidize iron and sulphur compounds under acid conditions at temperatures ranging from about 55°C to near 85°C (Brierley and Brierley 2002).

Microbial eukaryotes likely play critical, but as yet only partially determined roles in AMD communities (Baker et al. 2004). Among eukaryotic organisms, acidophilic algae and protozoan have received more attention than fungi and yeast although fungi have long been recognized as active participants in acid wastewaters (Gross and Robbins 2000). Two extremely acidophilic (pH <1) and metal tolerant mitosporic fungi, *Scytalidium acidophilium* and *Acontium velatum* were isolated by Starkey and Waksman (1943). Johnson and Rang (1993) cultured unidentified protists from an AMD site and showed that they were able to graze on acidophilic bacteria in culture. Fungal hyphae comprise a significant but variable portion of total biomass in many biofilm communities in the Richmond mine, USA, particularly those growing in flowing solutions. The hyphae may contribute to the anchoring of biofilm to pyrite sediments and may confer structure, especially to slime streamers. Relatively large fungal filaments also provide surfaces for the attachment of prokaryotes (Baker and Benfield 2003). In addition they keep organic carbon levels low and produce dissolved carbonate ions, which are significant for the growth of chemolithoautotrophic acidophilic prokaryotes.

Fungal filaments of the families *Eurotiomycetes* and *Dothideomycetes* have been isolated from Richmond mine at Iron Mountain. These isolates provide rigidity and

organization to the biofilm and anchor them to pyrite sediment in flowing AMD solutions. Both these families are belonging to the phylum *Ascomycota* (Baker et al. 2004). Very recently *Candida digboiensis* is isolated from lignite mine and is found to remain live in this acid mine drainage having pH <2.0 for more than 10 years (Patel et al. 2009).

32.5 Field Indicators and Environmental Impacts of ARD

32.5.1 Field Indicators

Any seepage water flowing from a mine, mine waste pile, tailing dam or pond may be acidic. The most common indicators in the field for the presence of AMD waters are:

1. pH values less than 5.5: Mine waters with a pH of less than 5.5 may have obtained their acidity through the oxidation of sulphide minerals.
2. Disturbed or absent aquatic and riparian fauna and flora: AMD waters have low pH values and can carry high levels of sulphate, salinity and total dissolved solids and even presence of some amount of heavy metals and metalloids. This results in the degradation or even death of aquatic and terrestrial ecosystems.
3. Yellow-brown precipitated mineral efflorescence covering stream beds and banks: The observation of colourful yellow-brown precipitates, which discolour seepage points and stream beds, is typical characteristic for the AMD process. The sight of such secondary iron-rich precipitates is a signal that AMD generation is well underway.
4. Discoloured, turbid or exceptionally clear water: AMD water can have a distinct yellow-red brown colouration, caused by an abundance of suspended iron hydroxide particles. The turbidity of the AMD water generally decreases downstream as the iron and aluminium flocculate, and salts precipitate with increasing pH. As a result, acidic waters can also be exceptionally clear and may give the wrong impression of being of good quality.
5. Abundant algae and bacterial slimes: Elevated sulphate levels in AMD waters favour the growth of algae and acidic waters may contain abundant slimy streamers of green or brown algae (Lottermoser 2007b).

32.5.2 Environmental Impacts

AMD waters from tailings dams, mine waste dumps, heap leach pads and ore stock piles should not be released from the mine site due to the presence of suspended solids and dissolved contaminants such as acid, salts, heavy metals, metalloids and sulphate. The uncontrolled discharge of AMD waters into the environment may

have adverse impact on surface waters, aquatic life, soils, sediments and ground waters.

1. Surface Water Contamination
The release of AMD waters with their high metal and salt concentrations impacts on the use of the water ways downstream for fishing, irrigation and stock watering. Potable water supplies can be affected. Seasonally high concentrations of H^+ ions and metals and increased conductivity, turbidity, total dissolve and suspended solids can be observed in AMD waters at the beginning of the wet season or spring (Lottermoser 2007b). The first flash can cause distinct impacts on downstream ecosystems with potentially severe effects on biota.

2. Impact on Aquatic Life
The high acidity of AMD waters can destroy the natural bicarbonate buffer system, which keeps the pH of natural waters within a distinct pH range. The destruction of the bicarbonate system by excessive hydrogen ions results in the conversion of bicarbonate to carbonic acid and then to water and carbon dioxide reaction (32.13). Photosynthetic aquatic organisms use bicarbonate as their inorganic carbon source; thus the loss of bicarbonate will have an adverse effect on these organisms. They are not able to survive in waters below a pH value of less than 4.3. A reduction in biodiversity, depletion of numbers of sensitive species, or even death of fish and other species are possible. Heavy metals and metalloids, at elevated bioavailable concentrations, are lethal to aquatic life and of concern to human and animal health (Lottermoser 2007b).

3. Sediment Contamination
Improper disposal of contaminated water from mining, mineral processing and metallurgical operations releases contaminants into the environments. Precipitation of dissolve constituents may result in abundant colourful mineral coatings.

4. Ground Water Contamination
AMD influence more frequently on the quality of ground waters than on that of surface waters. Ground water contamination may originate from mine working, sulphidic tailings dams, waste rock piles, heap leach pads, ore stock piles, coal spoil heaps, ponds and contaminated soils (Lottermoser 2007b). Contaminated water may migrate from working and waste repositories into aquifers. Significant concentration of sulphate, metals, metalloids and other mine contaminant have been found in ground water plumes migrating from mine working and sulphidic waste repositories and impoundments. Conservative contaminants e.g. SO_4^{2-} move at ground water velocities. However, reactive contaminants e.g. heavy metals and metalloids move more slowly than the ground water velocity and a series of different pH zones may be present in the contaminant plume. In such type of contaminated plume pH zones of different minerals such as aluminosilicates, $Fe(OH)_3$, $Al(OH)_3$, siderite and calcite is formed from the top to bottom respectively. In these pH zones the pH will shift towards the alkaline site from acidic as the water will pass from the top to bottom. The occurrence of these zones is attributed to the successive weathering of different pH buffering phases

in the aquifer. Neutralizing minerals such as carbonates may be contained in the aquifers and these minerals buffer acidic ground water. Depending on the neutralization property of the aquifer through which this water moves, it could be many years before significant impact on ground and surface water quality is detected (Lottermoser 2007b). AMD affects lotic (river) systems in numerous and interactive ways. AMD act on the organisms comprising the community structure of the ecosystem.

The effects of AMD can be loosely categorized as chemical, physical, biological and ecological. Overall impact of AMD on the community structure is the elimination of species, simplifying the food chain and so significantly reducing ecological stability. The effects of AMD are so multifarious that community structure collapses rapidly and totally, even though very often no single pollutant on its own would have caused such a severe ecological impact. Recovery is suppressed due to habitat elimination, niche reduction, substrate modification, the toxic nature of sediments and bioaccumulation of metals in the flora and fauna (Gray 1997).

32.6 ARD Management Strategies

In order to develop cost effective and environmentally sound strategies for the management of AMD discharges from abandoned and active mines, a protocol has been adopted, depending on the location and climate of the mine site (Lottermoser 2007b).

Initially various techniques can be used to reduce mine water volumes are:

- Diversion of runoff from undisturbed catchments
- Maximization of recycling and reuse of water
- Segregation of water types of different quality
- Controlled release into nearby waters
- Sprinkling of water over dedicated parts of the mine site area
- Use of evaporation ponds
- Installation of dry covers over sulphidic wastes in order to prevent infiltration of meteoric water

These water management strategies will reduce the potential AMD water volume.

Discharge of AMD water in ocean is one of the options for AMD disposal, because sea water has a strong buffering capacity due to the abundance of bicarbonate where as ground and surface waters in a carbonate terrain have similarly a significant natural buffering capacity. Releasing wastewaters during periods of high rainfall or peak river flow may also achieve dilution and reaction of the effluent to pollutant concentrations below water quality standards (i.e. dilution is the solution to pollution). But in all cases this disposal technique cannot be adapted due to location of mining site, environmental situation and political interference.

32.7 Treatment of ARD

Once started, AMD is a persistent and potentially severe source of pollution from mine sites that can continue long after mining has ceased. Abandoned historic mine sites still releasing AMD waters are a large liability for governments. The total worldwide liability related to AMD is likely to be in excess of 10,000 million US dollars. In the United States alone, the mining industry spends over US $ 1 million every day to treat AMD water (Lottermoser 2007b). It is costly and difficult to treat developed AMD than to control the generation process through sulphide oxidation prevention technologies.

AMD treatment technologies are site specific and multiple remediation strategies are commonly needed to achieve successful treatment of AMD waters. Established treatment processes include evaporation, neutralization, wetlands and controlled release and dilution by natural waters. More technologically advanced processes involved osmosis, electrolysis, ion exchange, biosorption, bioprecipitation (i.e. vessels that contain sulphate reducing bacteria causing the metal to precipitate as sulphides), biotransformation (aerated bioreactors and rock filters), lime stone reactors and solvent extraction (Greben and Maree 2005; Johnson and Younger 2005).

Both established and innovative AMD treatment techniques are generally designed to:

- reduce volume
- raise pH
- lower dissolved metals and sulphate concentration
- lower bioavailability of metals in solution
- oxidize or reduce the solution
- collect, dispose or isolate the mine water or any metal rich sludge generated

Mine site pollution prevention and remediation are carried by three different stages.

At source control
Mine water pollution at source control could be carried out by sulphide removal or separation, exclusion of water, exclusion of oxygen, pH control and control of microbial action.

Migration control
When it is not possible to prevent acid production, the next step of control is to prevent or reduce the migration of the contaminated mine water into the surrounding environments. This process is performed by

- Diversion of all surface water flowing towards the sulphidic rocks
- Prevention of ground water flow into area of sulphidic rocks
- Controlled placement of acid generating waste

Collection and treatment
When mine water pollution is not controlled and already started accumulating at mine site the third step of treatment is applied which includes,

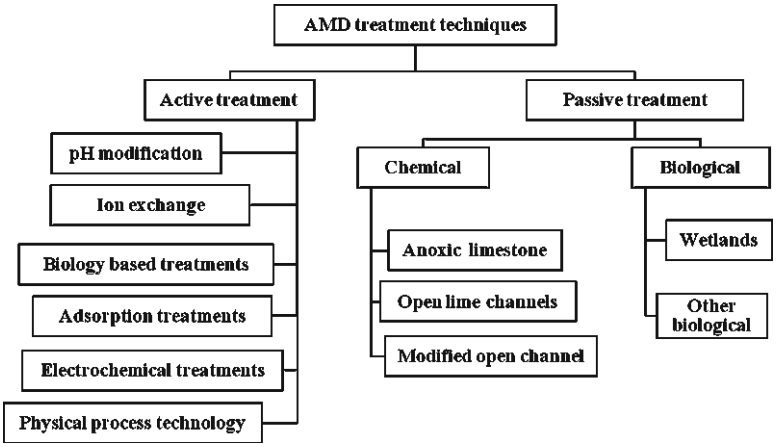

Fig. 32.3 Generalised treatment techniques for acid mine drainage

- Neutralisation of acidity
- Removal of metals
- Removal of suspended solids
- Prevention of discolouration of receiving water

All above specified AMD treatment techniques can broadly be classified as active and passive treatments (Johnson and Hallberg 2005).

32.7.1 Active Treatment

Active treatment of AMD is subdivided into: pH modification; ion exchange; biology based treatments; adsorption treatments; electrochemical treatments and physical process technology (Fig. 32.3).

The addition of neutralising agent reduces the acidity and dissolved heavy metal concentrations of mine waters. In the reaction of lime stone with AMD waters hydrogen ions are consumed, bicarbonate ions generated and dissolved metals are converted into sparingly soluble minerals such as sulphates, carbonates and hydroxides. The sludge that formed by alkaline treatment is generally of a low density, with a solids content of as little as 2% and very bulky not convenient for disposal purpose. The high density sludge process (HDS) is a process that has been developed to achieve greatly increased sludge densities as compared to conventional pH modification treatments (Zick et al. 1998).

Biology based treatments include techniques that harness the remediation ability of microorganisms in bioreactors, thereby facilitating a greater degree of control than is possible in passive wetland treatment process. Biological process also includes use of biologically produced materials as a sorbents or product, which precipitate the pollutants. Sulphate reducing bacteria (SRB) are used for amelioration of contaminated mine water by numerous laboratories to quantify the rate of

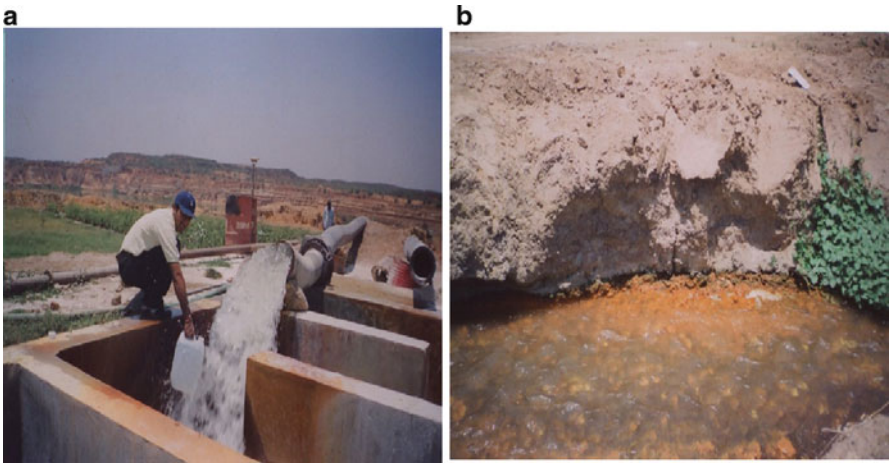

Fig. 32.4 (**a**) Bhuri pumping point, acid neutralization and (**b**) Influence of neutralization at Bhuri, Rajpardi mine

sulphate reduction and precipitation of metals (Kar et al. 1992; Drury 1999; Chang et al. 2000). Various workers have developed pilot scale rectors for H_2S production and metal precipitation using straw, wood shavings, spent mushroom compost and gravel substrates (Bechard et al. 1989; Dvorak et al. 1991; Lyew et al. 1994). The proprietary processes currently available in market are PAQUES THIOPAQ process and NTBC Research Crop- the biosulphide process. Glombitza (2001) has described a pilot scale process for the treatment of mine water from a lignite mine in Germany. In this process SRB were immobilized on porous media and methanol was used as a carbon source. During process, metal removal ability reached to 100% and pH increased from 3.0 to 6.9. Apart from strictly anaerobic process aerobic bioreactors are used to oxidize ferrous iron, as a part of multi-stage process employed for the treatment of mine water. Microbial biomass dead or alive is used as biosorbent for the sorption of metals from mine drainage (Tsezos 1990; Gadd 1992).

The simple active treatment is carried out by modification of pH. Chemical compounds commonly used in AMD treatment are lime stone ($CaCO_3$), caustic lime (CaO), hydrated lime [$Ca(OH)_2$], dolomite [$CaMg(CO_3)_2$], magnesite ($MgCO_3$), soda ash (Na_2CO_3), caustic soda (NaOH), ammonia (NH_3), kiln dust (largely CaO and $Ca(OH)_2$) and coal fly ash ($CaCO_3$ and CaO). Simple active treatment with addition of calcium carbonate practiced at Rajpardi lignite mine is shown in Fig. 32.4a. The formation of yellow brown iron precipitate is clearly seen in Fig. 32.4b.

32.7.2 Passive Treatment

Passive treatments are highly desired option for mine drainage treatment, as they do not required ongoing input of materials and financial resources as in the case of active process. Passive process also works on chemical as well as biological system.

32.7.2.1 Chemical Passive Treatment

Passive chemical process comprises a means of neutralizing acidity and metal precipitation without active addition of lime or other alkaline material. In this process acidic mine water is allowed to flow through a bed of limestone (Lapakko and Antonson 1990). In passive treatment, iron may need to remain in its reduced form (Fe^{2+}) until the precipitation of other metals has occurred and additional alkalinity has been dissolved in the AMD waters. Otherwise the neutralising solids may be coated with Fe^{3+} reaction products and rendered ineffective. Anoxic limestone drains are generally relied upon to keep iron in solution and to add alkalinity to the system. The precipitates may settle very slowly because of their small particle size. Settling of precipitates can be speed up by using flocculants and coagulants. Inorganic Fe and Al salts, as well as organic polymers are used to form large aggregates of solids. As a result, voluminous sludge, composed mainly of solid sulphates, hydroxides and carbonates as well as amorphous and poorly crystalline material is produced, which in most of the cases needs disposal. Depending on the mineralogical and chemical characteristics of the AMD sludge, metals can be recovered from the sludge using strong acids. Very pure Fe^{3+} hydroxides sludge may be used as pigments in the production of coloured bricks and concrete.

32.7.2.2 Anoxic Limestone Drains

In oxidizing environment, lime stone becomes coated with Fe^{3+} reaction products and rendered ineffective in the production of bicarbonate ions. Anoxic lime stone drains consist of shallow trenches backfilled with crushed limestone and covered with plastic and impermeable soil or sediment. These backfilled trenches are sealed from the atmosphere in order to maintain iron as dissolved Fe^{2+} species. This prevents oxidation of Fe^{2+} to Fe^{3+} and hydrolysis of Fe^{3+}. The effluent pH of anoxic limestone drains is typically between 6 and 7. Once the pH has been adjusted and the drainage has exited from the channel, controlled aeration permits oxidation of dissolved metals, hydrolysis and precipitation of metal hydroxides or carbonates. Alkali may also be added in so called successive alkalinity producing systems, where by water passes vertically through successive layers of organic matter and limestone chippings. These vertical flow systems have a layer of organic substrate, which reduces Fe^{3+} to Fe^{2+} and eliminates the oxygen dissolved in the water. The reduced water then enters an alkalinity generating layer of lime stone before it is finally discharged (Lottermoser 2007b).

32.7.2.3 Open Limestone Channels

Laboratory and field studies are conducted by passing acid mine drainage through open limestone channels. In this process the influent mine water contained 1.0–4.0 mg/L

oxygen. This process requires less land area as compared to anoxic limestone process. Further work is needed to optimise the process.

32.7.2.4 Biological Passive Treatment

Wetlands

Wetlands are organic rich, water saturated shallow ponds. The treatment is based on a number of physical, chemical and biochemical processes, which purify the AMD waters (Gibert et al. 2002; Karathanasis and Johnson 2003; Weber et al. 2008). These processes include sulphide precipitation, oxidation and reduction reactions, cation exchange, and adsorption of metals on the organic substrate, neutralization of proton acidity, adsorption of metals by precipitating Fe^{3+} hydroxides and metal uptake by plants. A basic design scheme for constructing wetlands includes an organic substrate and discrete, controlled in and out flow locations (Lottermoser 2007b).

In addition, plants such as reeds, sphagnum moss and cattails may be grown in wetland, which replenishes the organic substrate and support naturally occurring bacteria, vertebrates and invertebrates. Two types of wetlands, aerobic and anaerobic, are used for AMD water treatments.

Aerobic Wetlands

Aerobic wetlands are generally used for net alkaline waters. These wetlands are with or without vegetation and relatively shallow (~0.3 m). Water flows above the surface of an organic substrate or soil. Aerobic wetlands use oxidation and hydrolysis reactions to treat mine waters (Iribar et al. 2000). The hydrolysis of iron produces acidity (reaction 32.8 and 32.9) and lowers the pH of the mine water in the wetlands. Surface flow wetlands do not produce enough alkalinity that is require to buffer the acidity produced from sulphide oxidation at AMD source and hydrolysis reactions in the wetland. Alkalinity may have to be added to such waters. This can be achieved by growing certain plants in wetlands (e.g. reeds). Reeds are capable of passing oxygen through their root zone and the organic substrate. As a result, oxygen is converted to carbon dioxide, which will dissolve in the mine water, consume hydrogen and adds alkalinity in the form of bicarbonate. Alternatively anoxic limestone drains may have to be installed at the inflow location of the wetland.

Anaerobic Wetland

Anaerobic wetlands are generally used for net acid waters. Water flows through a relatively deep (~1 m), permeable and anoxic organic substrate. Placement of organic waste (e.g. mushroom compost, saw dust, manure) into wetlands helps to

establish the reducing conditions. Anoxic conditions favour the proliferation of sulphate reducing bacteria (SRB) (Gibert et al. 2002). SRB are a group of anaerobic bacteria e.g. *Desulfovibrio simplex*, *Desulfotomaculum geothermicum, Desulphobacterium autotrophicum* etc. that can reduce sulphates to form sulphide without significant assimilation of the sulphur in to cell biomass. The overall sulphate reducing process can be represented by equation

$$2CH_2O + SO_4^{2-} + 2H^+ \rightarrow H_2S + CO_2 + H_2O \quad (32.19)$$

$$2CH_2O + SO_4^{2-} \rightarrow H_2S + 2HCO_3^- \quad (32.20)$$

Where, CH_2O represents an organic compound. The sulphide can precipitate with many of the metals present in an AMD, such as Fe, Zn, Cu, Pb, Ni etc.

$$H_2S + M^{2+} \rightarrow MS + 2H^+ \quad (32.21)$$

where M = metals such as Cu, Fe, Ni, Zn

Precipitation of metal sulphides results in the production of hydrogen ions. The sulphate reduction reaction generates more alkalinity and will neutralize the proton generation (Gibert et al. 2002).

32.8 Liability of ARD

Concentrations of common elements such as Al, Cu, Fe, Mn, and Zn all dramatically increase in waters with low pH. Logarithmic increases in metal levels in waters from sulphide-rich mining environments are common where surface or groundwater pH is depressed by acid generation from sulphide minerals. These environmental, human health and economic consequences, if not mitigated, can have long-lasting effects. Acid mine drainage continues to emanate from mining since the first exploitation of the Rio Tinto ores 5000 years ago, the Greek philosophers around 325 B.C. also recognized production of acid (Lottermoser 2007c). Georgius Agricola's *De Re Metallica* (1556), the first and seminal treatise on mining exhibits detailed woodcut illustrations not only of the known mechanics of sixteenth century mining, but also depictions of the devastation of streams. The cost of mitigation of environmental damage from acid mine drainage is great. The U.S. Forest Service (USFS) estimates that between 20,000 and 50,000 mines are currently generating acid on lands managed by that agency; with negative impacts from these mines affecting some 8,000–16,000 km of streams. Many of these mines are small abandoned facilities located in remote areas of the western United States and originating prior to modern environmental controls. However, several large scale mines developed in the later half of the twentieth century have declared bankruptcy and left tax payers with the responsibility of treating acid waters in perpetuity. Examples include the Zortman Landusky Mine in Montana, the Summitville Mine in Colorado, and the Brohm Mine in South Dakota. The largest and most expensive sites that EPA (Environment Protection Agency) has listed under the Comprehensive Environmental Resource

Compensation and Liability Act (CERCLA) are mining sites in the West, including Iron Mountain Mine in California, Bunker Hill in Idaho, and the Butte-Clark Fork River complex in Southwestern Montana. Human health risks and ecological injury, chiefly from elevated metals, have been identified by EPA and natural resource trustees at many of these mega-mining superfund sites.

Acidic drainage has been identified as the largest environmental liability facing the Canadian mining industry and is estimated at $2–$5 billion dollars. Coal and lignite are the most essential minerals having large reserves in India. It's mining and beneficiation produce a variety of pollutants. The main pollutants emitted during the processing of coal and lignite are green house gases, fly ash and acid mine drainage. These pollutants are having harsh effect on environment as well as on economy. It was found that green house liabilities, coal dust/fly ash liability and sulphur liability are accounted for 12.07, 5.0 and 101.97 million US$, making an overall 2.4% of the total economic gains due to coal mining (Sharma et al. 2009).

32.9 Future Perspectives

The presented information provides a broad overview of the way in which coal mine water pollution can affect the environment globally. The available methods are unable to provide solutions for these problems. Thus, proper implementation of combination of at source control, mitigation control, and treatment of collected mine water with the application of sulphate reducing bacteria along with other biological supports need to be optimized both for passive and active treatment in controlled reactors and wetland process. Certain bacteria are responsible for tremendous acceleration of ARD/AMD generation which need to be control, thus development of environmental friendly "bactericides" need to be developed and their field application are to be designed and optimize.

32.10 Conclusions

The pollution problems of mining environments are far from being fully elucidated. Till today, very less is known for microbial activities in mining environments. Microorganisms catalyze the oxidation of sulphidic minerals, elemental sulphur and ferrous iron, which results in ARD generation. The generated extreme acidic condition stimulates dissolution of metals. Different autotrophic and heterotrophic bacteria are involved in this process. Very little is known about activity of microbial consortium. Thus, the further study of the microbial community structure of these ecosystems will provide detail information on role of organisms in acid generation or its neutralisation. Such study will help in correct prediction of the ARD production and acid consumption ability of mining materials, waste and material at the site so that the preventive and control measures can be developed for ARD and are applied throughout the world by coal mining industry.

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Chapter 33
Poultry Waste Management Using Microorganisms

Richa Jain, Swetlana Nagal, and P.C. Jain

Abstract Several million tonnes of feather are produced annually as a byproduct of poultry processing industries and poultry farms which apart from polluting soil and water also plays important role in spread of various human ailments such as dermatophytic infections, chlorosis, mycoplasma fowl cholera and avian influenza. Feather waste being rich in keratin protein are difficult to degrade as the polypeptide are densely packed and stabilized with numerous hydrogen bonds, disulphide bonds and hydrophobic interactions. Despite of recalcitrant nature of keratin proteins, keratin waste do not accumulate in nature confirming the presence of natural decomposers. A number of dermatophytes, saprophytic fungi, bacteria and actinomycetes are known to degrade keratin by virtue of a particular class of endoproteases called keratinase(s) that display the capability to degrade structure conforming keratin protein.

Feathers are composed of approximately 90% keratin protein thus can be a potential source of proteins and amino acids. Application of keratin degrading microorganism have provided appropriate technology for bioconversion of poultry feather into nutritionally enriched feed stuffs and essential amino acids. Waste feathers have also been used as substrate for production of proteolytic enzymes including keratinases. Enzymes produced by keratinolytic microorganisms have also find applications in prion degradation and peptide synthesis. Recent researches on utilization of feather waste are on the way to develop low cost hydrogen storage system from carbonized chicken feather fiber.

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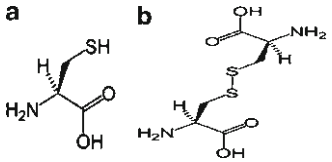
The conventional methods employing incineration or chemical transformation of feather usually resulting in production of low grade feather meal thus the methods appear to be inadequate. In continuation of these developments, a new improved process has been suggested for obtaining biodiesel fuel in addition to the production of feather meal from chicken feathers. However, biotransformation process can be further improved by involving certain steps of biofuel production to make the process cost effective for the production of high grade feather meal from waste feather.

Keywords Keratinases • Keratin • Leather processing • Keratin hydrolysate • Prion degradation

33.1 Structure of Keratin

Keratin is an insoluble structural protein found in mammal hair, reptile scales, (though not fish scales), bird feathers, claws (including nails and hooves), horn (but not antlers), baleens (the sieve-like structures with which whales filter food out of water), the enamel of the teeth etc. It is characterized by long polypeptide chains joined by hydrogen bonds, salt cross linkages and disulphide bridges (Lehninger et al. 1993). Amino acid composition of keratin varies with organisms but all of them contain sulfur-containing amino acids, largely cysteine (Routh 1942; Crewther et al. 1965). Two cysteine (a) molecules join via disulphide bonds to form cystine (b), which account for approximately 24% of keratin. The numerous disulfide bonds formed by cystine are responsible for the great stability of keratin. Diverse functions played by keratin are due to its supermolecular aggregation. Soft keratin contains approximately 2% cystine and is found in skin while hard keratin such as hair may contain approximately 14% cystine (Jain and Jain 2005). It is completely insoluble in hot and cold water and is not attacked by commonly known proteolytic enzymes. Its molecules are helical and fibrous, twisting around each other to form strands called intermediate filaments.



It contains alpha-helix, beta sheet motif, disulfide bridges which play a crucial role in its functional property. Keratins are divided into two main types i.e. α-keratin and β-keratin depending on type of linkages and extent of disulphide bridges (Voet and Voet 1995). The α-keratins are formed primarily as helical fibers, while the β-keratins are formed primarily in beta sheets. Some beta sheets are also found in α-keratins

(Kreplak et al. 2004). It is also known to contain high percentage of glycine and alanine and in beta sheets these two amino acids support stericaly-unhindered hydrogen bond between amino and carboxyl group of adjacent peptide bonds.

33.1.1 α-Keratin

α-Keratin is mainly found in hair, wool, horns, nails, claws and hooves of mammals. Mammals have approximately 30 variants of keratin that are expressed in a tissue-specific manner. Keratin is rich in hydrophobic amino acids such as phenylalanine, isoleucine, valine, methionine, alanine (Lehninger et al. 1993). The X-ray diffraction pattern of a keratin resembles that expected for α helix (hence the name a keratin). However, keratin exhibits a 5.1-Å spacing rather than the 5.4-Å distance corresponding to the pitch of normal α-helix. This discrepancy arises because of two α keratin polypeptides, which forms an α-helix, twisting around each other to form a left-handed coil. Normally, α helix have 3.6 residues per turn whereas keratin has 3.5 residues per turn which makes it a non true alpha helix. In it every 7th residue is leucine which helps in sticking of strands with the help of hydrophobic interactions. They are tough, insoluble proteins which may differ in hardness and flexibility. The α keratins are classified as "hard" or "soft" according to their sulfur content (Nickerson 1947). Hard keratins, such as those of hair, horn, and nail, are less pliable than soft keratins, such as those of skin and callus, because the disulfide bonds resist deformation.

33.1.2 β-Keratins

It is rich in stacked β pleated sheets and is found in birds and reptiles. It adds much more rigidity to reptilian skin than alpha-keratin does to mammalian skin while in birds, beaks, claws and feathers also contain β-keratin. They are principle constituents of the feathers of birds, corneous material of the carapace and plastron of turtles and the epidermis of snakes (Filshie and Rogers 1962). Recently, it has been demonstrated that the framework of β-keratin filaments is composed of twisted β-sheets, each containing four segments of polypeptide chains. β-keratin IF are 4.0 nm. β-Keratin is impregnated into the stratum corneum of the reptilian skin, providing waterproofing and the prevention of desiccation. Feathers represent over 90% protein, the major component being keratin, a fibrous and insoluble structural protein extensively cross-linked by disulfide, hydrogen and hydrophobic bonds. Owing to their insoluble nature, feathers are resistant to degradation by common microbial proteases (Papadopoulos 1989).

33.2 Sources of Keratin Waste

The leather and slaughter houses throw away considerable amount of materials containing mainly keratin protein such as hairs, bristle, horns, feathers, hoofs, etc. such waste is utilized for the preparation of 'animal flour' for its use as animal feed supplement. The leather industry is considered as one of the polluting industries because of generation of huge amount of liquid and solid waste, and emission of obnoxious smell because of degradation of proteinaceous material of skin and generation of gases such as NH_3, H_2S and CO_2. Solid wastes generated by these industries include raw trimmings, chrome shavings, buffing dusts and keratin wastes. Accumulation of these wastes lead to sludge problem and choking of treatment pipes and finally results in reduction in efficiency of treatment plants. Treatment of this waste is not cost effective thus posing an economic burden to the tanners. When the protein constituent of the waste is not utilized properly, it poses hazardous pollution problem to the environment. Slaughter houses also through away a large amount of keratin containing waste during dehairing of animal skin. Goat hair, sheep wool and buffaloes horns are the major source of keratin waste.

Poultry farms are another major source of keratin waste. Approximately 15–20% of the by-products of meat and poultry industry that are unsuitable for human consumption contains keratin mainly feathers. Feathers are composed of over 90% protein and produced in large amounts as a waste by poultry processing worldwide. Accumulations of feathers in nature lead to environmental pollution and wastage of feather protein (Onifade et al. 1998; Gousterova et al. 2005). Feathers represent 5–7% of the total weight of mature poultry birds and constitute a sizable waste disposal problem. In order to solve the problem of poultry waste disposal several different approaches including land filling, burning etc. are adopted (Tapia and Contiero 2008). However, most feather waste is land filled which causes contamination of air, soil and water. The burning of such waste causes production of foul odour. The chemical and thermal treatment of feathers for production of feather meal is generally resulting in loss of its nutritive value and thus some alternative strategies are needed for keratin waste disposal (Kim et al. 2002).

33.3 Keratin Waste Disposal

A large amount of keratinic waste generated by leather industries in the environment, this includes both liquid and solid waste, mostly of animal origin. The poultry industries are also contributing thousand's million tons of keratin waste as a byproduct of poultry processing plants annually. This waste is posing a lot of problems relating to environmental pollution in addition to the environmental contamination, as it serves as a nutrient reservoir for the growth and proliferation pathogenic microorganisms (Jain 2000). A large part of this waste is dumped in soil to minimize the immediate risk of environmental contamination. Burial of such waste in soil may contaminate our soil and water as seen in most slum areas of developing nations. Burning of such

waste is another option for its disposal but causes emission of foul odor in the atmosphere. The biological treatment of this waste to generate value added products is the best and feasible option as it contains a lot of animal proteins, although hard to degrade by most proteolytic enzymes (Kim et al. 2002; Suzuki et al. 2006; Bradelli 2008).

33.4 Keratin Waste Management

Feathers are produced in large amounts as a waste byproduct of poultry processing plants. Feathers contain large amounts of cystine, glycine, arginine, and phenylalanine (Bielorai et al. 1982). They can be a significant source of protein for livestock because of their high protein content (MacAlpine and Payne 1977; Baker et al. 1981). Raw feathers, however, are very poorly digested by nonruminant animals because they contain a high proportion of keratin protein that has cystine disulfide bonds (Papadopoulos et al. 1985; El-Boushy and Van der Poel 1990). The indigestible structure of raw feather must be hydrolyzed to be used as a feed ingredient. However, feather meal prepared using these methods can destroy certain amino acids and decrease protein quality and digestibility (Moritz and Latshaw 2001). A current value-added use for feathers is the conversion to feather meal, a digestible dietary protein for animal feed, using physical, chemical and biological treatments.

The waste generated by leather industries mainly contains animal proteins. Till recent years these materials together with other animal waste were backed at high temperature then milled to the so called animal flour and used as protein supplement in to the feed mixtures of domestic animals. Similarly, feather waste is converted to feather meal through chemical processes that requires high energy input and results in loss of essential amino acids (Baker et al. 1981; Papadopoulos et al. 1985). Although, keratinases from dermatophytic fungi and other related microorganisms have long been known but studied mainly to understand the role of these enzymes in pathogenesis and etiology of mycoses (Brasch and Zaldua 1994; Meevotisom and Niederpruem 1979; Grzywnowicz et al. 1989; Muhsin et al. 1997; Minocha et al. 1972; Ragot et al. 1973; Calvo et al. 1985). The earlier reports made on keratinolytic potential of actinomycetes (Noval and Nickerson 1959) and some soil inhabiting keratnophilic fungi (Jain and Agrawal 1980) triggered interest in the biotechnological applications of keratinolytic microorganisms and their enzymes for management of keratinic waste (Fig. 33.1).

33.4.1 Importance of Microorganisms in Keratin Waste Management

Importance of fungi in keratin waste management have been recognized when a number of saprophytic microorganisms have been recorded growing on keratinic baits during isolation of soil inhabiting dermatophytic fungi using keratin bait

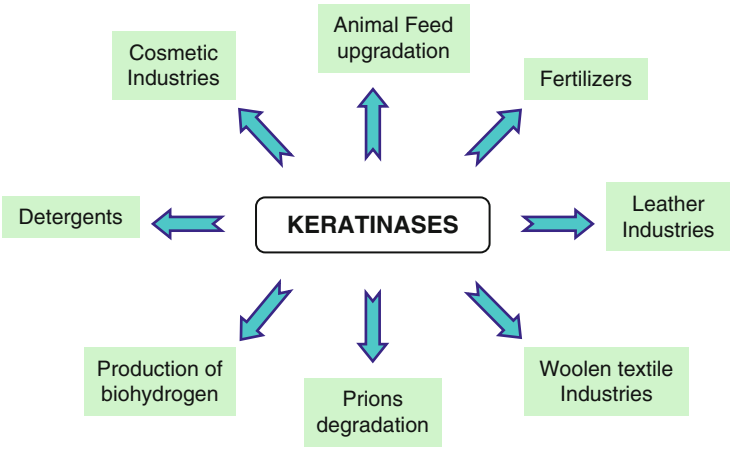


Fig. 33.1 Application of keratinases

technique of Vanbreusheghem (1952). Using this technique a number of keratinophylic fungi were isolated world wide from soils of potential habitats, presumably rich in keratinic matter (Ajello 1953; Griffin 1960; English 1963; Randhawa and Sandhu 1965; Garg 1966; Kunert 1966; Alteras 1967; Ajello and Padhye 1974; Kushwaha and Agrawal 1976; Mercatin et al. 1980; Deshmukh and Agrawal 1982; Singh and Agrawal 1983). Most of these workers have ignored reporting of saprophytic fungi during surveys under taken by them, possibly because of non availability of their pathogenic record to man and animals. A few reports made by later workers have recognized the importance of saprophytic fungi in keratin waste management and reported their isolations along with well known kratinophilic fungi (English 1965; Jain and Agrawal 1977; Jain 1982; Jain 1983; Pugh 1965; Khanam and Jain 2002; Khanam et al. 2002; Lachoria et al. 2004). Many of these saprophytic fungi have been reported keratinolytic and degraded bird's feathers efficiently (Hubalek 1976; Jain and Agrawal 1980; Khanam et al. 2004). *Aspergillus fumigatus* has been found to possess high keratinolytic potential (Santos et al. 1996) while , *Alternaria tenussima* K2 and *Aspergillus nidulans* K7 have been recently reported to degrade chicken , duck, goose and turkey feathers efficiently (Saber et al. 2010).

Burtt and Ichida (1999a) showed that the keratin hydrolyzing bacteria could also occur in the plumage of living birds. The presence of feather degrading bacteria on the feathers would be very interesting for the treatment of these wastes (Kim et al. 2001). They also demonstrated that inoculation may enhance keratin degradation in poultry compost, and such controlled system is very tangible for an accelerated process for feather digestion. A feather degrading *Bacillus licheniformis* PWD61 was isolated from the aerobic portion of a poultry waste digester (Williams et al. 1990) and bioengineered by Lin et al. (1995) to enhance degradation of β-keratin.

Bacillus strains are ubiquitous microorganisms, which can grow on natural media without any special requirements. These properties were exploited in the feather degradation. Moreover, *Bacillus* strains are thermophilic and hence this property has been exploited for development of efficient processes for degradation of feathers. Other bacterial strains known by their keratinolytic activity include *Streptomyces pactum* (Bokle et al. 1995) and *Streptomyces fradiae* (Kunert 1989; Sinha et al. 1991). *B. licheniformis* grows well at high temperatures (50–65°C) and hence, found very convenient to base feather degrading processes. Some reports have described thermoactive keratinolytic proteases produced by mesophilic microorganisms. These enzymes show keratinolytic activity at temperatures above 70°C, whereas most of the other keratinases from mesophilic bacteria and fungi are active at a rather alkaline pH but show optimal activity at lower temperatures. Although, mesophilic bacteria and dermatophytes are producing strong keratinases are mostly pathogenic, they are undesirable for application (Gradisar et al. 2005).

33.4.2 Animal Feed Upgradation

Several researchers have investigated chemical or enzymatic methods for the hydrolysis of feathers (Belewu et al. 2008; Steiner et al. 1983; Papadopoulos 1984; Papadopoulos et al. 1984b; Papadopoulos et al. 1985; Latshaw 1990; El-Boushy and Van der Poel 1990). Steiner et al. (1983) indicated that enzyme or NaOH treatment cleaved cystine disulfide bonds and improved feather solubility and susceptibility to digestive proteolytic enzymes.

Resistance to proteolytic enzymes has been attributed to the complex structure of keratin filaments. In addition, disulfide cross-links produce a compact three dimensional network, as a result of intermolecular disulfide bonds between rod domains and terminal domains of the constituent molecules. The nutritional upgrading of feather meal through microbial or enzymatic treatment has been described. Feather meal fermented with *Streptomyces fradiae* and supplemented with methionine resulted in a growth rate of broilers comparable with those fed isolated soybean protein (Elmayergi and Smith 1971). The use of feather-lysate from *Bacillus licheniformis* with amino acid supplementation produced a similar growth rate in chickens when compared to chickens fed with a diet that included soybean meal (Williams et al. 1991). The crude keratinase enzyme produced by *B. licheniformis* significantly increased the total amino acid digestibility of raw feathers and commercial feather meal. This enzyme increased the digestibility of commercial feather meal and could replace as much as 7% of the dietary protein for growing chicks. Keratinolytic microorganisms and their enzymes may be used to enhance the digestibility of feather keratin.

33.4.3 Production of Fertilizers

Looking to the large amounts of feather waste generated by growing poultry industries scientists have also find application of processing this waste as organic fertilizer.

The poultry waste generated as poultry feed can also be applied for organic farming as a semi-slow-release nitrogen fertilizer (Hadas and Kautsky 1994; Choi and Nelson 1996). Feather contains some amounts of fat approximating to as much as 12% of its dry weight. Fat content of feathers have been reported to hinder its colonization by the microorganisms (Baxter and Trotter 1969; Pugh and Evans 1970; Deshmukh et al. 1981). Above reports also indicated the need of fat extraction from the poultry feathers to make the microbial processes more quick and economical. Narsimharao Koundamudi and his colleagues suggested a process of feather treatment for extraction of fat from chicken feather meal using boiling water and processing it to biodiesel which is estimated to generate approximately 593 million gallons of biodiesel worldwide, in addition to have a higher grade of animal feed and a better nitrogen source for fertilizer applications (Kondamudi et al. 2009).

33.5 Production of Keratinase and Other Proteases

Poultry waste particularly the feather waste is a significant source of keratinic substrate for the production of enzyme keratinase and similar proteases. With the advances in knowledge about the mechanism of keratinase production and their properties, a number of novel applications have also emerged (Jain 2000). Some of the novel applications of keratinases include their use in prion degradation, pharmaceutical and cosmetic preparations (Bradelli 2008). Some keratinases which do not hydrolyse gelatin and its synthetic substrate collagen are the preferred enzymes for dehairing of bovine pelts and attracted considerable interest of cosmetic industries (Gradisar et al. 2000; Riffel et al. 2003; Bradelli 2008). The keratinases with above properties could be incorporated in shaving creams (Neena 1993; Slavtcheft et al. 2004). Production of keratinases by bacteria has been worked out extensively probably to identify the most efficient organism for feather hydrolysis to obtain feather hydrolysate rich in proteins and amino acids. Keratinase production by bacteria using feather substrate and their production conditions are summarized in Table 33.1. Complete degradation of feathers have been achieved in 24 h using *Bacillus licheni-fomis* RG1 (Ramnani and Gupta 2004) in shake cultures and 48 h using *Fervido-bacterium pennavorans* (Friedrich and Antranikian 1996) and *Fervidobacterium islandicum* AW-1 (Nam et al. 2002) in static cultures. *Bacillus licheniformis* PWD1 showed optimum keratinase production with in 30 h but complete degradation could be achieved by the end of incubation period i.e., 10 days when poultry feathers were used as production substrate (Williams et al. 1990; Lin et al. 1992). The molecular diversity existing among the keratinase(s) of different origin has also attracted attention of many workers possibly to find their novel applications. keratinases from different organisms showed a great variation in their molecular weight and ranges from 18 to 200 kDa (Table 33.2). *Streptomyces albidoflavu*s SK1-02 produced keratinase having molecular weight 18 kDa and the highest molecular weight 240 was reported for Kocuria rosea (Bernal et al. 2003). The molecular weight of keratinase of *Fervidobacterium islandicum* was recorded 200 kDa (Nam et al. 2002).

Table 33.1 Conditions used for processing of feathers hydrolysis

S.No.	Microorganism	Substrate (feathers) used in the medium	Cultural parameters for feather hydrolysis			Reference
			pH	Temp (°C)	Shaking (rpm)	
1	*Bacillus licheniformis* PWD-1	Hammer-milled-feather	7.5	50	–	Williams et al. (1990)
2	*Serratia marcescens* SB08	Yeast extract 3.0 g/l.	6.0		100	Venil and Lakshmanaperumalsamy (2009)
3	*Bacillus licheniformis* RG1	Feather 0.5%	7.0	37	250	Ramnani and Gupta (2004)
4	*Kocuria rosea*	Finely milled feathers 30 g.		40	400	Bernal et al. (2006)
5	*Bacillus licheniformis* RPk.	Chicken feathers 7.5 g/l		37	200	Fakhfakh et al. (2009)
6	*Streptomyces gulbargensis*	3 g starch (per liter)	9.0	45	–	Syed et al. (2009)
7	*Aspergillus clavatus ES1*	Starch 5 g/l, yeast extract 2 g/l	8.0	30	–	Hajji et al. (2008)
8	*Chrysosporium georgiae*	Chicken feathers 1% glucose	6–8	30	–	el-Naghy et al. (1998)
9	*Cytophaga-Flavobacterium group strain kr8, kr9 and kr14*	Feather meal 10 g/l		30	–	Riffel and Brandelli (2006)
10	***Bacillus*** sp.	Feather meal 1% (w/v)	7.5	37	150	Pissuwan and Suntornsuk (2001)
11	*Bacillus subtilis*	Feathers 10 g/l	7.5	23	–	Cai et al. (2008b)
12	*Streptomyces* spp. *E4* and *E5*.	Feather 20.0 g/l,	8.0	30	120	Kansoh et al. (2009)
13	*Alternaria tenussima* K2	Chicken feather powder 1% (g/l)	7.5	35	120	Saber et al. (2010)
14	*Aspergillus nidulans* K7	Chicken feather powder 1% (g/l)	7.5	35	120	Saber et al. (2010)
15	*Pseudomonas aeruginosa* KS-1	Feather 0.5%		37	250	Sharma and Gupta (2010)
16	*Bacillus pumilus* KS12	Chicken feather 5.0 g/l	7.0	37	200	Rajput et al. (2010)

Bressollier et al. (1999) identified at least six extracellular proteases from *Streptomyces albidoflavus* cultured on feather based medium. The major keratinolytic enzymes have been found to be serine protease has a molecular weight of 18 kDa and were optimally active at pH values ranging from 6.0 to 9.5 and temperatures from 40°C to 50°C (Deshmukh and Kushwaha 2005). Jain et al. (2009) screened a number of

Table 33.2 Keratinase producing microbes and the characteristics of their keratinase

S.No.	Organisms	Mol. wt. (kDa)	Optimum pH	Optimum temp.	Properties	Reference
1	*Bacillus subtilis* S14	27	8.0–9.0	–	Metalloprotease	Macedo et al. (2008)
2	*Bacillus sp.*	32	8.0	–	–	Deivasigamani and Alagappan (2008)
3	*Bacillus sp. P-45*	26	8.0	55	Serine	Daroit et al. (2010)
4	*B. pumilus*	34	9.0	60	Serine Metallo	Fakhfakh-Zouari et al. (2010)
5	*B. subtilis* KS-1	25.4	–	–	–	Suh and Lee (2001)
6	*Scopulariopsis brevicaulis*	40–45 24–29	7.8 7.8	40 40	Serine	Malviya et al. (1992)
7	*Pichia pastosis*[a] *B. megaterium*[a]	39 30	–	–	Metalloprotease Metalloprotease	Radha and Gunasekaran (2009)
8	*B. licheniformis* PWD-1	33	7.5	50		Lin et al. (1992)
9	*B. licheniformis* PWD	31.4	8.5	45		Cheng et al. (1995)
10	*B. licheniformis* RPK	32	9.0	>60		Fakhfakh et al. (2009)
11	*B. licheniformis* MZK-3	–	9.0	40	Metalloprotease	Hossain et al. (2007)
12	*B. subtilis KD- N2*	30.5	8.5	55		Cai et al. (2008a)
13	*B. subtilis RM01*	20.1 In SSF	8.0	50	Serine	Rai et al. (2009)
14	*Fervidobacterium pennavorans* K02	130	10	80		Friedrich and Antranikian (1996)
15	*Kocuria rosea* LPB-3	240	10	40		Bernal et al. (2006)
16	*Crysobacterium* Kr6	64	8.5	50		Riffel et al. (2007)
17	*Streptomyces thermoviola-ceous* SD8	40	8	55		Chitte et al. (1999)
18	*Streptomyces sp.* 594	In SSF	5–10	55–80	Serinemettalo	De Azeredo et al. (2006)
19	*Lysobacter* NCIMB 9497	148	–	50	Metalloprotease	Allpress et al. (2002)

[a]Recombinant

actinomycetes for production of proteolytic enzymes and recorded *Streptomyces exfoliates* CFS 1068 and *Streptomyces sampsonii* GS1322 with 115.2 and 116.0 U/ml protease activity in culture filtrate when grown on nutrient broth containing gelatin as substrate. Latter, S. *exfoliates* CFS 1068 was found to degrade poultry feathers completely and produced 40.43 U/ml/min collagenase activity in its culture filtrate when feathers were used as substrate (Jain and Jain 2010). However, Collagenase activity is often found associated with keratinase activity in keratinolytic microorganisms. In Streptomycetes, the collagenase and keratinase activity was reported to be of inherent nature (Ivanko et al. 2002).

33.5.1 Application in Leather Industries

Leather industries are among the major pollution causing industries. The chemicals mainly responsible for pollution in pre-tanning process are lime, sodium sulphide, caustic soda, salts and degreasing chemicals (Taylor et al. 1987; Marsal et al. 1999; Sivasubramanian et al. 2008). About one third of the total pollutants generated by leather industries are resulting from the dehairing operations. Thus, enzymatic dehairing has been recognized as a reliable alternative to avoid the problems created by sulphide in tanneries in addition to improvement in leather qualities (Puvanakrishnan and Dhar 1986, 1988). Nagal et al. (2010) showed the production of an alkaline protease by a bacterium *Elizabethkingia meningoseptica* KB042 using waste chicken feathers. The enzyme was found to promote dehairing. The experimental pelts treated with enzyme were whiter and more uniform in texture than those treated with chemicals solution having 0.5% sodium sulphide and 1.5% lime (Fig. 33.2). The hairs recovered from enzyme treated skin were intact as compared to chemical treated skins (Fig. 33.3). Biocatalytic leather processing involves the use of a mixture of enzymes, among which proteases, lipases and carbohydrases are well exploited by the leather processing industries (Saravanabhavan et al. 2004; Thanikaivelan et al. 2004). Proteases possessing keratinolytic activity with mild elstolytic activity are preferred by tanning industries. Importance of Keratinases as depilating agents has also been reported (Letourneau et al. 1998; Bressollier et al. 1999; Allpress et al. 2002; Friedrich and kern 2003). A keratinase from *Bacillus subtilis* S14 was also reported to replace the conventional chemical treatment process completely.

33.5.2 Biotransformation of Poultry Waste into Proteins and Amino Acids

Keratinase producing microorganisms have the important industrial application in fermentation technology. Submerged fermentation of poultry waste by microorganism producing keratinase helps in the conversion of non-soluble keratin (feather) into

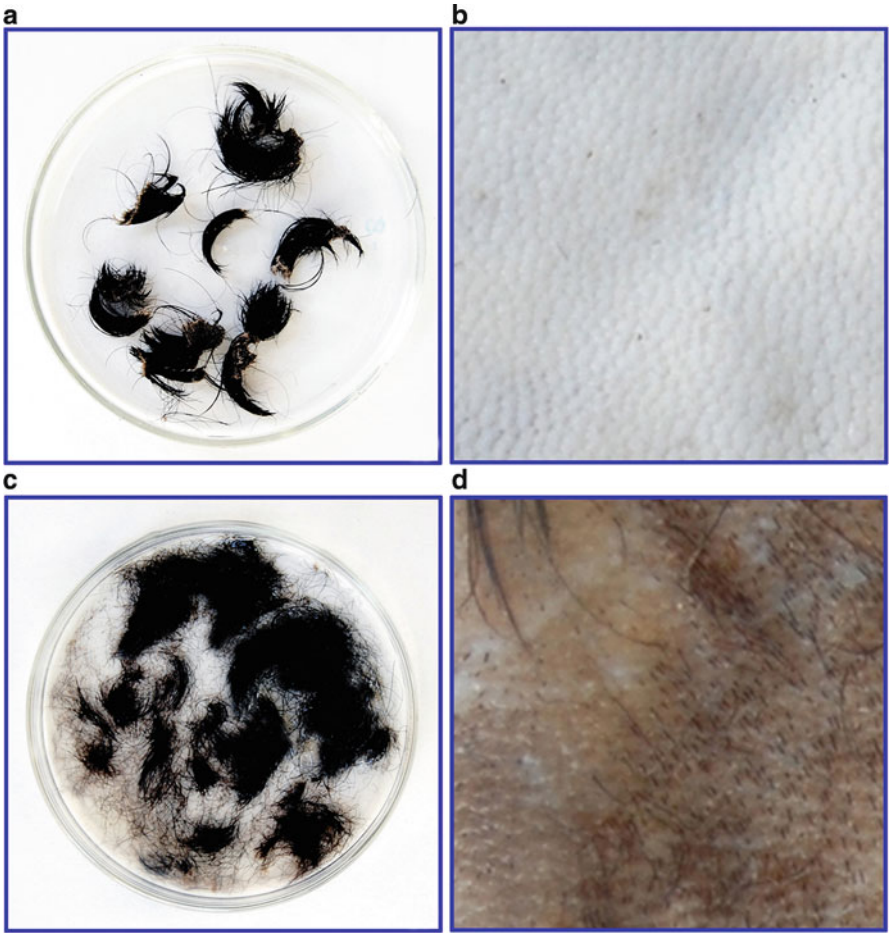


Fig. 33.2 Quality of hairs and pelts obtained after dehairing of goat skin: (**a**) and (**b**) Using alkaline protease from *E. meningoseptica* KB042; (**c**) and (**d**) with 0.5% sodium sulphide and 1.5% lime

soluble protein or polypeptide (Sangali and Brandelli 2000; Shih 1993; Suntornsuk and Suntornsuk 2003). Worldwide, more than 10,000 t of keratin is generated as a waste by-product at poultry processing plants annually. Two types of keratins, α-keratins and β-keratins, consist of tightly packed protein chains in α-helices and β-sheets, respectively. Furthermore, keratin filament structures are stabilized by their high degree of cross-linking of disulfide bonds, hydrophobic interactions, and hydrogen bonds. Due to their extremely rigid structures, keratins are insoluble and hard to degrade. On the other hand, since poultry feathers contain a great deal of potentially useful proteins and amino acids and keratins to some extent has been used in cheap animal feedstuffs as

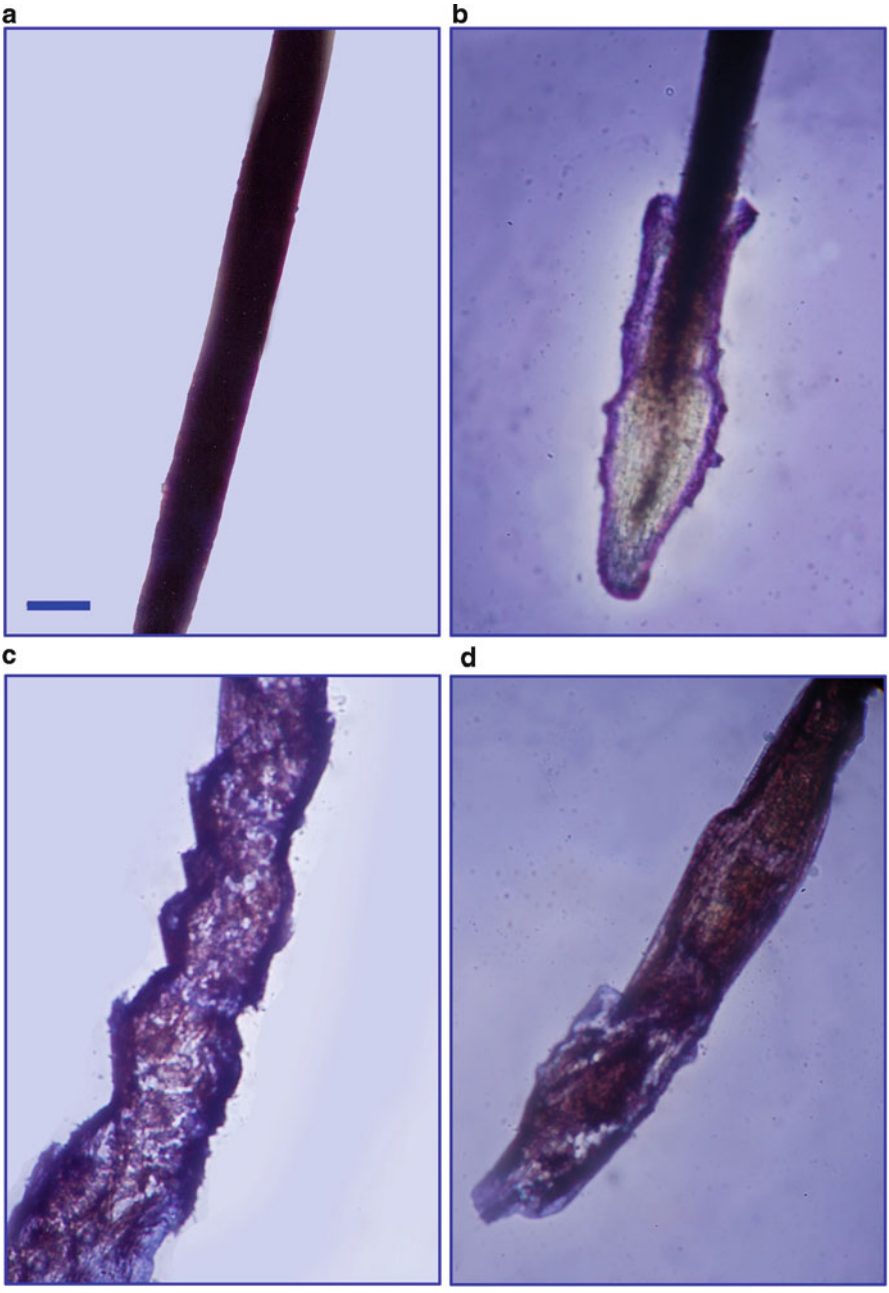


Fig. 33.3 Microscopic structure of hair obtained from goat skin at 100× magnification: (**a**) and (**b**) Enzymatic dehairing, (**c**) and (**d**) chemical dehairing

feather meal. However, the conversion of feathers to feather meal results in the loss of nutritionally essential amino acids, such as methionine, lysine, and tryptophane, and the formation of non-nutritive amino acids, such as lysinoalanine and lanthionine (Papadopoulos 1989; Latshaw 1990; Wang and Parsons 1997). This is because the process in conversion of feathers to feather meal includes severe treatments under high temperature and pressure. Therefore, an alternative method of efficiently degrading keratins without this conventional defect has been sought (Onifade et al. 1998; Graazziotin et al. 2006). Although a limited number of potentially useful microorganisms have been found, such microorganisms must have the capacity to utilize keratin for growth, and specific and strong keratinases must be able to degrade the keratin. For more practical use bearing commercial benefits, the development of a durable and fast keratin-degrading system using suitable microorganisms is essential.

Feather hydrolysis can be achieved by cultivating keratinolytic microorganisms on feathers. The production of extracellular keratinases by the growing microorganism may result in the production of feather hydrolysate rich in proteins and amino acids. Reports made by many workers (Nam et al. 2002; Dalve 1994; Deivasigamani and Alagappan 2008; Jeong et al. 2010a, b; Nagal and Jain 2010a, b) have shown a considerable amount of proteins and amino acids in the feather hydrolysate as a consequence of keratinolytic activity of microorganisms. Nagal and Jain (2010b) reported Elizabethkingia meningoseptica KB042 as a better candidate of interest to base process for the production of feather hydrolysate using waste feathers (Fig. 33.4) rich in proteins and amino acids.

The use of keratinolytic bacteria and fungi for the production of feather hydrolysate has been the subject of some patented processes (Shih and Williams 1990; Burtt and Ichida 1999b, c). The strategies adopted and production of proteins and amino acids using certain microorganisms is summarised in Table 33.3. Jeong and his colleagues showed the production of 19 amino acids including all the essential amino acids in the feather hydrolysate using *Bacillus subtilis* S8 (Jeong et al. 2010a). The bacterium completely degraded intact feathers resulted in the production of free –SH group, soluble proteins and aminoacids. The concentration of total amino acid produced was 3360.4 μM. Proline, histidine and phenylalanine were the major amino acids released in the culture medium. In addition to these the strain also showed the properties related to plant growth promotion i.e., production of hydrolytic enzymes, indole acetic acid, and phosphate solubilization and broad spectrum antibiotic activity. Thus the strain was found suitable not only to produce proteins and amino acids as nutrient but can also be implicated in-situ biodegradation of feather waste. *Xanthomonas* sp. P5 (Jeong et al. 2010b) and *Fervidobacterium islandicum* (Nam et al. 2002) have also been worked out for production of free amino acids using feathers as substrate.

33.5.3 Prion Degradation

Prions are the proteins in a misfolded form, like β-sheet structure of keratin thus it is hypothesized that keratinases would also be istrumenta in degradation of prions

Fig. 33.4 Degradation of hen feathers by *E. meningoseptica* KB042: (**a**) Control, (**b**) Feather hydrolysate obtained after 6 days in flask inoculated with *E. meningoseptica* KB042

and other β-sheet structured proteins such as β-amyloids. The infective prions (PrP^{Sc}) also has high proportion of β-sheet structures and thus resistant to digestion by most proteases (Caughey et al. 1991; Pan et al. 1993). They cause a variety of diseases in man and animals (Table 33.4) and that the transmissible prions are believed to be the cause of progressive neurological diseases leading to brain damage and death (Belay 1999; Hussen and Al-Mufarrej 2004). All prion diseases collectively called transmissible spongiform encephalopathies (TSEs) and it is believed that feed made from scrapie infected animals becomes contaminated with prions ($OvPrP^{Sc}$) and the scrapie prion converted to Bovine spongiform encephalopathy (BSE) on feeding upon contaminated feed. Similarly, BSE is converted to Creutzfeldt-Jacob disease (CJD) in humans. The report made by Lin et al. (1997) that PWD-1 keratinase is capable of decomposing pathogenic form of prion protein, triggered interest in application of proteolytic activity of bacterial proteases to the treatment and degradation of pathogenic PrP^{Sc} and its isoforms. Recent studies have shown that keratinase produced by Bacillus licheniformis PWD-1 can effectively

Table 33.3 Release of amino acid and proteins from feathers using microorganisms

S. No.	Organism	Feather % in medium	Free amino acids	Soluble protein	Amino acid composition in feather hydrolysate	Reference
1.	*Streptomyces fradiae* Var S-221	(g/100 ml): feather powder, 19.504	6.13 g/100 ml			Cheng et al. (2010)
2.	*Meiothermus ruber* H328	3% (*w/v*) intact chicken feathers	1.89 mmol	55% of total keratin proteins		Matsui et al. (2009)
3.	*Fervidobacterium islandicum*				Histidine, Cysteine, Lysine Tryptophan Methionine	Nam et al. (2002)
4.	*Xanthomonas* sp. P5	0.1% (w/v) feather	188.6 μM		Asparagine methionine Histidine Threonine	Jeong et al. (2010a)
5.	*Bacillus subtilis*	0.1% (w/v) feather	3360.4 μM		19 amino acids including all essential amino acids: proline (2809.9 μM), histidine (371.3 μM) phenylalanine (172.0 μM)	Jeong et al. (2010b)
6.	Bacilus sp	Feather 1%		1.44 mg/ml		Deivasigamani and Alagappan (2008)
7.	*Vibrio* sp	60 g/l raw feathers		2.5 g/l		Grazziotin et al. (2007)
8.	*Bacillus cereus* KB043	1% hen feathers		1206.15 ± 14.7 μg/ml		Nagal and Jain (2010a)
9.	*B. licheniformis 511* *B subtilis 1-1* *B subtilis 717* *B subtilis 103*	10 g/l raw feathers		1.17 mg/ml 1.17 mg/ml 1.17 mg/ml 1.17 mg/ml		Matikeviciene et al. (2009)

Table 33.4 Prion related diseases of man and animals

Host	Diseases	Prion name	PrP isoform
Cat	Feline spongiform encephalopathy (FSE)	FSE Prion	$FePrP^{Sc}$
Cattle	Bovine spongiform encephalopathy (BSE), mad cow disease	BSE Prion	$BovPrP^{Sc}$
Sheep and goat	Scraple	Scrapie Prion	$OvPrP^{Sc}$
White-tailed deer, Elk, Mule deer, Moose	Chronic wasting disease (CWD)	CWD Prion	$MDePrP^{Sc}$
Mink	Transmissible mink encephalopathy (TME)	TME Prion	$MkPrP^{Sc}$
Nyala, Oryx, Greater Kudu	Exotic ungulate encephalopathy (EUE)	EUE Prion	$NyaPrP^{Sc}$
Humans	Creutzfeldt–Jakob disease	CJD Prion	$HuPrP^{Sc}$
	Iatrogenic Creutzfeldt-Jakob disease	iCJD Prion	
	Variant Creutzfeldt-Jakob disease	vCJD Prion	
	Familial Creutzfeldt-Jakob disease	fCJD Prion	
	Sporadic Creutzfeldt-Jakob disease	sCJD Prion	
	Gerstmann–Sträussler–Scheinker syndrome (GSS)	GSS Prion	
	Fatal familial insomnia (FFI)	FFI Prion	
	Kuru	Kuru Prion	

digest prion proteins in infected cow or sheep brain stem (Langeveld et al. 2003) but require the use of the denaturing pretreatment or other conditions for decontamination of PrP^{Sc} in animal feed (Langeveld et al. 2003). The enactivation of PrP^{Sc} is important to prevent propagation of the prion diseases (Taylor 1991, 2004; Taylor et al. 1995). The attempts are on the way to find suitable enzyme for the decontamination of animal meal to minimize the risk of TSE (Leo et al. 2004). *Bacillus* sp. MSK 103 produces a keratinase that possessed activity for degrading PrP^{Sc} (Yoshioka et al. 2007) while Suzuki et al. (2006) and Bradelli (2008) reported useful enzymes suitable for degradation of resistant animal proteins including prions. A recombinant enzyme, PURE100 keratinase, commercial enzyme might find application in the decontamination of precision instruments that are susceptible to prion contamination (proteosebiotech.com/shop/keratinase/pure100keratinase-21). Recently, Sharma and Gupta (2010) have also reported a keratinase from *Pseudomonas aeroginosus* KS-1 as a prospective decontaminating agent possessing thermostability at 50°C.

33.5.4 Medical and Pharmaceutical Application

For application of enzymes in Medical and Cosmetic industries the enzymes are required in almost pure form. Protease Biotech's keratinase proceed from *B. lichiniformis* (PWD) and produced in *E. coli* has been reported to possess high affinity to

keratin. It is produced under recombinant conditions and purified to a level 99% by chromatography and hence pure 100 keratinase is suitable for its application in biomedicine, pharmacy and cosmetics. This enzyme can be used for treatment of acne, psoriasis, human callus and degradation of skin, depilation and treatment of scars and epithelium regeneration. The enzyme is also reported as good for preparation of vaccine for dermatophytosis therapy (http://www.proteosebiotech.com/shop/keratinase).

33.6 Future Perspectives

Decomposition methods of keratinic waste like incineration or chemical treatments (Onifade et al. 1998) are rather expensive or environment-polluting. In contrast, Present day biotechnology offered an environmentally sound two stage fermentation system for conversion of keratinic waste into a useful product, biohydrogen (Bálint et al. 2005). A keratin-degrading *Bacillus* strain (Perei et al. 2000) was used to obtain fermentation product which was rich in amino acids and peptides and subsequently used as major nutrient source for an anaerobic hyperthermophilic archaeon, *Thermococcus litoralis*, which produced hydrogen gas as a physiological byproduct. Besides *T. litoralis, E. coli* and *Caldicellusiruptor saccharolyticus* capable of producing hydrogen were also examined but neither of them could utilize the keratin hydrolysate for biohydrogen production (Bálint 2006). The application of keratinase as a detergent additive has also been suggested (Gupta and Ramnani 2006). Another field of interest is the application of keratinolytic enzymes in woolen textile industries for shrink proofing and to improve wool dyeing (Sousa et al. 2007). The scientists are also of opinion that the liquid nutrient residues from feather composting could be used for aquaculture and hydroponic crops (Ichida et al. 2001). A new process for extracting fat from chicken feather meal using boiling water and processing it in to biodiesel has been described. The removal of fat from feather meal results in both high grade animal feed and better nitrogen source for fertilizer application (Kondamudi et al. 2009).

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Chapter 34
Cyanoremediation: A Green Clean Technology

Ragini Gothalwal and Srikanth Chillara

Abstract Industrialized societies and those with mechanized agricultural production are now be set by a number of pollution problems that were largely unknown prior to world war II. The properties that make cyanobacteria generally undesirable are also the qualifications for possible positive economic use. These tinny plants are distributed all over the world and found is all types of habitats. Cyanobacteria will be strong contenders in the future for integrated system level analysis because of their potential in a wide variety of process and product fields, ranging from non-green house energy (H_2), through to pollution abatement and therapeutics.

The main cyanobacterial strain viz sps. of *Anabena, Nostoc, Phormidium, Aphanocapsa, Oscillatoria, Lgngbya, Spirulina, Aulosira, Anasystis* appears to be promiring bioremediators for the effluent rich in nitrates and phosphates. Cyanobacteria metabolize organic compounds through ring hydroxylation. The cyanobacteria produce siderophores which may be responsible for absorption of metal ions into organism. The metal accumulating algal biomass can either be disposed off or incinerated for metal recovery. It was also studied that cyanobacteria accelerate transformation and degradation of certain polycyclic aromatic hydrocarbons, organo phosphorous, in water by the sun light. Thus algal detoxification, and degradation of environmental pollutants could help in controlling the pollution of the aquatic and terrestrial habitats.

"Green clean technology" has been clearly identified as the answer to some of the problems related to the industrial pollution of aquatic and soil environment. Alongside this, biomonitoring of environment may be followed by targeted bioremediation. Clean processes are designed to lead to the discharge of cleaner aqueous effluents.

Keywords Cyanobacteria • Remediation • Bioremediation • Detoxification • Biomonitoring • Pollution

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34.1 Introduction

Rapid industrialization and quest for more comforts have led to overexploitation of the nature, polluting the niche we dwell in and contaminating the air we breathe. Environmental management aspect was ignored in order to achieve the target of industrial development and large number of hazardous chemicals and substances have found their way into aquatic ecosystem.

The industrial effluents contain several types of chemicals such as dispersants, livening agents, acids, alkalies, carriers and various dyes, phenol, carbonate, alcohol, cyanide, heavy metals etc. (Cooper 1995). Leather industries and tanneries generate massive byproducts, solid waste, high amount of waste water rich in organic wastes with different loads of pollutants and, emissions into air.

Treating water bodies as sinks for wastes and indiscriminate use of water in industrial and agricultural activities has affected the quality and quantity of drinking water. Conventional treatment methods not only requires relatively large amount of space and produce considerable bulky sludge. The problem is safe disposal of such materials. With the advances in biotechnology, bioremediation has become one of the major fields of environmental restoration, utilizing microorganisms to reduce the concentration and toxicity of various chemical pollution such as heavy metals, dyes, pesticides etc. Due to the inadequacy and increased cost of physical and chemical methods, need for friendly and cheaper methods have been strongly realized.

Biological treatment especially using algae or cyanobacteria for treatment of water in aquatic environment which is known to accumulate high levels of metal/pollutants, therefore, has been sought as relatively cheap and environmental friendly methodology. Such a technology can be integrated with other processes eg. aquaculture, pisiculture, feed production, soil conditioner production, fine chemical production and recovery of metals.

Selective uptake, accumulation and biodegradation of the pollutants by algae like *Chlorella*, C*halymydomonas*, *Scenedesmus* and several marine algae has gained importance in remedying the situation. Many microalgae (Cyanobacteria) like *Microcystis, Oscillatoria, Lyngbya*, and Diatom act as bioindicators of degree and type of pollution (Gunale 1991). Algae-mediated harvesting of nutrients like phosphorus and nitrate from the polluted water has become an important part of the integrated recycling system.

Cyanobacterial strains viz. species of *Anabaena, Nostoc, Phormidium, Aphanocapsa*, *Oscillatoria, Lyngbya, Spirulina, Aulosira,* and *Anacystis*, appear as promising bioremediators for the effluents rich in nitrates and phosphates, though there are reports on the use of cyanobacteria for the treatment of sewage and simple effluent but its application in complex, industrial effluents is comparatively small. Cyanobacteria metabolize aromatic compounds through ring hydroxylation, O-methylation, side-chain hydroxylation, alkylation, and formylation (Mc Eldowney et al. 1993; Narro 1985). It is demonstrated that *Anabaena fertillisma* and *Nostoc* sp. are able to degrade, detoxify and use pesticides as sole source of phosphorus through the production of phosphate solubilising enzyme (Subramanian et al. 1994)

Cyanobacteria produce siderophores which may be responsible for absorption of metal ions into organisms. The metal accumulated algal biomass can either be disposed off or incinerated for metal recovery. It has also been reported that blue green algae accelerate transformation and degradation of certain polyaromatic hydrocarbons, organophosphurous compounds in water by sunlight. Thus algal detoxification of environmental pollutants could help in controlling the pollution of the aquatic and terrestrial habitats.

The technique of bioremediation is not only useful and economic but also practicable (Wang et al. 1998). In this context polluted water treated with cyanophycean forms offers advantages as under:

1. Some filamentous forms i.e., *Spirulina, Oscillatoria, Phormidium, Anabaena* and *Nostoc* have the capacity of bloom formation and make its harvesting easier and less costly than other microalgae such as *Chlorella, Ankistrodesmus* and *Scenedesmus.*
2. They absorb sufficient amount of heavy metal from the contaminated environment.
3. By the use of genetic engineering it is feasible to manipulate the potential of the organism to tolerate, accumulate and metabolize pollutant, and thus to create an ideal cyanobacterial form for environmental cleanup.

"Green clean technology" has been closely identified as the answer to some of the problems related to the industrial pollution of aquatic and soil environment. Alongside this, bio monitoring of environment may be followed by targeted bioremediation. Clean processes are designed to lead to discharge of cleaner aqueous effluents.

34.2 Cyanobacterial Diversity in Varied Niches

The first use of cyanobacteria by humans dates back 2,000 year to the Chinese who used *Nostoc, Spirulina* and *Aphanizomenon* sp. to survive during famine. Cyanobacteria are special creation of nature for the welfare of mankind. They are most simple, photosynthetic, O_2 evolving, non-vascular plants having characteristics of both microbes and higher plants. It is important to transform the cyanobacteria genetically in a stable facile predictable manner in order to explore the full biotechnological potential of these organisms to mankind in various ways. Cyanobacteria constitute a vast potential resource in varied areas such as mariculture, food, feed, fuel, fertilizer, medicine, industry, combating pollution and value added products of commercial importance. (Gustafson et al. 1989; Carmichael 1994; Bender et al. 1994a, b; Sundaraman et al. 1996; Kulik 1995; Subramanian and Uma 1996; Jaki et al. 2001).

Filamentous cyanobacteria including N_2 fixing strains that combine aerobic metabolism in their vegetative cells with anaerobic metabolism in heterocyst (Wolk et al. 1995) are widespread in many ecosystems including polluted ones (Fogg 1987; Gibson and Smith 1982; Sorkhoh et al. 1992). The viability and metabolic activity of these cyanobacteria unlike those of heterotrophs, is not subject to reduction by the decrease in the concentration of pollutants that they may be broken down.

The ability of algae to survive through earlier geological period was the consequence of widespread compatibility and adaptability to the extreme of temperature, desiccation, illumination, radiation, salinity, pH, toxicants and nutrient availability. These attribute have been retained through the evolutionary sequence and have conferred cosmopolitan nature on algae especially cyanobacteria. The continuum has helped them acquire attributes which have far reaching implications in maintaining ecological balance making the agriculture sustainably organic and industrial production of variety of value added products.

Biodiversity of cynaobacteria can be considered as an indicator of primary producer population under stressed conditions. Also, cyanobacteria have gained prime importance in recent biotech advances due to their high potential. The group algae comprises of both prokaryote and eukaryote thallophytes commonly found in aquatic habitat with quite a few abounding sub-aerial and aerial niche. They are found growing in hot water springs at temperatures as high as 85°C as well as on snow. They are able to survive in various extreme environment via the production of organic compounds known as compatible solutes. In particular, cyanobacteria are capable of inhabiting hyper saline environments such as those found in intertidal regions (Goh et al. 2010). Their thallus organization ranges from a motile unicell to most complicated polyoxial type. The algae were treated as laboratory curiosities presenting beautiful pattern as agents disfiguring surfaces by slimy growth and causing nuisance in water supply plants. They have now become endorsed as sentinels of environment, protecting the aquatic and terrestrial habitats. They are now well documented and the performance at the base of the tropic pyramid is well recognized. The less investigated beneficial effects of cyanobacteria include curbing of ammonia volatilization, suppressing weeds, reducing methane emission, transformation of P, Fe, Mn, Tn, Cu, pesticide degradation and reclamation of waste lands/degraded soil (Mandal et al. 1998; Kuritz and Wolk 1995; Prasanna et al. 2002).

34.3 Metal Accumulation and Detoxification by Cyanobacteria

Heavy metals are considered to be major environmental pollutant and are regarded to be cytotoxic, mutagenic and carcinogenic, prominent source contributing to contamination of soil are geogenic and anthropogenic, which includes, mining, smelting, disposal of municipal and industrial wastes, pesticides and automobile exhaust. Heavy metal ions are of two types: essential and non essential. The essential metal ions are required at trace level; above certain concentration, the status of these heavy metals changes from an essential growth promoting element to inhibitor/toxin. The non-essential metal ions exhibit toxicity even at relatively low levels.

Microbes can detoxify metals by valence transformation, extracellular chemical precipitation or volatization. Such microbes combat high concentration of heavy metals by inactivation of metals, alternation of the site of inhibition, impermeability of the metals and, other bypass mechanism (Bellivean and Treyors 1989).

Cyanobacteria can use N_2 and phosphorous in the salt form, during treatment of polluted water, thereby decreasing their level below accepted standard (Garbisu and Hall 1993; Hashimoto and Furukawa 1989). Heavy metals have atomic number greater than iron (atomic no.26) or having density more than 5 g/cm^3. The important heavy metals which are responsible for pollution are Cu, Zn, Cd, Hg, Ni, Ag, Cr and Pb. Cyanobacteria are able to tolerate high concentration of toxic metals (De Filippis and Pallaghy 1994). The cyanobacteria can be used in bioremediation process for making metal-free environment. Metal tolerance in cyanobacteria may be genetical or physiological. The efficiency of bioremediation depends on the selection of cyanobacteria (Gamila and Naglaa 1999; Nakanishi et al. 2004). For effective bioremediation process it is essential to know the protein/enzyme and gene involved in this process.

Cyanobacteria can remediate heavy metals by a variety of mechanisms including bioaccumulation, biosorption and bioreduction. Cyanobacterial cells have developed natural methods of responding to metals such as Cu, Pb and Cd through passive accumulation in cells and through surface binding to various functional groups. *Spirulina platensis* was shown to contain detectable levels of Hg and Pb when grown under contaminated conditions (Slotton et al. 1989), implying that this cyanobacterium was taking up the toxic metal ion from its environment both by adsorption and takes up metal ion (Bender et al. 1994a, b). Reports also indicate that carboxyl groups on algal cell biomass are responsible for binding to various ions (Gardea-Torresdey et al. 1990). Live algae posses intracellular polyphosphates which participate in metal sequestrations, as well as a algal extracellular polysaccharide that serves to chelate or bind metal ions (Zhang and Majidi 1994; Kaplan et al. 1987). Strains of *Synechocystis,* spp. have been shown to develop a thickened calyx when exposed to Cu stressed growth conditions (Gardea-Torresdey et al. 1996) *Synchococcus* sp. PCC 7942 was found to possess a Cu transporting p-type ATPase in the thylakoid membrane (Bonilla et al. 1995). *Synchococcus cedrorum* 1991 was shown to be tolerant to heavy metal and pesticides (Gothalwal and Bisen 1993). Several other investigators have reported on biosorption of heavy metals by algae biomass (Volesky and Helan 1995; Volesky and Schiewer 1997). Such findings show the probability of manipulating or overexpressing the existing resistance mechanism and the use of such organism to remove heavy metal from the environment. Green algae are capable of synthesising on exposure to heavy metals, thiol rich peptide called phytochelatin, where cyanobacteria are able to bind with metallothioneins, which helps sequester and buffer cell intracellular zinc.

Average yearly emission of Ni in the atmosphere was $55,650 \times 10^3$ kg; 113×10^6 kg was located in aquatic ecosystem and 4.16×10^6 kg in soil (Nriagu and Pacyna 1988). Ni has been an essential micronutrient for many microorganisms as it plays important role in microbial enzyme activity (Walsh 1995). Algae play an important role in monitoring the equilibrium of aquatic ecosystem (Campanella et al. 2000). They are often used in phytotoxicity test for environmental monitoring (Boswell et al. 2002). The cyanobacteria are able to bind large amount of metal due to the presence of mucilaginous sheath (Wang et al. 1998; Tien 2002). Mario Garcia et al. (2000a, b) reported a gene cluster composed of nine ORFs involved in Ni^{2+}, CO^{2+} and Zn^{2+} sensing and tolerance in the cyanobacterium *Synechosytis* sp. strain PCC6803 (Fig. 34.1).

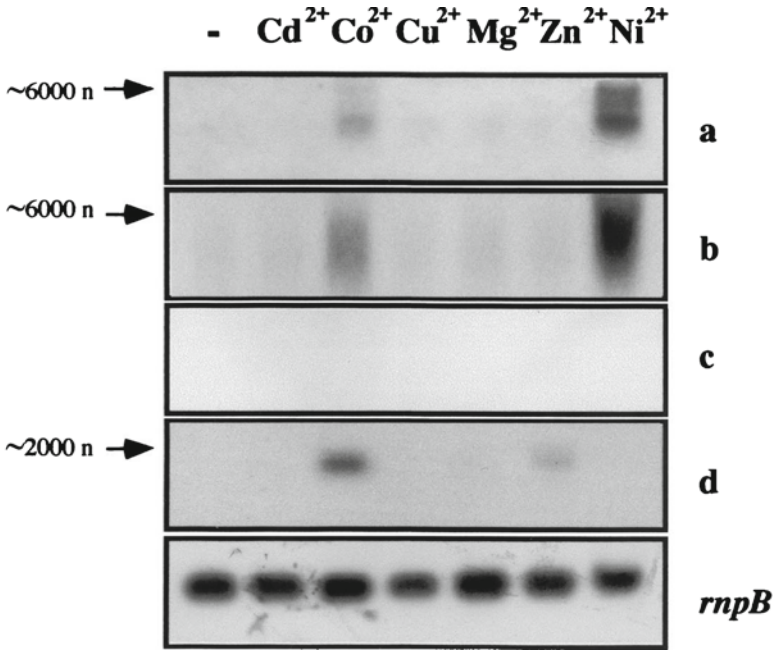

Fig. 34.1 Metal-dependent expression of the *Synechocystis* transition metal-resistant cluster. Total RNA was isolated from mid-log-phase *Synechocystis* sp. strain PCC 6803 cells exposed for 1 h to a 15 mM concentration of the indicatedmetal ions. Control cells were not exposed to added metals (2). Fifteen micrograms of total RNA was denatured, separated by electrophoresis in a 1.2% agarose gel, blotted, and hybridized with probes (**a**–**d**) as the filters were stripped and rehybridized with an *rnpB* gene probeas a control. Estimated sizes of the transcripts (in nucleotides [n]) are indicated (Mario Garcia et al. 2000a, b)

The cluster includes Ni^{2+} response operon and a CO^{2+} response system, as well as Zn^{2+} response system. Previously described expression of Ni^{2+} response operon (nm) was induced in the presence of Ni^{2+} and Co^{2+}. Reduced Ni^{2+} tolerance was observed following disruption of two ORFs of the operon nmA and nmD. nm D gene encodes a putative Ni^{2+} permease whose carboxy terminal region is a metal binding domain. Cor R in the CO^{2+} sensing transcription factor has been described. This region of S*ynechocsystis* sp. strain PCC 6803 genome is involved in sensing and homeostasis of Ni^{2+}, CO^{2+} and Zn^{2+}. How and why 09 gene related functions have been clustered in a region of *Synechocystis* genome is interesting question that remain to be addressed.

Cr (VI) is soluble, toxic and carcinogenic, whereas Cr (III) is less soluble and less toxic (Imai and Crkoyna 1990). Thus, it is desirable to change Cr (VI) into Cr (III); chromate reducing activities can be found in the cell extract of many bacteria, (Campose et al. 1995; Deo Leo and Ehrlick 1994; Gopalan and Veeramani 1994; Llorera et al. 1993). Some cyanobacterial species are known to reduce toxic Cr (VI) into less toxic Cr (III) (Meenakshi et al. 2004).

Metallic mercury in part due to its low solubility is relatively non toxic, compared to oxidized form of Hg. Ionic, partially covalent inorganic salts and many of the organic alkyl and amyl derivative of Hg are highly toxic (Zalups and Lash 1996; Karunasagar et al. 2003). Some organomercurial compounds are lipid soluble and all mercury compound bind to lipid and nucleotide to various degree. In addition, mercury compounds are genotoxic (Akiyama et al. 2001). Both *Aphanothece flocculosa* and *Spirulina platensis* are excellent biosorbents for uptake of mercuric ion. At room temperature and initial Hg^{2+} concentration of Ca-1,000 ppm at pH6, *A. flocculosa* and *S. platensis* had maximum uptake of 456 and 428 mg Hg^{2+}/g dry biomass, respectively. *A. flocculosa* has the ability to regenerate, more than 90% of Hg using ammonium chloride as a regenerant. Regenerant using HCl for *S. plantensis* 100% Hg recovery is attained after one cycle. The presence of dissolved CO^{2+}, Ni^{2+} and Fe^{3+} was found to play a synergistic role in Hg^{2+} uptake by both the strains.

34.4 Metallothioneins and Other Protective Mechanisms

An important protective mechanism used by cells in response to a variety of stress is the expression of heat shock genes. These proteins are present in highly conserved forms in all organisms studied including bacteria, plant and animals.

Metallothioneins are low molecular weight proteins or polypeptides (6,000–8,000 Da) which bind metal ions in metal thiolate clusters. These polypeptides are abundant in cysteine residues (cys) and often possess a characteristic pattern of 'S' containing amino acids (Turner and Robinson 1995). They are commonly found in association with essential metal ions like zinc and copper, but also shown binding capacity towards toxic metals like cadmium, mercury, and lead. These metal binding proteins are mediated via the abundant cysteine residues and this characteristic organisation into – cys-cys-, cys-x-cys-, or cys-x-x-cys- sequences (X corresponds to any amino acids in the protein sequence). Metallothioneins can be characterized into three specific classes:

Class-I – containing most animal metallothioneins.
Class-II – consists of metallothioneins where cysteine location in the polypeptide is distinctly related to those found in archetypal equine renal metallothionein.
Class-III – metallothioneins are characterized by a typical non-translationally synthesised metal, thiolate polypeptides.

Cyanobactrial metallothioneins belong to Class-II. They possess approximately 56 aminoacids including greater cysteine residues. This number of cysteine residues less than 20 cysteine residue out of 75 amino acids for the plant class-I MTs (Silver and Ji 1994).

The metallothionein genes are arranged as an opreon called Smt locus containing both smt A (MT protein) and smt B (repressor regulating protein) gene. smtB is a transacting repressor of expression of smtA operator/promoter region. Metallothionein expression from gene to the functional protein is induced by the

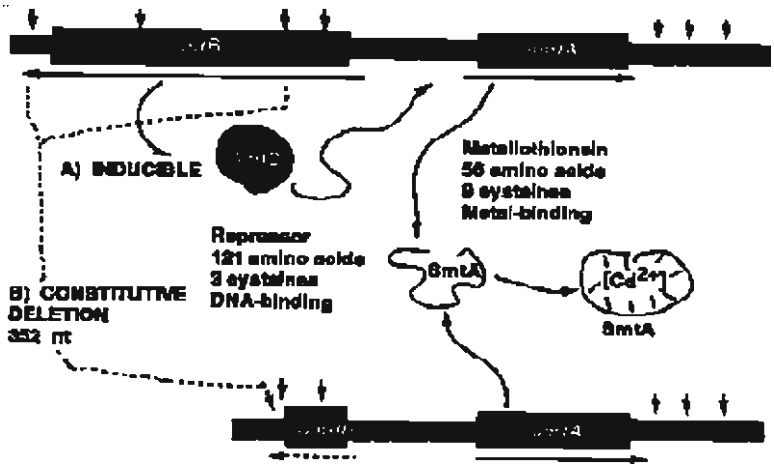

Fig. 34.2 The metallothionein genetic determinant of Synechococcus. (*A*) Wild-type, repressible system with divergentlytranscribed smtA (metallothionein) and smtB (regulatory) genes. Arrows at top, locations of seven HIPM (highly iteratedpalindromic sequences 5′GCGATCGC3′). The repressor protein SmtB and bacterial metallothionein SmtA (hypothesizedas gaining a more rigid structure with bound Cd2'). (*B*) Genetic determinant (constitutive metallothionein synthesis) afterdeletion of 352 nucleotides from the first to the third HIPM, from the *left* as shown (Silver and Ji 1994)

metal ions and the regulation of transcription to mRNA is dependent upon the interaction between these metal ions and the reprossor protein regulatory transcription, via interaction with thiol group present on the repressor protein and all of the smtB repressor gene (Fig. 34.2). Subsequent unregulated transcription of smtA may be advantageous to organism constantly stressed with high level of cadmium, copper, arsenic, and lead. Cells devoid of functional repressor (smtB) show elevated concentration of smtA mRNA transcripts even in the absence of inducer.

At present vast majority of the available experimental data relates to cyanobacterial metallothionein, smtA from Synchococcus PCC 7942 (Fig. 34.3); smtA is required for normal reaction to zinc and smtA mediated zinc resistance has been used as a selectable marker (Morby et al. 1993; Silver and Phung 1996). The imidazole group of histidine residue in addition to thiol groups of CYS residues co-ordinates zinc in bacterial metallothionein. The structure of bacterial metallothionein facilitates some discrimination between the "adventitious" and "advantageous" zinc binding sites such that under excess zinc conditions, metal is predominantly scavenged from the former. Expression of smtA is induced in response to elevated concentration of Zn^{2+} via the action of smt B; smt B has some sequence similarity to the arsenic responsive repressor, Ars R^2 gene encoding related protein present in several bacterial genomes. Metal perception by smt B differ from Ars . Some smtB Ars R family proteins including the zinc sensor Zia R from the cyanobacteria *Synechococcus* PCC 6803, have the metal sensory motifs of both smt B and Ars R (Robinson et al. 1990).

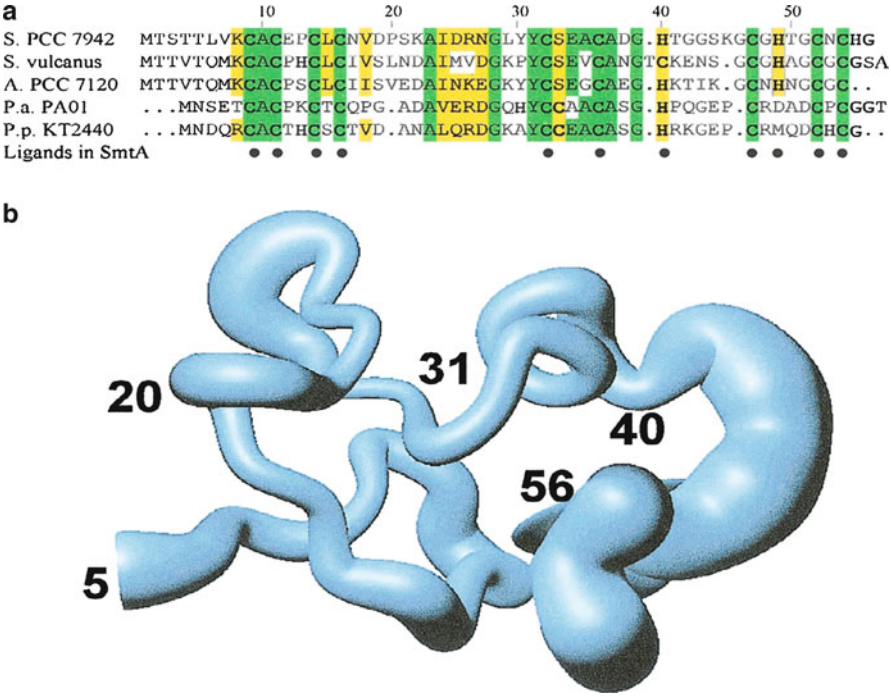

Fig. 34.3 Sequence and backbone structure of SmtA. (**a**) Sequence alignment ofSmtA homologues from various strains of *Synechococcus* (S.), *Anabaena* (A.),*Pseudomonas aeruginosa* (P.a.), and *P. putida* (P.p.). Green, conserved residue; yellow, semiconserved residue. The sequences for the Pseudomonads are truncatedat the C terminus. (**b**) "Sausage" representation of a superposition of the backbone traces (N, Ca, C) of the 20 best NMR-derived structures of holo-SmtA without specific incorporation of Zn21. The *cylindrical rod* represents a trace through the Ca atoms of the protein. Its radius is proportional to the mean global backbone displacement per residue. The rmsd about the mean coordinates for residues 5–56 is 1.36 6 0.35 Å for a best fit of the backbone atoms. (Blindauer et al. 2001)

Gardea-Torresdey et al. (1990) performed experiments to investigate the ability to recover bound metals. *Synchococcus* sp. PCC 7942 biomass was immobilised in a silica polymer matrix and was then tested for its ability to bind Cu (II) and Pb (II). Additionally a genetic investigation was performed to identify genes for class-II metallothionein in *Synechococcus* PCC 7942, 2070 and in other strains of cyanobacteria. Genes for the metallothionein were wide spread among the various groups and species of cyanobacteria (Gardea-Torresdey et al. 1990). The derived amino acid sequence resulted from further characterization of these genes will also elucidate the structure and function of these proteins. Protein amino acid sequences is not strictly conserved in this protein class but shown striking similarity in the functional groups. The number of thiol containing cysteine residues are less in cyanobacterial proteins. However, these positions within the protein are well conserved. The position of acidic amino acid residues such as those containing carboxylate

(Aspartic acid = D, Glutamic acid = E) as well as hydroxyl containing residues(Serine = S, Threonine = T, Tyrosine = Y) are also well conserved. These residues may also involve in metal binding.

34.5 Effluent Treatment

The use of algae and cyanobacteria in waste treatment could prove beneficial in different ways since they are able to bring about oxygenation and mineralization in addition to serving as food source for aquatic species Microbial mats dominated by cyanobacteria are effective in removing metals (Cd, Cu, Zn, Cr and Mn) from water by a mechanism involving exopolysaccharides (Bender et al. 1994a, b).Utilization of cyanobacteria in effluent treatment is a relatively recent phenomenon and , since 1980, momentum of using cyanobacteria in waste water treatment has picked up. It has great potential to take up external nutrients such as ammonium, nitrate, nitrite, orthophosphates, and heavy metals. The common cyanobacterial strains used in waste water treatment include, *Anabaena doliolum*, *Anabaena.*CH3. *Lyngbya gracilis, Phormidium foveolatum, Oscillatoria animalis, O.pseudogerminata*, *Phormidium laminosarum* and *Spirulina maxima* (Canizares et al. 1991; Grabisu et al. 1991; Rai and Mallic 1992; Lu et al. 1994; Meghraj et al. 1994;). Cyanobacterial strains comprising of *Oscillatoria annae* and *Phormidium tenue* proved to be suitable for sewage water treatment.

34.6 Why Cyanobacteria in Waste Water Treatment

Cyanobacteria have been recognised as having enormous potential for use in biotechnology especially in agriculture and are now slowly catching up towards their use in waste treatment. The following tenets have been suggested by Prakasham and Radhakrishnan (1998):-

- Cyanobacterial growth does not require energy rich compounds like other non-photosynthetic microorganisms.
- Cyanobacteria have simple growth requirements which use water as a source of reductant. This character gives them an edge over other photosynthetic bacteria.
- Many cyanobacteria combine photosynthesis and nitrogen fixation, which is an added advantage over other eukaryotic organisms.
- Cyanobacterial biomass production is in abundance and this can be used as an animal feed, an important source for extraction of high value substances like vitamins and drug intermediates, nitrogen fixation, hydrogen production, light energy photo conversion and amino acid production.
- They are environmental friendly and do not cause toxicity to other biotic component.
- Separation of cyanobacterial biomass is much easier than other microbial biomass due to their size

Algae also play a key role in disinfection of waste water treatment by converting light energy into heat which in turn accelerate the death rate of colon form bacteria. At 4°C these bacteria have a half life of 20 days whereas at 30°C this drops to less then a day. By metabolising bicarbonate and nitrate, algae often increase the pH to10 or more. This accelerates the process of auto flocculation of algal biomass in accompanying bacteria and polyvalent cation such as copper, chromium, iron, manganese, lead, strontium, and zinc along with calcium and magnesium which settles to the bottom. The algal biomass so accumulated can be collected and used in the production of combustible gases and organic manure (Goyal 1982, 1989; Prakasham and Radhakrishnan 1998). Based on their study, it was concluded by Shukla et al. (2009) that exponentially growing cells of *Anabaena doliolum* were unable to tolerate nickel up to 10 mM concentration, probably through production of thiolic peptides and metallothioneins. The cyanobacteria may be used as a tool for the removal of nickel from moderately uncontaminated waste water. As it is a high biomass producing strain, it can be used conveniently separated from the solution by filtration.

Majority of tanneries world wide use chromium salts (Cr III and Cr IV) which poses a serious threat to the environment on account of improper disposal of the waste water .Release of the effluent into aquatic ecosystem alters the pH, increases the BOD, COD and gives the water an intense colour (Ajayi and Osibarjo 1980). Conventional treatment of tannery effluents for the purpose of detoxification requires application of physical and chemical methods which involves chromium precipitation and sulphide treatment. It is now apparent that cyanobacteria also play a major role in degrading organic materials in the ecosystem. Nanda et al. (2010) have employed *Nostoc* in bioremediation of tannery effluents. As the chief ingredients of BG-11 media are salts, hence the supplementation of tannery effluents into minimal medium acted as the carbon source for the cyanobacteria to metabolise it and reduce its concentration in the medium.

34.7 Pesticide, Oil Degradation and Remediation by Cyanobacteria

Many chemicals are released into surface water either as a method of disposal or as a consequence of the technology of the utilization. In particular, the use of pesticide many of which are toxic or contain toxic contaminants, is central to high yields of modern agriculture. Lindane is a toxic compound with potential long term persistence (Meister 1993; Alexander 1994). *Anabaena* sp.PCC71208 and *Nostoc ellipsosporum* co-metabolise lindane. Stimulation of the rate of degradation of lindane by nitrate may be attributable to increased availability of nitrogen to nitrate supplemented culture. Fleming and Haselkorn (1974) found that atleast eight protein molecules were synthesised by nitrate-grown culture of *Anabaena* sp. strain PCC 7120 that were not derived from nitrate nitrogen growing cultures. Alternatively, same proteins were involved in the transport or metabolism of lindane. *Anabaena* sp. strain

PCC 7120 and *N. ellipsosporum* supplied with fcb ABC operon from *Arthrobacter flobiformii* (Tsoi et al. 1991) developed the capacity to dechlorinate 4CB; higher concentration of 4CB (2.5 mM) exhibited growth of the culture. Also, genetic engineering by addition of linA gene enhanced the degradation of lindane by two cyanobacterial strains at least when they were grown with nitrogen. It appears likely that other biodegradable operon will also be expressed in cyanobacteria. Cyanobacteria have been shown to degrade both naturally occurring aromatic hydrocarbons (Eillis 1977; Cerniglia and Gibson 1979; Cerniglia et al. 1980a, b; Narro et al. 1992) and xenobiotics (Meghraj et al. 1987) Wolk et al. (1995) have reported that cyanobacteria can be genetically engineered to enhance the degradation of organic pollutants such as highly chlorinated aliphatic pesticide , lindane (γ-hexachloro cyclohexane) *Oscillatoria sps.* could tolerate upto 500 mg/l endosulfan.

Cyanobacteria metabolize organic compounds through ring hydroxtlation. This has been demonstrated in *Aulosira fertilissima* and *Nostoc* sp. which were able to degrade, detoxify and use the pesticide as the sole phosphorous source through the production of phosphate solubilizing enzyme (Subramanian et al. 1994). It was also found that cyanobacteria accelerated transformation and degradation of certain polycyclic aromatic hydrocarbons, organophosphorous compound in water by the sunlight. Thus algal detoxification of environmental pollutant could help in controlling the pollution of the aquatic and terrestrial habitat**.**

Salinity and temperature are important key environmental parameters that influence the degradative process of petroleum components. These parameters influence the structure and physiology of existing microbial communities and result in change in physical and chemical properties of pollutants (e.g. solubility and viscosity) including the diversity; the metabolic potential of degrading bacteria is considered to decrease as environmental conditions become more extreme (Foght and Mc Farlane 1999; Margesin and Schinner 2001). Earlier studies showed that the rate of hydrocarbon degradation decreased with increased salinity and biodegradation could not be detected above 15% salinity (Ward and Brock 1976; Rhykerd et al. 1995). Nevertheless bacterial strains capable of performing pollutant degradation at high salt concentration were isolated (Oren et al. 1992). The increase in temperature was shown to enhance biodegradation (Ward and Brock 1976; Margesin and Schinner 2001), although high temperature is known to reduce the diversity of microbes.

Cyanobacterial mats develop well under extreme conditions where the abundance and activity of grazing organism is limited (Javor and Castenholz 1984; Cohen 1989; Farmer 1992). These mats are of special interest because of the frequent exposure to oil pollution from nearby terminals. Indeed some of these mats became dominant only after the gulf war of 1991, when more than 10.8 million barrels of crude oil was released into the Arabian gulf (Sorkhoh et al. 1992; Fayad and Overton 1995).The cyanobacterium *Microcoleus chloroplastes* found in these mats was detected in many hyper saline environments (12%) (Prufert-Bebout and Garcia-Pichel 1994; Garica-Pichel et al. 1996; Karsten 1996). The detection of relative sequences indicates that these mats contain bacterial population that is resistant to UV and solar radiation (Rainey et al. 1997). Salinity influences biodegradation rates either by reducing bacterial activity (Walker and Calwell 1975) or by limiting the

solubility of hydrocarbons. This inhibitory effect of salinity was shown to be more pronounced for aromatic then for aliphatic compounds (Mille et al. 1991).

Optimum temperature for degradation was 28°C which is close to ambient temperature. Degradation was possible at 40°C but not at 50°C nor below 15°C. The metabolism of polycyclic aromatic hydrocarbon degradation under thermophilic and mesophilic condition is shown to be different as a result of the influence of temperature on enzyme activity (Muller et al. 1998; Annweiler et al. 2000). Higher temperature also reduces the viscosity of crude oil and thus increases its diffusion through sediment, Raeid et al. (2006) showed that hypersaline polluted site could be enriched in oil degrading bacteria and that the addition of new bacteria was not needed. Therefore, attempt at the bioremediation of such sites should consider ways of stimulating existing bacteria to degrade oil compounds rather than introducing new strains.

Using marine cyanobacteria *Oscillatoria* sp. BDU10742 and *Aphanocapsa* sp. BDU 16 a halophilic bacterium *Halobacterium D5101* Uma and Subramanian (1990) could treat Ossein factory effluents and reduce calcium and chloride levels significantly. This enabled 100% survival of Tilopia fish with cyanobacteria as the only feed. Shashirekha et al. (1997) found another marine cyanobacterium *Pharmidium valderianum* BDU 30501 that was able to tolerate and grow at a phenol concentration of 50 mg^{-1} and removed 38 mg^{-1} with a retention period of 7 days. These results open the possibility of treating a variety of phenol containing effluents. The same organism was used to study the condition and regenerate optimal sorption/desorption of heavy metal ions cadmium and cobalt (Karna et al. 1999). Marine *Oscillatoria boryana* BDU92181 was found to effectively degrade and metabolize melanoidin, a pigment which is abundant in distillery effluents (Kalavathi et al. 2001). Studies at National facility for marine cyanobacteria(NFMC) have identified suitable cyanobacteria for treating number of noxious effluent containing organophosphate pesticides, detergent, antibiotics etc. and even degradation of solid waste like coir pith by the lignolytic action of certain cyanobacteria (Malliga et al. 1996).

Some non-heterocystous cyanobacterium such as *Oscillastoria, Microcoleus, Plectonema, Porphyrosiphon, Lyngbya and Trichodesmium* have been reported to fix atmospheric nitrogen under anaerobic and micro aerobic conditions (Ohki et al. 1992; Tiwari et al. 2000) and they also produce a remarkable tolerance to the biocide viz., 2,4-D, Malathion and dimecron at doses higher than those recommended for rice crops (Table 34.1). Few strains of *Anabaena* could tolerate 100 ppm. ceresan M (N-Sulphanilideethylmercur-p-toulene) and sulphanilamide, while strain of *Tolipothrix ternurs* have been found to be sensitive up to a concentration of 0.1 ppm ceresan M (Venkatraman and Rajayalakshmi 1972). Effect of different concentration of malathion (10–200 ppmv/v) on the growth of chlorophyll a content of *N.linkia* and *Westiellopsis* sp. has been reported by Bastia and Adhikary (2001); lethal dose of the pesticide was 200 ppm(v/v).

Bioremediation offers many interesting avenues from a bioinformatics point of view although it is still little explored. This discipline requires the integration of organic compounds, sequence, structure and function of proteins, comparative

Table 34.1 Summary of the studies examining the response (s) of blue green algae to pesticides (Adhikary 2006)

Sl No	Organism	Insecticide/herbicide/ fungicide tested	Concentration	Findings	Reference
1	Species of blue green algae	Ceresan, Dithane, Delapon	0.1–100 ppm	Most of the species of *Anabaena* tolerated 100 ppm of Ceresan. Dithane was lethal to some species of *Anabaena* and *Nostoc even at the lowest concentrations. At 100 ppm of Delapon.* Almost all species grew well.	Venkatraman and Rajayalakshmi (1972)
2	*Cylindrospermum sp. Aulosira fertilissima Plectonema boryanum*	BHC Lindane Diazinon Endrin	10–500 ppm	BHC was more toxic among all the pesticides tested. A *fertilissima* and *P.boryanum* were more resistant than *Cylindrospermum* sp.	Singh (1973)
3	*Nostoc muscorum*	Carbofuran	25–1,000 µg/ml	Higher concentration of the pesticide was more toxic at the acidic pH and the toxicity reduced at the alkaline pH	Kar and Singh (1977)
4	*Nostoc muscorum Wollea bhardlvajae*	Carbofuran HCH	4–30 µg/ml 4 µg/ml	Toxicity of both the insecticides could be removed by repeated cultivation of the alga	Kar and Singh (1979a)
5	*Nostoc musorum*	Hexachloro cyclo hexane (HCH)	4 ppm	Toxicity of HCH was reduced by increasing the concentration of the nutrients K_2HPO_4 (10–40 ppm) and$CaCI_2$ (55–330 ppm) in the Culture medium	Kar and Singh (1979b)
6	*Mastigocladus laminosus Tolypothrix tenuis*	Tolkan, Fluchloralin	50–500 ppm	*M. laminosus* tolerated both the pesticides up to 100 ppm, where as *T. tenus* tolerated Tolkan and Fluchloralin up to 500 and 50 ppm, respectively	Khalil et al. (1980)
7	*Anabaena* doliolum *Nostoc muscorum*	Butachlor	2.5–20 ppm	*Anabaena doliolum* was found to be more tolerant than either *Nostoc muscorum* or *Anacystis nidulans* to Butachlor;	Pandey and Kashyap (1986)
8	*Anabaena* ARM 286 *Anabaena* ARM 310	BHC Ekalux	1–10 ppm	10 ppm of BHC, was more stimulating for oxygen evolution.	Subramaniam et al. (1987)

9	*Anabaena variabilis* *Aulosira fertilissima* *Scytonema chiastum* *scytonema stuposum*	Bavistin Eenlate captan Dithane Cyathion	100–400 ppm	Lethal effect was observed in *Anabaena variabilis* at 100 ppm of Captan, 400 ppm of Cyathion, in *Aulosira fertilissima* at 400 ppm of Dithane, 200 ppm Cyathion, *chiastum* at 100 ppm of Bavistin, 400 ppm of Cyathion and in *Scytonema stuposum* at 400 ppm of Bavistin, Beniate , Dithane and 200 ppm of Capton and Cyathion.	Dikshit and Tiwari (1992)
10	*Anabaena khannae* *Calothrix marchica* *Nostoc calcicola* *Tolypothrix limbata*	Butachlor Benthiocarb Pandimethalin Oxadiazon	0.5–1.5 ppm	*Anabaena khannae* and *Calothri marchica* were proved to be more resistant.	Kolte and Goyal (1992)
11	25 species of rice field blue green algae	Furadan (3%G) commercial grade	10–80 mg/ml	Among the Test organisms, all, the species of *Calothrix* tolerated up to 70 mg/ml of Furadon while *Aulosira* sp. was most sensitive. Sheathed forms of blue green algae were more tolerant.	Rath and Adhikary (1994)
12	*Aulosira fertilissima* ARM 68, *Nostoc muscorum* ARM 221	Monocrotophos Malathion Dichlorovos Phosphomidon Quinolphos	1–250 ppm	The optimal concentration for growth of monocrotophos, Malathion, Dichlorovos, phosphomdon and Quinolphos was 100,75,25 and 1 ppm respectively. Both the species grew maximally with the pesticides in the absence of inorganic phosphate suggesting their utilization as the sole source of phosphorus.	Subramanian et al. (1994)
13	10 species heterocystous blue green algae	Sevin Rogor Hildan	0.1–500 ppm	Among the species tested, *Calothrix parietina* UU 1423 and, *Calothrix* sp. UU2427 possessing a well defined sheath were more tolerant to all the three pesticides.	Das and Adhikary (1996)

genomics, and environmental microbiology and so on. Data related to bioremediation (genome sequence, structure of chemical compounds, enzymes sequence and structure etc.,) are being accumulated in public databases (Eillis et al. 2003).

The bioinformatics resume devoted to bioremediation is still scarce. The University of Minnesota Biocatalysis/Biodegradation database (UMBBD) is among the more prominent resource. Pazos et al. (2005) have developed Meta router, a system for maintaining heterogeneous information related to bioremediation in a frame work that allows its query, administration and mining(application of methods for extracting new knowledge). Among the data mining features is a programme included for locating biodegradative pathways for chemical compounds, according to a given set of constraints and requirements. The interpretation of biodegradative information with the compounding protein and genome data provide a suitable framework for studying the global prospective of the bioremediation network. The full featured system (except administration facility) is freely available at http://pdg.cnb.uam.es/Metarouter. One of the reasons, our knowledge of microbial degradative pathways is so incomplete is the immense complexity of microbial physiology that allows response and adaptability to various internal and external stimuli (Fulekar 2007).

Bioinformatics provide database for microarrays, gene identification and microbial degradation pathways of compounds (Eillis et al. 2001). Bioinformatics analysis will facilitates and quicken the analysis of cellular process to understand the cellular mechanism to treat and control microbial cells as factories. Bioinformatics has wide application in bioremediation for the structure determination and pathways of biodegradation of xenobiotics (Fulekar and Sharma 2008).

34.8 Future Perspectives and Conclusions

Increasing awareness of anthropogenic environmental pollution and its implications for human and environmental health, has led to continuous development of two complementary approaches for assessing the degree of contamination such as quantification of total contaminants and its bioavailable fraction. Physicochemical analysis using a wide spectrum of analytical instrumentation, allow highly accurate, sensitive determination of sample composition, it is essential for regulatory compliance monitoring as well as for understanding the source of pollution and the means for its remediation . However, the array of analytical process necessary for a complete analysis of environmental sample in often costly, time consuming, highly complex and requires trained personnel.

Thorough understanding of all various parameters that affect bioremediation has become very crucial for its successful implementation in field. Environmental pollution and contamination has become a key focus of relief to these problems. However, some of the present methods of environmental cleanup result in production of harmful by-products. Environmentally friendly processes need to be developed to clean up the environment without creating harmful waste products.

Cyanobacteria are phototrophic organisms and ecologically important and are therefore, ideally suited for monitoring of compounds that inhibit photosynthesis activity such as herbicides. Cyanoabacteria have been used for the detection of phototoxic pollutants based on amperometric sensors employing shuttled or direct (Croiseliere et al. 2001) electron transfer.

It is the demand of time that cyanoremediation technologies are developed through selection and isolation of suitable strains of cyanobacteria having high potential for nitrogen fixation, hydrogen production, bioremediation and nutritional value.

Cyanoremediation is increasingly being recognised as a promising technology. Studies on indigenous rhizosphere microflora, genetically modified microbes and improvement in techniques for monitoring and tracking bioinoculants have opened the gateways for exploring efficient cyanobacteria for improving bioremediation process as an alternative to chemical and physical strategies. Furthermore, systemic and concerted efforts are required for assessing the effects of cyanoremediation on the food chain and natural recycling as well as for making the process safer, economical and ecofriendly. Cyanobacteria with suitable and appropriate genetic traits and efficient, effective biodegradation processes would be helpful for clean and green environment.

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Chapter 35
Microbial Decolorization of Colored Industrial Effluents

Ramesh Chander Kuhad, Rishi Gupta, and Yogender Pal Khasa

Abstract Rapid industrialization and urbanization results in the discharge of large amount of organic wastes not easily degradable into water bodies and thereby polluting the environment. The effluent from textile industries itself is of major concern, which contains variety of dyes. The dye wastes being toxic affect the aquatic life and eventually the humans. Therefore, the degradation and/or decolorization of these dyes has become a necessity. The physical and chemical methods used for treating industrial effluents containing dyes are costlier and require high energy. In contrast, various biological methods have been studied to minimize pollution along with the toxicity in the industrial effluents. This chapter will discuss the status about the possibilities of developing various the status about the possibilities of developing various methods or technologies for treating dyes containing waste waters.

Keywords Dyes • Decolorization • Biosorption • Biodegradation • Biotransformation • Textile effluents

35.1 Introduction

Textile industries are one of the oldest and technologically complex industries worldwide and their numbers have been increasing rapidly over the years. Since ages these industries have been using colorants for dyeing the desired materials. Earlier in nineteenth century, the colorants used were of natural origin i.e., from plants (e.g. the red dye alizarin from madder and indigo from wood), insects (e.g. the scarlet dye kermes from shield-louse *Kermes vermilio*), fungi (anthraquinone

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from *Aspergillus cristatus* and cynodontin from *Curvularia lunata*) and lichens (archil from *Roccella tinctoria* and cud bear from *Ochrol echia*) etc. (Wisinak 2004). Later on in 1856, the advent of synthetic dye took place accidentally by an English chemist W. Henery Perkin, where, in an attempt to synthesize quinine he obtained a bluish substance with good dying properties that is known as Mauveine, and with the development of synthetic chemistry by twentieth century, synthetic dyestuffs had almost completely supplanted natural dyes (Welham 2000).

At present over 100,000 dyes are commercially available with millions tons of their production annually (Pandey et al. 2007; Leena and Selva 2008; Szygułaa et al. 2008; Jaikumar and Ramamurthi 2009; Diwanian et al. 2010). Since, the dyes do not bind completely to the fabric, therefore depending on their class, their loss in waste waters could vary from 2% for basic dyes to as high as 50% for reactive dyes (O'Neill et al. 1999; Pandey et al. 2007). The discharge of these colored industrial effluents in the water bodies is supposed to be the major water pollutant (Willmott et al. 1998; Khehra et al. 2005; Pandey et al. 2007; Jaikumar and Ramamurthi 2009). Even the presence of very small amounts of dyes is highly visible in water and affects the aesthetic merit, water transparency and gas solubility in water bodies (Pandey et al. 2007; Diwanian et al. 2010). The synthetic origin and complex aromatic structure of dyes are often a big constrain for their fading on exposure to sweat, soap, water, sunlight and oxidizing agents (Ramalho et al. 2004; Khehra et al. 2005; Pandey et al. 2007; Srinivasan and Thiruvenkatachari 2010) and this renders them more stable and less amenable to biodegradation. There are many reports on the use of physical or chemical treatment processes for color removal from dyes containing effluents. These include flocculation combined with flotation, electroflocculation, membrane filtration, electrokinetic coagulation, electrochemical destruction, ion-exchange, irradiation, precipitation, ozonation, and katox treatment method involving the use of activated carbon and air mixtures (Vandevivere et al. 1998; Robinson et al. 2001; Swaminathan et al. 2003; Behnajady et al. 2004; Wang et al. 2004; Golab et al. 2005; Lopez-Grimau and Gutierrez 2005; Pandey et al. 2007; Srinivasan and Thiruvenkatachari 2010). However, these methods have high operating costs and limited applicability and also produce large quantities of sludge, which again creates a problem in its disposal (Diwanian et al. 2010).

Recently, the reports about the capability of microbes for biodegradation of xenobiotics, aromatic compounds, dyestuff and other recalcitrant pollutants have generated a considerable research interest in this area of effluent treatment (Asgher et al. 2008). The development of microbiological or biological methods is considered attractive due to their low cost, eco-friendly nature and public acceptability. A wide variety of microorganisms including bacteria, fungi and algae, are capable of decolorizing a diverse range of dyes (Crini 2006; Pandey et al. 2007; Srinivasan and Thiruvenkatachari 2010). Biological dye removal techniques are based either on microbial biotransformation of dyes (e.g. activated sludge, anaerobic digestion, trickling filter etc.) or on biosorption process. In this chapter we focus on chemical constituents of textile industrial wastes with special attention to the dyes, their possible health hazards to human being as well as animals and various microbial techniques for treatment of dye containing textile effluents.

35.2 Effluents from Textile Industries

Among many industries, textile industry has a direct connection with environmental aspects to be explicitly and abundantly considered. It is well known that textile mills such as cotton mills consume large volume of water for various processes such as sizing, desizing, scouring, bleaching, mercerization, dyeing, printing, finishing and ultimate washing (Fig. 35.1), and as a result of these various chemical processing of textile, large volume of waste water with numerous pollutants is discharged (Bisschops and Spanjers 2003).

These chemicals range from simple inorganic compounds to polymeric organic products. As an estimate, more than 8,000 chemical products associated with the dyeing processes has already been listed in the color index (Society of Dyers and Colorists 1976). A case study of the effluent discharge from the cotton textile industry is shown in Table 35.1.

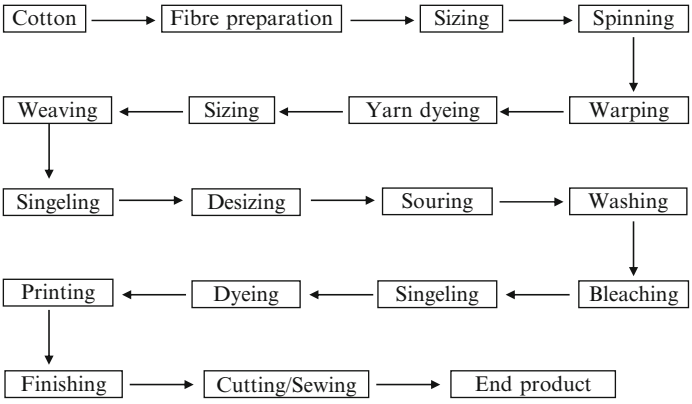

Fig. 35.1 Flow chart diagram of complete processing used in the textile industry (Adapted and modified from Bisschops and Spanjers (2003))

Table 35.1 Characteristics of textile waste water for the processing of cotton fabrics (all values are in mg/L unless otherwise stated)

Parameters	Desizing	Scouring	Bleaching	Dyeing
COD	950–20,000	8,000	288–13,500	1,115–4,585
BOD	–	100–2,900	90–1,700	970–1,460
Color	64–1,900	694	153	1,450–4,750
TSS	18–800	184–17,400	130–25,000	120–190
Carbon	250–2,750	–	320	–
Nitrogen	70–89	–	40–60	–
Phosphorous	10-Apr	89.3	Jun-60	–
Sulphur	–	–	90–100	1,750–4,000
Chlorine	–	–	–	20,000–26,000
Turbidity	900–950	–	–	–
Water usage (L/kg)	–	2.5–43	30–50	38–143

There are numerous varieties of synthetic dyes used for textile dyeing and other industrial applications. The structural diversity of dyes derive from the use of different chromophoric groups for example azo, anthraquinone, triarylmethane, phthalocyanines etc., and from the use of different application technologies for example reactive, direct, disperse and vat dyes etc. Moreover it is assumed that out of total world colorant estimated (800,000 ton/year), 69–70% is azo dyes (Easton 1995). But due to poor fixation of dyes various reactive azo textile dyes like orange, reactive dyes, food yellow etc., occur in waste water in a concentration ranging from 5 to 1,500 mg/ml (Pierce 1994). Besides different dyes, the materials that may be expected to predominate in textile waste water may be:

(a) **Nitrogen compounds**: The compounds used as dye bath additives containing nitrogen are the main source of nitrogen in textile effluent. Besides ammonia sources are the chemicals used in printing, coating preparation and dyeing processes, while the printing pastes contain large quantities of urea, another source of nitrogen (Delée et al. 1998; Bisschops and Spanjers 2003).
(b) **Phosphorus compounds**: In textile wastewaters, dye bath effluents containing phosphate buffers are the main source of phosphorus. Phosphates are used in different steps in textile wet processing. For instance in buffers, scouring, water conditioners, surfactants, and flame-retardant finishes (Delée et al. 1998; Bisschops and Spanjers 2003).
(c) **Sulphur Compounds**: Depending on the process, high levels of sulphur compounds can be present in textile effluent. For instance, if sodium sulphate is used in reactive dyeing, sulphate levels can be as high as 20–42 g/L. Moreover, sodium sulphide and sodium hydrosulphite are commonly used as reducing agents when sulphur or vat dyes are used. While sulphuric acids used for pH control are also a source of sulfur in the dye bath (Delée et al. 1998; Bisschops and Spanjers 2003).
(d) **Chloride**: Chloride ions from bleaching and dyeing processes are major source of their abundant presence in textile wastewater. In bleaching, amongst other chemicals, chloride containing compounds are used most often. Sodium chloride for instance, is used in large quantities in reactive dyeing (EPA 1997; Bisschops and Spanjers 2003).
(e) **Oil and Grease**: The grease content is generally a characterization of wool scouring wastewater (APHA 1998; BTTG 1999; Bisschops and Spanjers 2003).
(f) **Metals**: Metals enter the wastewater in many ways; the incoming water supply, metal parts (like pumps, pipes, valves, etc.), oxidizing and reducing agents, electrolyte, acid and alkali, dyes and pigments, certain finishes, herbicides and pesticides, and maintenance chemicals (Smith 1988; Bisschops and Spanjers 2003). The main source of heavy metals is the dyeing process, because most used dyes contain chromium, cadmium, zinc or other metal atoms.
(g) **Surfactants**: Surfactants are widely used in textile processing as they lower the inter-facial tension of water and other materials at phase boundaries. The most widely used are the anionic surfactants and their general important types are carboxylates, better known as soaps (alkali metal salts of fatty acids), sulfonates, sulphates and phosphates (Bisschops and Spanjers 2003).

35.3 Dyes Classification

Generally a dye can be described as a colored substance that has an affinity to the substrate to which it is being applied. The dye is usually used as an aqueous solution and may require a mordant to improve the fastness of the dye on the fiber. Dyes are comprised of two main groups i.e., chromophore and auxochrome (PPAH 1998). A correlation of chemical structure with color has been accomplished in the synthesis of dyes using a chromogen-chromophore with auxochrome. Chromogen is the aromatic structure containing benzene, naphthalene or anthracene rings. A chromophore group is represented by the following radicals, which form a basis for the chemical classification of dyes when coupled with the chromogen; azo (–N═N–); carbonyl (═C═O); carbon (═C═C═); carbon-nitrogen (>C═NH or –CH═N–); nitroso (–NO or N–OH); nitro ($-NO_2$ or ═NO–OH); and sulfur (>C═S, and other carbon-sulfur groups) (PPAH 1998). While, the auxochrome or bonding affinity groups are amine, hydroxyl, carboxyl, and sulfonic radicals, or their derivatives and their main impact was to intensify the color of the chromophore. The common structures of various dyes are shown in Fig. 35.2.

Based on the dyeing mode, main structural moieties and chromophores, dyes can be divided into various different groups, of which azo (monoazo, diazo, triazo, polyazo), anthraquinone, phthalocyanine, cyanine, diazonium, nitro, nitroso, quinine, thiazin, xanthene and arylmethane dyes etc. are the most important (PPAH 1998). However, according to color index, there are 15 different classes of dyes, which are Acid dyes, Reactive dyes, Basic dyes, Direct dyes, Metal complex dyes, Mordant dyes, Disperse dyes, Pigment dyes, Vat dyes, Sulphur dyes, Solvent dyes, Fluorescent brighteners and classes which include Food dyes and Natural dyes (Table 35.2).

35.4 Environmental Impact of Textile Dyes and Their Toxicity

Dye wastewater from textile and dye based industries is one of the most complex industrial wastewaters to treat. It is known that 90% of reactive textile dyes entering the activated sludge sewage treatment plants discharge into rivers and cause problems such as (a) reduction in penetration of sunlight in the streams, which is essential for photosynthesis and consequently, the ecosystem of the stream is seriously affected (b) toxicity to fish and mammals life (c) inhibition of the activity and the growth of microorganisms particularly at high concentrations (d) some cationic species (mostly triphenylmethanes) affect the flora and fauna even at lesser concentrations (Verma 2008).

Interest in the pollution potential of textile dyes has been primarily prompted by concern over their possible toxicity and carcinogenicity (Maas and Chaudhari 2005; Salony and Bisaria 2006; Revankar and Lele 2007). This is mainly due to the fact that many dyes are made from known carcinogens, such as benzidene and other

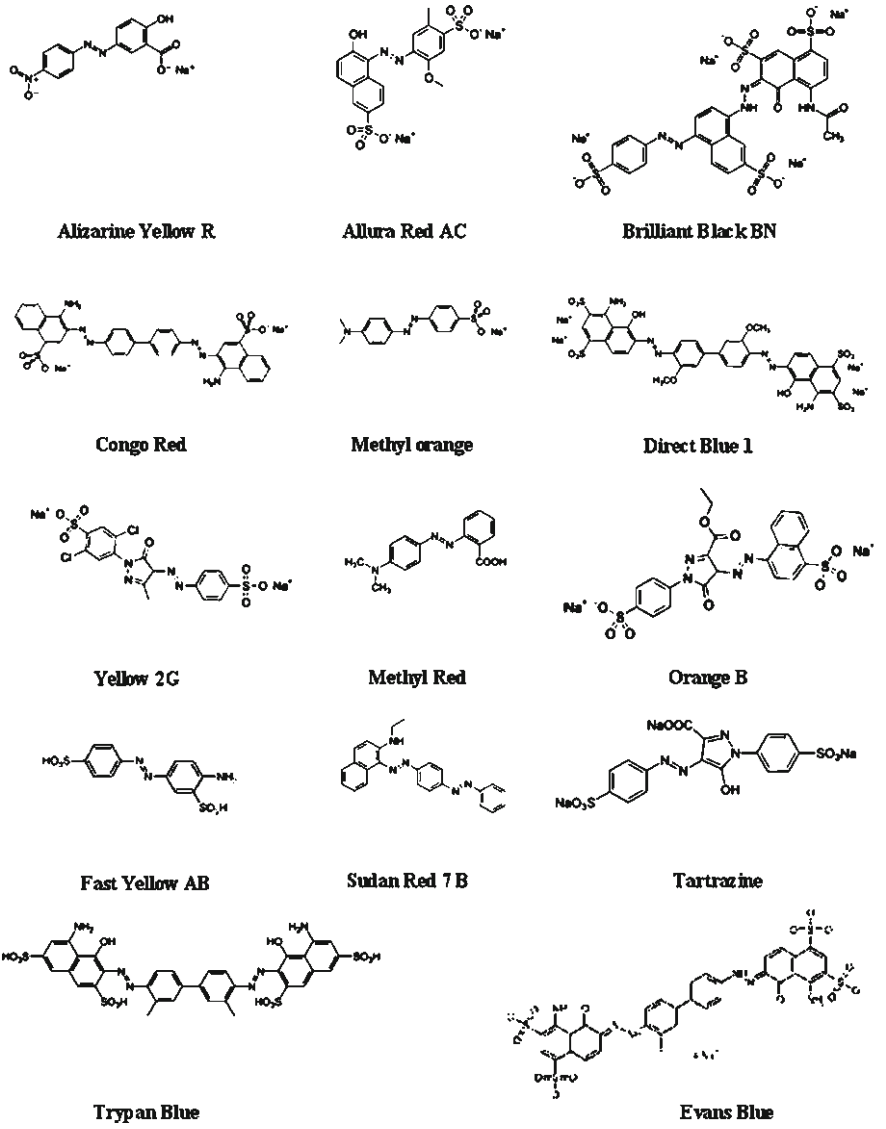

Fig. 35.2 Chemical structure of common dyes used in various industries (www.wikipedia.com)

aromatic compounds (Clarke and Anliker 1980; Levin et al. 2005). Chronic effects of dyestuffs, especially of azo dyes, have been studied for several decades. Azo dyes in purified form are seldom directly mutagenic or carcinogenic (Brown and DeVito 1993), however, reduction of azo dyes, leads to formation of aromatic amines, which are known mutagens and carcinogens (Dawkar et al. 2009). Exposure to aromatic amines may cause methaemoglobinemia in which the amine oxidize the heme iron

Table 35.2 Important classes of dyes and their characteristics

Dye class	Color index	Fiber	Type of interaction/reactions with fiber	Examples
Acid dyes	Largest class ~2,300 different dyes listed ~40% of them are in current production	Mainly wool, Nylon, Polyamide, Modified acryl	Ionic interactions, anionic compounds of dyes bind with the cationic NH_4^+ ions.	Azo dyes like Orange II, Orange G and Carbonyl like Indigo Carmine, Anthraquinone etc.
Basic dyes	Represent ~5% of all dyes listed in color index	Mainly synthetic fibers like modified polyacryl	Ionic interactions	Triphenyl like Crystal violet and Malachite green, and anthraquinone, triaryl-methane etc.
Reactive dyes	Second largest dye class	Cotton, wool, silk and nylon etc.	Covalent interactions with fibers	Reactive Orange II, Reactive Black5, Reactive Red198, and Quinine type Remazol blue
Direct dyes	~1,600 are listed and about 30%are in current production	Mainly cellulose fibers like cotton	Vanderwaal forces	Mostly are Azo Congo red, Direct Blue86, Phthalocyanine etc.
Disperse dyes	Third largest group of dyes	Synthetic fibers like cellulose acetate, polyester, Polyacrylamide.	These dyes penetrate synthetic fibers	Azo compounds like Methyl yellow, Disperse Yellow 1 etc.
Vat dyes	–	Cellulose fibers mainly cotton and wool	Dyeing method based on the solubility of vat dyes in their reduced (Leuco) form	All vat dyes are anthraquinone or indigoid like indigo etc.
Mordant dyes	~600 Mordant dyes are listed in the color index	Wool, Leather, Silk, Paper and modified cellulose fibers	Mordant dyes fixed to fabric by the addition of a mordant.	These are Azo, Oxazine or Triarylmethane but usually these are dichromates or chromium complexes.
Pigment dyes	About 25% of all commercial dyes name listed in the color index	Used for printing diverse fibers.	To interact with fiber required the help of dispersing agent.	Mostly are Azo (Yellow to Red) or metal complexes and Quinacridone like Acridine

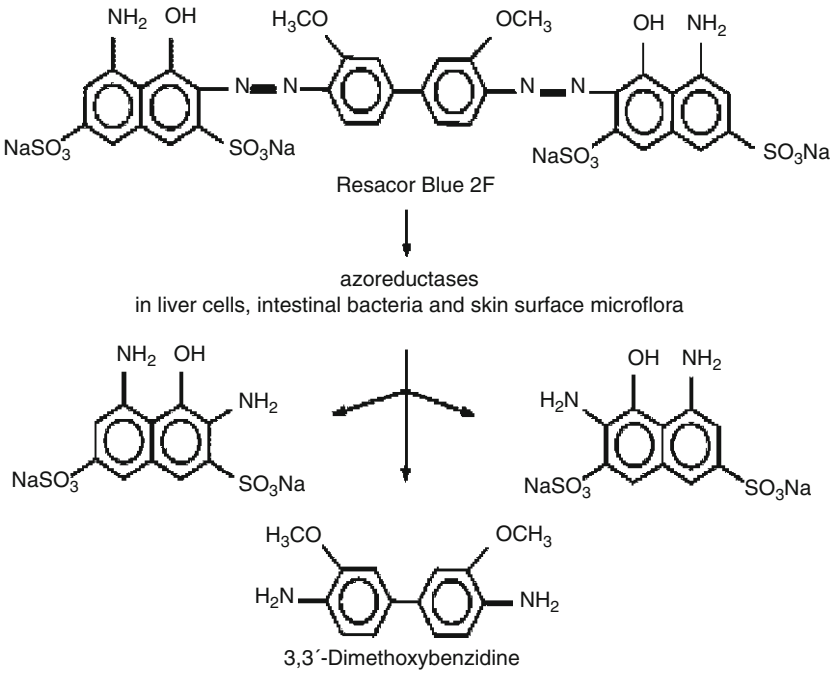

Fig. 35.3 Reaction mechanism of degradation of dye Resacor Blue 2F to a carcinogenic compound 3,3—Dimethoxybenzidine (Hildenbrand et al. 1999)

of hemoglobin from Fe (II) to Fe (III), blocking the oxygen binding. In mammals, reduction of azo dyes is mainly due to bacterial activity in the lower gastrointestinal tract, liver and the kidney. The carcinogenicity mechanism probably includes the formation of nitremium and carbonium ions from acyloxy amines that bind to DNA and RNA, which induce mutations and tumour formation (Van der Zee 2002; Golka et al. 2004). A study carried out by Hildenbrand et al. (1999) on reduction of azo dyes in cell cultures showed that the addition of a dye, Resacor Blue 2F, to kidney and liver cells produced a carcinogenic aromatic amine 3,3′-Dimethoxybenzidine (Fig. 35.3).

Dermal and immunological effects have also been reported in workers exposed to benzidine and cancer is the documented toxic effect of benzidine in both human and animals. Direct brown, Direct black, Direct blue, are the dyes generally used in textile industries and the workers exposed to these compounds excrete high level of benzidine into their urine (Van der Zee 2002; Golka et al. 2004). Metabolic transformation of benzidine results in the formation of reactive intermediates which are thought to produce DNA adducts, which may initiate carcinogenesis by producing mutations that become fixed before DNA can be repaired (Van der Zee 2002; Golka et al. 2004). Moreover, anthraquinone based dyes, which are the most resistant to degradation when form metal -based complex can also be carcinogenic, in water supplies (Van der Zee 2002; Golka et al. 2004).

Other compounds like formaldehyde, carbon disulphide, some phenolic and sulphur containing compounds are also used in textile industries. Formaldehyde is generally used in textile industries to attain a permanent press finish. It is a well-known cause of ocular and airway irritation and it can cause certain skin reactions including contact dermatitis via either allergic or irritant mechanism (Van der Zee 2002; Golka et al. 2004). Carbon disulphide (CS_2) is prominent in the production of the synthetic fiber viscose rayon. It is used in the conversion process of cellulose to rayon fibers and cellophane and high level of CS_2 resulted in the severe central nervous system (CNS) disorders including acute mania and narcosis (Van der Zee 2002; Golka et al. 2004).

35.5 Effluent Treatment Techniques

Due to the alarming environmental problem of textile effluents, economic removal of polluting dyes is gaining great importance (Tunay et al. 1996; Robinson et al. 2001; Van der Zee 2002; Khehra et al. 2005; Pandey et al. 2007; Asgher et al. 2008; Srinivasan and Thiruvenkatachari 2010). There are several factors that determine the technical and economic feasibility of each single dye removal technique: (1) dye type (2) waste water composition (3) dose and cost of required chemical (4) operation costs (5) environmental fate and handling cost of generated waste products (Van der Zee 2002; Husain et al. 2009). In a broad term, methods of effluent treatment for dyes may be classified into three main categories: physical, chemical and biological as indicated in Table 35.3. The physical and chemical techniques were numerous including anion exchange resins (Kracher et al. 2002), floatation (Lin and Lin 1993), electroflotation (Ogfitveren and Koparal 1994), electrochemical destruction (Ulker and Savas 1994) and the use of activated charcoal (Pala and Tokat 2002).

Earlier, municipal treatment systems were mainly used for the purification of textile mill wastewaters. These systems, however, depended mainly on biological activity and were mostly found to be efficient in removing the more resistant synthetic

Table 35.3 Different commonly used methods of effluent treatment

Physical	Chemical	Biological
Adsorption	Neutralization	Stabilization
Sedimentation	Reduction	Aerated lagoons
Floatation	Oxidation	Trickling filters
Flocculation	Electrolysis	Activated sludge
Coagulation	Ion exchange	Anaerobic digestion
Foam fraction	Wet air oxidation	Bioaugmentation
Reverse osmosis		
Ultrafiltration		
Ionization		
Radiation		

dyes. Primarily they depend upon using physical or chemical treatment processes in conjunction with biological treatment (Mishra and Tripathy 1993; Banat et al. 1996; Supaka et al. 2004). Although the physical and chemical treatment techniques are effective for color removal but they consume more energy and chemicals than biological processes, however, among the low cost viable alternatives available for effluent treatment and decolorization, the biological systems seem to be more superior. Biological systems are recognized by their capacity to reduce biological oxygen demand (BOD) and chemical oxygen demand (COD) by conventional and aerobic biodegradation. Moreover, there are a wide variety of microorganisms including bacteria, fungi, actinomycetes and algae etc. and their enzymes are capable of decolorizing a wide range of dyes. Earlier, the drawback with microbial decolorization was with its inability to remove color (O'Neill et al. 2000), but now a days potential microbial decolorization systems have been developed with total color removal, in some cases within few hours (Balan 1999; Balan and Moteiro 2000; Nyanhongo et al. 2002; Nilsson et al. 2006; Asgher et al. 2008; Srinivasan and Thiruvenkatachari 2010).

35.6 Microbial Decolorization

35.6.1 Biodegradation of Dyes

Biological dye removal techniques are mainly based on microbial biotransformation of dyes. As dyes are designed to be stable and long-lasting colorants, they are not easily biodegraded. Many microbes have been isolated which can catalyze anaerobic reductive fission of the azo linkage resulting in formation of colorless aromatic amines. Nevertheless, many researchers have demonstrated partial or complete biodegradation of dyes by pure and mixed cultures of bacteria, fungi and algae (Ramalho et al. 2004; Khehra et al. 2005; Pandey et al. 2007; Diwanian et al. 2010).

35.6.1.1 Bacterial Biodegradation

Investigations to bacterial dye biotransformations have so far mainly been focused to the most abundant chemical class, that of the azo dyes. However, extensive studies have been carried out to determine the role of the diverse groups of bacteria in the decolorization of azo dyes (Table 35.4). The electron withdrawing nature of the azo linkages obstructs the susceptibility of azo dye molecules to oxidative reactions (Fewson 1988; Pagga and Brown 1986; Jimenez et al. 1988; Shaul et al. 1991; Pagga and Taeger 1994; Ganesh et al. 1994). In contrast, there are many reports of successful breakdown of azo linkages by reduction under anaerobic (methanogenic) conditions, which require an organic carbon energy source. (Chinwetkitvanich et al. 2000; Talarposhti et al. 2001; Isik and Sponza 2005; van der Zee and Villaverde 2005). Carliell et al. (1996) and Razo-Flores et al. (1997a) have associated the decolorization

Table 35.4 List of bacterial strains used commonly for dye decolorization

Cultures	Dyes	Reference
Aeromonas hydrophila	Various azo dyes	Idaka and Ogawa (1978), Chen et al. (2003), and Ren et al. (2006)
Bacillus subtilis	2-Carboxy 4′dimethylaminobenzene, p-Aminoazobenzene	Horitsu et al. (1977), Yatome et al. (1991), and Dawkar et al. (2009)
Klebsiella pneumoniae	Methyl red	Wong and Yuen (1996)
Mixed aerobic culture	*Mordant yellow*	Haug et al. (1991) and Khehra et al. (2005)
Mixed anaerobic culture	Diaza—linked chromophores	Knapp and Newby (1999)
Pseudomonas cepacia	C.I. Acid orange 12, C.I. Acid orange 20, CI Acid Red 88, Orange I, p-Aminoazobenzene	Yatome et al. (1991) and Ogawa and Yatome (1990)
Pseudomonas luteola	Red G, RBB	Hu (1990) and Hu (1994)
Pseudomonas putida	Tectilon blue	Walker and Weatherley (2000) and Chen et al. (2007)
Pseudomonas stutzeri	Orange I	Yatome et al. (1991)
Citrobacter sp.	Crystal violet, Gentian violet, Malachite green, Brilliant green, Basic fuschine, Methyl red, Congo red	An et al. (2002)
Enterobacter cloacae	Reactive Black 5	Wang et al. (2009)
Enterobacter sp.	Reactive Red 195	Jirasripongpun et al. (2007)
Yersinia sp.	Reactive Red 195	Jirasripongpun et al. (2007)
Serratia sp.	Reactive Red 195	Jirasripongpun et al. (2007)
Enterococcus gallinarum	Direct black 38	Bafna et al. (2008)
Kocuria rosea	Malachite green	Parshetti et al. (2006)
Micrococcus glutamicus	Scarlet R	Saratale et al. (2009a)
Nocardia corallina	Crystal violet	Yatome et al. (1993)
Proteus vulgaris	Scarlet R	Saratale et al. (2009a)
Shewanella decolorationis	Crystal violet	Chen et al. (2008)
Shewanella putrefaciens	Reactive Black 5, Direct red 81, Acid red 88, Disperse orange 3	Khalid et al. (2008)

with methanogens, whereas studies by other investigators showed that acidogenic as well as methanogenic bacteria can also be good candidate for the dye decolorization (Chinwetkitvanich et al. 2000; Bras et al. 2001).

The reduction of azo linkages of dyes under anaerobic conditions appears to be non-specific, as most of the varied group of azo compounds is decolorized; however the rate of decolorization is dependent on the organic carbon source, as well as on the dye structure (Bromley-Challenor et al. 2000). First-order kinetics with respect to dye concentration has been generally reported for the course of dye decolorization, although zero order was also observed in few cases (van der Zee et al. 2001; Isik and Sponza 2005). With a few specific dyes, such as acid orange 7 (AO7), autocatalysis by quinone-like compounds, formed during azo dye reduction, contributes to a significant extent to the overall reduction process (van der Zee et al. 2000; Mendez-Paz et al. 2005).

Interestingly, anoxic decolorization of various azo dyes by mixed aerobic and facultative anaerobic microbial consortia has also been reported (Nigam et al. 1996; Kapdan et al. 2000; Padmavathy et al. 2003; Khehra et al. 2005; Moosvi et al. 2005). However, a switch from anaerobic to aerobic conditions was also required to achieve complete degradation because the main azo bond cleavage needed the reductase enzymes, which are mainly functional under anaerobic conditions (van der Zee and Villaverde 2005). Haug et al. (1991) described a bacterial consortium capable of mineralizing the sulphonated azo dye mordant yellow. Khehra et al. (2005) reported a bacterial consortium consisting of *Bacillus cereus*, *Pseudomonas putida*, *P. fluorescence* and *Stenotrophomonas acidaminiphila* capable of completely decolorizing acid red 119. It was also able to decolorize 99% acid red 119, 94% acid red 97, 99% acid red 113% and 82% of reactive red 120 dye at an initial concentration of 60 mg L^{-1} of mineral salts medium in 24 h. In another report almost complete decolorization of mixture of dyes by *Ischnoderma resinosum* in liquid medium was achieved after 20 days (Eichlerova et al. 2006).

Moreover, it has also been observed that the decolorization of Mordant Yellow 3 by *Sphingomonas xenophaga* strain BN6 was greatly enhanced by glucose, whereas a significant decrease in azo dye decolorization in its presence was reported for *P. leuteola, Aeromonas* sp. and few other mixed cultures (Haug et al. 1991; Kapdan et al. 2000; Chen et al. 2003; Khehra et al. 2005). The negative effect of glucose on anoxic decolorization has been attributed either to a decrease in pH due to acid formation, or to catabolic repression (Chen et al. 2003). There are other reports on the "aerobic" metabolism of azo dyes, where the bacterial strains (e.g. *Aeromonas sp.*, *B. subtilis, Proteus mirabilis, P. pseudomalli BNA, P. luteola*) were grown aerobically with complex media or sugars, then incubated without shaking in the presence of different azo dyes (Horitsu et al. 1977; Chen et al. 1999).

Several bacterial strains that can aerobically decolorize azo dyes have been isolated during the past few years (Nachiyar and Rajkumar 2003). Coughlin et al. (1999) have reported that *Sphingomonas* sp, strain 1CX, an obligate aerobe, could grow on an azo dye AO7 as sole carbon, energy and nitrogen source. In another report, Tony et al. (2009) developed two bacterial consortia from textile waste water treatment plant and found that consortia were able to decolorize 50–96% of six individual azo dyes; Congo red, Bordeaux, Ranocid Fast Blue, Blue BCC, Levender Red and Remazol golden yellow. Recently a decolorization process by thermophilic bacterium, *Anoxibacillus* genus, isolated from Spanish hot springs has been developed to bioreactor scale, with more than 80% decolorization in less than 12 h (Deive et al. 2010).

35.6.1.2 Fungal Biodegradation

White-rot fungi are the most widely studied microorganisms for dye decolorization (Couto 2009). This property is mainly due to the relatively non-specific activity of their ligninolytic enzymes, such as lignin peroxidase, manganese peroxidase and laccase. The reactions catalyzed by these extracellular enzymes are oxidation reactions,

e.g. lignin peroxidase catalyzes the oxidation of non-phenolic aromatics, whereas manganese peroxidase and laccase catalyze the oxidation of phenolic compounds (Nozaki et al. 2008). The same unique non-specific mechanism that gives these fungi the ability to degrade lignin also allow them to degrade a wide range of pollutants and they possess a number of advantages not associated with other bioremediation systems (Kuhad et al. 2004; Enayatzamir et al. 2009; Couto 2009). Virtually all dyes from all chemically distinct groups are prone to fungal oxidation but there are large differences between fungal species with respect to their catalyzing power and dye selectivity (Fu and Viraraghavan 2001a, b). Fungal degradation of aromatic structures is a secondary metabolite event that starts when nutrients (C, N and S) become limiting (Kirk and Farrell 1987). Various fungal strains used for decolorization of dye containing have been listed in Table 35.5.

Most of the earlier dye decolorization studies were based mainly on *Phanerochaete chrysosporium* and *Trametes versicolor* (Toh et al. 2003). *P. chrysospoium* has proved its capability to biodegrade the azo- and heterocyclic dyes, Orange II, Tropaeofin O, Congo red and Azure G (Cripps et al. 1990). The extent of color removal during decolorization depends upon the complexity of dye, nitrogen availability in the media and ligninolytic activity in the culture. Veratryl alcohol is believed to stimulate the ligninase activity, which seems to be linked to decolorization (Paszczynski and Crawford 1991). Capalash and Sharma (1992) tested the biodegradation of 18 azo dyes using *P. chrysospoium* and only eight were degraded with 40–70% color removal. This degradation was mainly through the lignin degrading enzyme system or adsorption to cell mass. Substitution with sulfo groups on the aromatic component of some azo dyes did not seem to significantly affect the biodegradability of the azo dyes (Paszczynski et al. 1992; Pasti-Grigsby et al. 1992). Spadaro et al. (1992), in contrast, showed that when aromatic rings of dyes had substituted hydroxyl, amino, acetamide or nitro functional groups, mineralization was greater than those with unsubstituted rings. The different isoenzymes of lignin peroxidase produced by *P. chrysosporium* are able to decolorize several dyes with different chemical structures including azo, triphenylmethane, heterocyclic and polymeric dyes (Ollikka et al. 1993). The chemical steps involved in the degradation of azo dyes by LiP and MnP has been elucidated (Pasti-Grigsby et al. 1992; Goszczynski et al. 1994). Decolorization of Direct Blue 15 by *P. chrysosporium* immobilized on $ZrOCl_2$-activated pumice follows the first-order kinetics with respect to initial dye concentration and MnP plays the major role in decolorization with mycelial adsorption being the minor mechanism (Pazarlioglu et al. 2005). Recently our group has also tested *P. chrysospoium* for six different dyes and found 50–80% decolorization in most of the dyes (Diwanian et al. 2010).

Other than *P. chrysospoium*, several other basidiomycetes such as *Cyathus, T. versicolor, Bjerkandera adusta, Dichomitus squalens, Irpex flavus, Daedalea flavida, Polyporus sanguineus, Funalia trogii* ATCC200800*, Ganoderma sp.* WR-1*, Ischnoderma resinosum, Pleurotus, Phelbia* and *Thelephora* species have also been observed for their dye decolorization activity (Vasdev and Kuhad 1994; Heinfling et al. 1998; Swamy and Ramsay 1999; Kirby et al. 2000; Selvam et al. 2003; Chander et al. 2004; Ozsoy et al. 2005; Eichlerova et al. 2006; Chander and Arora 2007;

Table 35.5 List of bacterial strains used commonly for dye decolorization

Cultures	Dyes	Reference
Aspergillus sojae B-10	Amaranth, Sudan II, Congo Red	Ryu and Weon (1992)
Candida sp.	Procyon Black SPL, Procyon Blue MX2G, Procyon Red HE7B, Procyon Orange HER	De Angleis and Rodrigues (1987)
Coriolus versicolor	Malachite Gree, Azure B, Poly R-478, Anthraquinone Blue, Congo Red, Xylidine	Levin et al. (2004)
Cerrena unicolor	Acid Blue 62, Acid Blue 40, Reactive Blue 81, Direct Black 22, Acid Red 27	Michniewicz et al. (2008)
Daedalea quercina	Chicago Sky Blue, Poly B-411, Remazol Brilliant Blue R, Trypan Blue, Reactive Blue 2	Baldrian (2004)
Funalia trogii	Remazol Brilliant Blue Royal (RBBR), Drimaren Blue CL-BR	Erkurt et al. (2007)
Irpex lacteus	Reactive Blue 19 (RBBR), Reactive Black 5, Reactive orange 16, Remazol Brilliant Blue R	Ma´ximo and Costa-Ferreira (2004) and Svobodova et al. (2008)
Lentinula edodes	Remazole Brilliant Blue R	Boer et al. (2004)
Myrothecium verrucaria	Orange II, 10B (Blue), RS (Red)	Brahimi-Horn et al. (1992)
Myrothecium sp.	Orange II, 10B (Blue), RS (Red)	Mou et al. (1991)
Neurospora crassa	Vermelho Reanil P8B	Corso et al. (1981)
Phanerochaete chrysosporium	Amaranth, Orange G, Direct Blue 15, Methylene Blue, Direct Blue 15, Direct Green 6, Congo Red	Urek and Pazarlioglu (2005)
Pleurotus pulmonarius	Amido Black, Congo Red, Trypan Blue, Methyl Green, Remazol Brilliant Blue R, Methyl Violet, Ethyl Violet, Brilliant Cresyl Blue, Methylene Blue, Poly R-478	Tychanowicz et al. (2004)
Pleurotus ostreatus	Phenol Red, Orthocresol Red, Meta-cresol Purple, Bromophenol Red, Bromocresol Purple, Bromophenol Blue, Bromocresol Green	Shrivastava et al. (2005)
Pycnoporus cinnabarinus	Pigment plant effluent	Schliephake et al. (1993)
Scyzophyllum commune	Solar golden yellow R	Asgher et al. (2008)
Trametes trogii	Malachite Green, Xylidine, Ponceau 2R, Anthraquinone Blue	Levin et al. (2005)
Trametes versicolor	Remazol Brilliant Blue R, Remazol Brilliant Blue RR, Remazol Red RR, Remazol Yellow RR, Reactive Red 2, Reactive Blue 4	Christian et al. (2005), Toh et al. (2003), and Nilsson et al. (2006)

Revankar and Lele 2007). Vasdev et al. (1995) observed effective decolorization of three triphenylmethane dyes by *C. bulleri* and *C. stercoreus*. Heinfling et al. (1998) described the transformation of six azo dyes and phthalocyanine dyes by lignin peroxidases from *B. adusta* and *P. erngii.* Abadulla et al. (2000) reported the use of purified laccase from *T. hirsuta* to degrade triarylmethane, indigoid, azo and anthraquinonic dyes. Novotný et al. (2000) have reported that the white rot fungus *I. lacteus* and *P. ostreatus* are the potential candidates for the removal of dye remazol brilliant blue red present in the soil under *in vitro* conditions. Nyanhongo et al. (2002) reported the decolorization of synthetic dyes by *T. modesta* under acidic conditions. D'Souza et al. (2006) reported the decolorization of textile effluent by marine fungus NIOCC≠2a to various degrees within 6 h of incubation. Recently Diwanian et al. (2010) have tested eight fungal isolates for the decolorization of six synthetic dyes and reported that the percentage decolorization of all dyes by the new fungal isolate *Crinipellis sp.* RCK-1 was higher than the strains of *P. chrysosporium* and *P. cinnabarinus*.

Low molecular mass redox mediators like ABTS are necessary for laccase-catalyzed decolorization of most of the dyes (Lu et al. 2005, 2007). However, *P. pulmonarius* and *L. edodes* SR-1 have been reported to produce only extracellular laccase to decolorize dyes of different spectra without any mediators (Nagai et al. 2002). MnP mediated decolorization of azo dyes such as Direct Blue 15, Direct Green 6, and Congo red by *P. chrysosporium* can be enhanced by the addition of Tween-80 (Urek and Pazarlioglu 2005) and copper (Tychanowicz et al. 2006). Whereas, LiP produced by *T. versicolor* can decolorize Remazol Brilliant Blue R (RBBR) in the presence as well as in the absence of veratryl alcohol (Christian et al. 2005). The decolorization ability of WRF can be substantially increased by carefully optimizing the operational conditions such as initial dye concentration, nutrient content of the media, age of fungus and carbon as well as nitrogen sources (Ozsoy et al. 2005; Nilsson et al. 2006; Sanghi et al. 2006). Addition of glucose as carbon source caused a dramatic increase in decolorization of Solar golden yellow R by *Schyzophyllum commune* IBL-06, whereas additional nitrogen sources were inhibitory to MnP formation and dye decolorization (Asgher et al. 2008).

35.6.1.3 Degradation by Actinomycetes

Extracellular enzymes (peroxidases) from actinomycetes are well known for their participation in the initial oxidation of lignin, catalyze hydroxylation, oxidation and dealkylation reactions (Ball et al. 1989; Goszczynski et al. 1994; Mc Mullan et al. 2001). Species of *Streptomyces* and *Thermomonospora* are good examples of actinomycetes capable of effective dye decolorization. Pasti and Crawford (1990) investigated the ability of ligninolytic *Streptomyces* for the decolorization of textile dyes and hypothesized strong correlation between decolorization and ligninolytic ability. Decolorization of mono sulphonated monoazo dye derivatives of azo benzene by the *Streptomyces* sp. was observed to have the common structural pattern (Pasti and Crawford 1990). Paszczynski et al. (1992) compared the efficiency of a soil actinomycete culture, *S. chromofuscus*, to *P. chrysosporium* and concluded that the soil bacterium has less decolorization ability than the white-rot fungus. Several

other actinomycete strains have also been reported to decolorize various reactive dyes such as anthraquinone, pthalocyanine and azo dyes (Zhou and Zimmermann 1993). Other copper based dyes, such as formazan-copper complex dyes, were completely decolorized through degradation by the same actinomycete strains (Zhou and Zimmermann 1993). Ball and Cotton (1996) have also studied three well characterized lignocellulose degrading actinomycetes, *S. viridosporus*, *S. badius* and *T. mesophila* and showed that they decolorize the polymeric dye, Poly-R with a maximum decolorization rate of 0.1 unit/day. The potential of different *Nocardia* species, such as *N. corallina* and *N. globeurulla* for their ability to degrade crystal violet has also been demonstrated (Yatome et al. 1993).

35.6.1.4 Yeast Biodegradation

Only a limited studies are available in literature about yeast based dye decolorization (Kuhad et al. 2004; Jadhav et al. 2008; Saratale et al. 2009). The ability of *Kluyveromyces marxianus* IMB3 to decolorize Remazol Black-B dye was investigated and maximum color removal, 98% was achieved at 37°C (Meehan et al. 2000). Similarly, a number of simple azo dyes were degraded in liquid aerated batch cultures by *Candida zeylanoides*, with a color removal ranged from 44% to 90% after 7 days (Martins et al. 1999). Besides, *Saccharomyces cerevisiae* has also been reported to degrade dyes in molasses media (Aksu 2003).

35.6.1.5 Algal Biodegradation

Degradation of azo dyes by algae has been reported in only a few studies (Jinqi and Houtian 1992; Semple et al. 1999). Since, the degradation pathway is thought to involve reductive cleavage of the azo linkage followed by further degradation (mineralization) of the generated aromatic amines and algae have been demonstrated to degrade several aromatic amines, even sulphonated ones, hence they if used will be a potential candidate for dye decolorization (Luther and Soeder 1987; Soeder et al. 1987; Luther and Soeder 1991). Recently, Marungrueng and Pavasant (2006) had reported the adsorption of astrazo blue FGRL by *Caulerpa lentillifera*. In open wastewater treatments systems, especially in (shallow) stabilization ponds, algae may therefore contribute to the removal of azo dyes and aromatic amines from the water phase.

35.6.2 Biosorption of Dyes

35.6.2.1 Bacteria

The focus of dye decolorization majorly rely on studying the biodegradation/decolorization potential of bacteria and less attention has been given to the dead bacterial biomass for biosorption of dyes (Sheu and Freese 1973; Beveridge 1981;

Dijkstra and Keck 1996; Hu 1996; Beveridge 1999; Forgacs et al. 2004). The mode of solute uptake by dead/inactive cells is extracellular; the chemical functional groups of the cell wall play vital roles in biosorption (Vijayaraghavan and Yun 2007a, b; Srinivasan and Thiruvenkatachari 2010). The mode of solute uptake by dead/inactive cells is extracellular. The chemical functional groups present on the bacterial cell wall include carboxyl, phosphate, amine and hydroxyl groups play vital roles in biosorption (van der Wal et al. 1997). Several dye molecules, which exist as dye cations in solutions, are also attracted towards carboxyl and other negatively charged groups. Also, amine groups adsorb anionic dyes via electrostatic interaction or hydrogen bonding. Vijayaraghavan and Yun (2007a, b) observed that the amine groups of *C. glutamicum* were responsible for the binding of reactive dye anions via electrostatic attraction. Carboxyl, amine, phosphonate, sulfonate and hydroxyl groups have become well established as being responsible for dye binding.

With respect to the bacterial dye biosorption, Zhou and Zimmermann (1993) used the actinomycete *Streptomyces* BW130 as an adsorbent for the decolorization of effluents containing anthroquinone, phthalocyanine, and azo dyes and found that decolorization was through adsorption of these dyes instead of any degradation. Other Cu-based azo dyes, such as formazan-copper complex dyes, were completely decolorized through degradation by the same actinomycete strains (Zhou and Zimmermann 1993). Later on, in a study by Hu (1996), three Gram-negative bacteria (*Aeromonas* sp., *P. luteola* and *Escherichia coli*), two Gram-positive bacteria (*B. subtilis* and *Staphylococcus aureus*) and activated sludge (consisting of both Gram-negative and Gram-positive bacteria) were used as biosorbents for the removal of reactive dyes (Reactive Blue, Reactive Red, Reactive Violet and Reactive Yellow) and observed that dead cells of test genera showed a higher uptake of dyes than living cells. The observation was attributed to the increased surface area of Gram-negative bacteria. Won et al. (2005) identified *Corynebacterium glutamicum* as a potential biosorbent of Reactive Red 4, which can bind 104.6 mg/g at pH 1.

The bacterium used as biosorption agent for dye decolorization is listed in Table 35.6.

35.6.2.2 Fungi

A wide variety of fungal organisms are capable of decolorizing a wide range of dyes (Fu and Viraraghavan 2001a, b). Many genera of fungi have been employed either in living or inactivated form. The use of white-rot fungi such as *P. chryso sporium* in decolorizing textile wastewater has been widely reported in literature (Bilgic et al. 1997; Cammarota and Sant Anna 1992; Lankinen et al. 1991; Tatarko and Bumpus 1998; Young and Yu 1997; Gomaa et al. 2008; Sharma et al. 2009; Faraco et al. 2009). A list of fungi used for dye decolorization through biosorption is given in Table 35.6.

In addition to biodegradation, a biosorption mechanism might also play an important role in the decolorization of dyes by living fungi. For dead cells, the

Table 35.6 List of various microbes used in biosorption of different dyes

Culture	Culture name	Dyes	Reference
Fungi	*Aspergillus niger*	Acid Blue 29, Basic Blue 29, Basic Blue 9, Congo Red, Disperse Red 1, Gryfalan Black RL	Fu and Viraraghavan (2001a, b), Fu and Viraraghavan (2003), Khalaf (2008), and Aksu and Karabayur (2008)
	Aspergilllus foetidus	Reactuve Black 5	Patel and Suresh (2008)
	Cunninghamella elegans	Direct Red 80, Reactive Blue 214, Reactive Blue 19	Prigione et al. (2008)
	Dichomitus squalens	Coracryl Black, Coracryl Pink, Coracryl violet, Coracryl Red, Reactive Yellow, Reactive Red	Chander and Arora (2007)
	Funalia trogii	Astrazone Blue FGRL; Cibracron Red	Asma et al. (2006)
	Neurospora crassa	Acid Red 57	Akar et al. (2006)
	Phaenerochaete chrysosporium	Direct dyes; Astrazone Blue FGRL; Cibracron Red	Asma et al. (2006) and Pazarlioglu et al. (2005)
	Polyporus sanguineus	Coracryl Black, Coracryl Pink, Coracryl Red,	Chander and Arora (2007)
	Rhizomucor pusillus	Direct Red 80, Reactive Blue 214, Reactive Blue 19	Prigione et al. (2008)
	Rhizopus arrhizus	Reactive Orange 16, Reactive Red 4, Reactive Blue 19, Reactive Black 5; Germazol Torquoise Blue G; Gryfalan Black RL	O'Mahony et al. (2002), Aksu and Tezer (2000), Aksu and Cagatay (2006), and Aksu and Karabayur (2008)
	Rhizopus nigricans	Reactive Green, Reactive Blue	Kumari and Abraham (2007)
	Rhizopus stolonifer	Reactive Orange 16, Bromophenol Blue	Zeroual et al. (2006)
	Trametes versicolor	Direct Blue 1	Bayramoglu and Arica (2007)
Yeasts	*Debaromyces polymorphus*	*CI Reactive Black*	Yang et al. (2005)
	Kluvromyces marxianus	*Remazol Black B*	Meehan et al. (2000)
	Rhodotorula rubra	*Crystal violet*	Kwasniewska (1985)
	Candida guilliermondii	Reactive black	Aksu and Donmez (2003)
	Candida tropicalis	Remazol blue	Yang et al. (2003)
	Candida zeylanoides	Azo dyes	Martins et al. (1999)
	Saccharomyces cerevisiae	Reactive Green, Reactive Blue 38 and 3	Kumari and Abraham (2007)
Bacteria	Bacterial consortium	Reactive Black B	Kilic et al. (2007)
	Bacillus subtilis	Reactive dyes	Hu (1996)

(continued)

Table 35.6 (continued)

Culture	Culture name	Dyes	Reference
	Corynebacterium glutamicum	Reactive Black 5	Vijyaraghvan and Yun (2007)
	Streptomyces BW 130	Azo-reactive Red 147, Azo-copper Red 171	Zhou and Zimmermann (1993)
	Streptomyces rimosus	Methylene Blue	Nacera and Aicha (2006)
	Staphylococus aureus	Reactive dyes	Hu (1996)
Algae	*Azolla filiculoides*	Acid Red 88, Acid Green 3, Acid Orange 7, Acid Blue	Padmesh et al. (2006a)
	Azolla rongpong	Acid Green 3	Padmesh et al. (2006b)
	Caulerpa scalpelliformis	Basic Yellow	Padmesh et al.(2005)
	Chlorella vulgaris	Remazol Black-B	Aksu and Tezer (2005)
	Cosmarium sp	Malachite Green	Aravindhan et al. (2007)
	Enteromorpha prolifera	Acid Red	Ozer et al. (2005)
	Spirogyra sp.	Synazol	Khalaf (2008)

mechanism is biosorption, which involves physico-chemical interactions such as adsorption, deposition, and ion-exchange. Decolorization of dye wastewater by fungal biomass has been extensively reviewed by Fu and Viraraghavan (2001a), Singh (2006) and Kaushik and Malik (2009). However, limited information is available on interactions between dead fungal biomass and a variety of dyes with complex molecular structures. Fu and Viraraghavan (2002) studied the roles played by functional groups such as carboxyl, amino, phosphate and lipid fractions present in fungal biomass of *A. niger* in biosorption of four different dyes. The adsorption capacity of biomass can be increased (Aksu 2005) by certain physical or chemical pretreatments. Zeroual et al. (2006) reported enhanced biosorption capacity of *Rhizopus stolonifer* for the dye Bromophenol blue after NaOH pretreatment. Interestingly, Bayramoglu and Arica (2007) increased the biosorption capacity of *T. versicolor* from 101.1 to 152.3 mg/g after heat treatment suggesting the surface properties of fungal biomass was modified due to heat.

35.6.2.3 Yeast

Yeast decolorization and degradation of dyes has not been extensively studied. Biosorption of textile dyes has been found to occur by biomass derived from yeast *K. marxianus* IMB3 (Bustard et al. 1998). *K. marxianus* IMB3 was also found to decolorize Remazol Black-B through physical adsorption (Meehan et al. 2000). The oxidative yeasts *Rhodotorula* sp. and *R. rubra* were found to degrade crystal violet completely in 4 days (Kwasniewska 1985). The list of yeast used for dye biosorption is summarized in Table 35.6.

35.6.2.4 Algae

Algae have been found to be potential biosorbents because of their availability in both fresh and saltwater. The biosorption capacity of algae is attributed to their relatively high surface area and high binding affinity (Donmez and Aksu 2002; Tien 2002). Cell wall properties of algae such as electrostatic attraction and complexation are known to play a vital role in biosorption (Satiroglu et al. 2002). The dye decolorization by algae is mainly due to the accumulation of dye ions on their surface (Ozer et al. 2006). Extracellular polymers consist of surface functional groups, which enhance biosorption of the dye molecules onto the surface of the polymer (floc) during dye removal process (Mohan et al. 2002; Shukla et al. 2002). Moreover, it has been reported that the released metabolic intermediates, which have excellent coagulation capacity along with the dye remaining in the aqueous phase tend to adsorb and coagulate on surface (Mohan et al. 2002). While, removal of Acid Red 274 dye using inactivated *Spirogyra rhizopus* system was attributed to biosorption and biocoagulation (Ozer et al. 2006). A list of algae used for biosorption of dye containing effluent is presented in Table 35.6.

35.7 Mechanism of Dye Decolorization

The microbial dye decolorization studies have revealed that a two-step mechanism *viz* the physical adsorption and enzymatic degradation are involved in dye decolorization. Knapp and Newby (1999) observed that in many cases adsorption of dye on the microbial cell surface is the primary mechanism of decolorization, while, Young and Yu (1997), suggested the binding of dyes to the fungal hyphae and physical adsorption and enzymatic degradation by extracellular and intracellular enzymes as reasons for the color removal. Moreover, it has also been reported that the dyes with different structures are decolorized by different enzymes with varied different decolorization rates (Abadulla et al. 2000).

The inherent complexity of both the dye's structures and the enzymatic transformation mechanisms make the elucidation of the degradation pathways a difficult task. Enzymes such as lignin peroxidase, manganese peroxidase and laccase are involved in lignin degradation, which participate in the decolorization of the dyes (Conneely et al. 2002; Singh et al. 2004; Chen et al. 2005; Chander and Arora 2007; Diwanian et al. 2010), however the role of these enzymes in decolorizing process is not clear. Several works refer to the LiP of *P. chrysosporium* as being the main decolorizing agent, while in a recent contrast investigation of the degradation of selected phthalocyanin dyes and their degradation products showed the involvement of Lac and MnP (Conneely et al. 2002). The findings of Kirby et al. (2000) demonstrated that Lac is involved in the decolorization of textile dyes by *P. tremellosa*, however another process must account for the remaining color removal that is observed in the absence of detectable levels of this enzyme.

A direct correlation between lignin modifying enzyme (LME) production and industrial effluent decolorization was given by Wesenberg et al. (2002), suggesting

a differential LME production pattern. The mechanism of azo dye oxidation by peroxidases involves the oxidation of the phenolic groups followed by its cleavage to phenyl diazine, which further can be oxidized by one-electron reaction generating N_2 (Paszczynski and Crawford 1991; Paszczynski et al. 1992; Spadaro and Tenganathan 1994).

While, in bacteria that possess electron transport systems the transfer of electrons from the respiratory chain to appropriate redox mediators could take place directly. While, in obligate anaerobic bacteria the intracellular azoreductases showed high specificity to dye structures and reductively cleaved their carboxylated as well as their sulfonated structural analogs. Now the sequences of genes encoding these three specific azoreductases are known (Suzuki et al. 2001; Blumel et al. 2002; Blumel and Stolz 2003). Amino acid sequence alignments did not show any noticeable homology between this azoreductase and two other well-characterized azoreductases from *X. azovorans* KF 46 and *P. kullae* (Blumel and Stolz 2003). Interestingly, nonspecific enzymes catalyzing azo bond reduction have been isolated from aerobically grown cultures of *Shigella dysenteriae* (Ghosh et al. 1992), *E. coli* (Nakanishi et al. 2001), *Bacillus* sp. (Maier et al. 2004), *S aureus* (Chen et al. 2005) and *P. aeruginosa* (Nachiyar and Rajkumar 2005) have been shown to be flavoproteins, in which the cytosolic flavin-dependent reductases transfer electrons via soluble flavins to azo dyes. Russ et al. (2000) with a recombinant *Sphingomonas* strain-BN6 have shown that the reduction of sulfonated azo dyes by cytosolic flavin-dependent azo reductases is mainly observed in vitro and is of little importance in vivo.

Currently there appears to be no available information about the transport systems for these dyes, although there are few reports on systems which are involved in the transport into bacterial cells of other sulfonated substrates, such as p-toluene sulfonate, taurine and alkane sulfonates (Locher et al. 1993; Eichhorn et al. 2000). The ability of *X. azovorans* KF46F and *Sphingomonas* sp. strain ICX to take up AO7 and to reduce the dye in vivo shows the presence of transport as well as azoreductase enzymes in these organisms. Construction of recombinant organisms with sulfonated dye decolorizing ability, therefore, may require the transfer of the aerobic azoreductase gene into bacterial strains that are able to grow on sulfonated aromatics. These organisms generally exhibit narrow substrate specificity. Studies on 4-ABS degrading strains have also shown that they are highly specific as they can utilize only 4-ABS and not other benzenesulfonates (Feigel and Knackmuss 1993; Singh et al. 2004). This suggests the presence of highly specific transport systems for the uptake of aromatic sulfonates in these cultures. Thus the derived recombinants may still have restricted substrate specificity.

35.8 Conclusion and Future Prospects

The presence of dyes imparts and intense color to effluents, which leads to environmental as well as aesthetic problems. The treatment of dye containing wastewater and its decolorization is still an arduous task. As regulations are becoming even more stringent, there is an urgent need for technically feasible and cost-effective

methods. Among various methods used, the biological treatment appears to be the most economically viable choices available for effluent treatment. Critical analysis of the literature shows that decolorization of dyes by white rot fungi offers several advantages over the conventional treatments due to its potential ligninolytic enzyme system. It is forecasted that enzyme based treatment systems will be the technologies of the future. Besides decolorization, biosorption of dyes are also emerging as a promising alternative to conventional treatment system. The presence of variety of functional groups in the biosorbents makes them selective and highly capable of biosorbing and biodegrading dyes from textile effluents. A deeper examination of the biological process kinetics should be carried out to analyze the influence of operational variables on the decolorization to enhance their efficiency. The biodegradation ability can also be enhanced by adapting them to higher concentration of dyes, which will led to forced or directed evolution. In addition, genetically engineered strains, suitable for degradation process are still a need to assess the extent of mineralization of aromatic amines, as amines can undergo auto-oxidation, leading to the formation of soluble recalcitrant polymers, which may be toxic.

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