Bioremediation Technologies for the Removal of Pollutants

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Bhupinder Dhir

Abstract

Bioremediation technology involves the use of living organisms like microbes and plants to reduce/degrade, eliminate and transform contaminants present in soils, sediments and water. The technology has gained wider acceptance in the recent years because of its potential to remove various organic and inorganic contaminants from various components of the environment. The technology provides an effective treatment of inorganic and organic contaminants under in situ and ex situ conditions by natural means. Potential of microbes and plants both have been exploited to achieve maximum removal/remediation of inorganic and organic contaminants. The biotechnological approaches and genetic engineering strategies have been employed by researchers to improve the efficacy of this technique for achieving complete degradation of contaminants. Enhancement in potential of both plants and microbes for achieving complete remediation of one or more than one pollutant can prove an asset for remediating contaminated sites. The present chapter highlights the role of microbial and phytoremediation in removal of pollutants from the environment.

Keywords

Bioremediation • Contaminants • Microbes • Plants

5.1 Introduction

Environmental contamination with inorganic and organic toxicants has increased over the years due to rapid industrialization, urbanization and anthropogenic activities. The organic contaminants such as petroleum hydrocarbons, pesticides,

B. Dhir (✉)
Department of Genetics, University of Delhi South Campus, New Delhi 110021, India
e-mail: bhupdhir@gmail.com

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agrochemicals, pharmaceutical product and inorganic pollutants such as heavy metals are constantly added in the environment (Agarwal 1998; Zeyaullah et al. 2009). Most of the xenobiotic compounds resist degradation. The remediation or treatment of contaminants done by conventional methods (both physical and chemical) is a costly, time-consuming, invasive approach and causes environmental deterioration (EPA 1999, 2003). According to an estimate, the cleaning/restoring of the contaminated sites in the USA requires a capital investment of approximately US $1.7 trillion. Bioremediation has emerged as a safe, reliable, effective, low-cost and environmentally friendly alternative technology to achieve sustainable remediation of hazardous and recalcitrant pollutants. In this technique, treatment of contaminants can be done at site in a cost-effective, less disruptive, eco-friendly (no by-products, no requirement of complex setups and operations) manner.

The bioremediation technology uses biological processes and naturally occurring catabolic activity of microbes and plants to eliminate, attenuate, or transform inorganic and organic contaminants to less hazardous products such as carbon dioxide and water (Abruscia et al. 2007; Pandey and Fulekar 2012). Biological agents such as yeast, fungi, bacteria and plants remove contaminants by biotransformation and biodegradation mechanisms. The physiological and metabolic capabilities of organisms assist in degrading the pollutants converting them to nontoxic and environmentally safe products. In this technology, target compound is used as a carbon source. The complete mineralization of contaminants results in the formation of H_2O and CO_2 (Strong and Burgess 2008; Sharma and Fulekar 2009).

5.2 Bioremediation

Bioremediation processes have been broadly categorized into two groups.

5.2.1 Ex Situ Bioremediation

In this type of remediation, removal of the contaminant from soil and groundwater is done away from the site (Maheshwari et al. 2014). The treatment of contaminants has been done away from site. This includes bioreactors, biofilters, land farming, bioventing, biosparging, biostimulation and composting methods (Olaniran et al. 2006).

Ex situ bioremediation is of two types.

5.2.1.1 Solid Phase Treatment

It is a treatment process for land and soil contaminated with organic, industrial wastes, municipal wastes and sewage sludge. It includes:

- *Land Farming:* In this technique, contaminated soil is excavated and spread over a prepared bed and periodically tilled to achieve degradation of pollutants. Microorganisms facilitate aerobic degradation of contaminants.
- *Composting:* In this technique, contaminated soil is mixed with nonhazardous organic amendments such as manure or agricultural wastes. The presence of organic materials supports the growth of microbial population.
- *Biopiles:* Biopiles are a hybrid of land farming and composting. Engineered cells are constructed as aerated composted piles. Contaminated material is mixed with a bulking agent and aerobic, thermophilic bacteria are used in the treatment process.
- *Bioreactors:* In this technique, biodegradation is carried out by microbes in a container. It is used to treat organic contaminants from liquids or slurries.
- *Bioventing:* It involves supplying air and nutrients through wells to contaminated soil to stimulate the indigenous bacteria. The low airflow rates provide the amount of oxygen necessary for the biodegradation while minimizing volatilization and release of contaminants to the atmosphere. It is used to treat hydrocarbons.
- *Bioaugmentation:* It involves introduction of exogenic microorganisms (sourced from outside the soil environment) capable of detoxifying a particular contaminant. The addition of contaminant-degrading organisms accelerates the transformation rates (El Fantroussi and Agathos 2005; Thierry et al. 2008). Enhanced chlorpyrifos biodegradation has been reported via this process.
- *Biosparging:* This involves the injection of air under pressure to increase groundwater oxygen concentrations and enhance the rate of biological degradation of contaminants by naturally occurring bacteria.
- *Biostimulation:* This involves the addition of soil nutrients, trace minerals, electron acceptors, or electron donors to enhance the biotransformation of a wide range of soil contaminants (Li et al. 2010). Trichloroethene and perchloroethylene are reported to be completely converted to ethane by microorganisms in a short span of time with the addition of lactate as biostimulation (Shan et al. 2010). Electron shuttles such as humic substances (HS) stimulate anaerobic biotransformation of organic pollutants through enhancing the electron transfer speed.

5.2.1.2 Slurry Phase

In this type of bioremediation, contaminated soil is combined with water, other additives and microbes in a bioreactor. Nutrients and oxygen are added, and conditions are controlled to create the optimum environment for the microorganisms to degrade the contaminants. Slurry reactors are used for treatment of contaminated soil and water.

5.2.2 In Situ Bioremediation

In situ technique is applied to treat contaminated soil and groundwater. This involves addition of indigenous or naturally occurring microbial populations by feeding nutrients and oxygen to increase their metabolic activity. Oxygen, electron acceptors, and nutrients (nitrogen and phosphorus) promote microbial growth. The treatment is done on the site without any need to excavate or remove soils or water in order to accomplish remediation (Vidali 2001).

5.3 Microbial Remediation

5.3.1 Contaminants Removed by Microbes

Naturally occurring bacteria and fungi degrade/detoxify hazardous substances. Aerobic and anaerobic bacteria degrade various inorganic and organic contaminants (Kumar et al. 2011). Aerobic bacteria such as *Pseudomonas*, *Alcaligenes*, *Sphingomonas*, *Rhodococcus* and *Mycobacterium* degrade pesticides, hydrocarbons, alkanes and polyaromatic compounds and use the contaminant as the sole source of carbon and energy. Anaerobic bacteria degrade polychlorinated biphenyls (PCBs) and organic solvents such as trichloroethylene (TCE) and chloroform. Dioxygenases and monooxygenases are two of the primary enzymes employed by aerobic organisms during transformation and mineralization of xenobiotics, while anaerobic microbes use range of electron acceptors such as NO_3-, Fe, Mn, SO_4^{2-} and CO_2 depending on their availability and the prevailing redox conditions. Methane monooxygenase degrade various substrates such as chlorinated aliphatic trichloroethylene and 1,2-dichloroethane.

Microbes form an important part of consortium that assist in degrading contaminants. These include *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Beijerinckia*, *Flavobacterium*, *Methylosinus*, *Mycobacterium*, *Myxococcus*, *Nitrosomonas*, *Nocardia*, *Penicillium*, *Phanerochaete*, *Pseudomonas*, *Rhizoctonia*, *Serratia*, *Trametes* and *Xanthobacter* (Table 5.1). The complete mineralization involves synergism and cometabolism. Cometabolism of xenobiotics is required when the compound cannot serve as a source of carbon and energy. Hydrocarbons and persistent organic pollutants (POPs) such as dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyls (PCBs), dioxins etc. are degraded in the soil by bacteria present in the rhizosphere (Olson et al. 2003). Acidophilic bacteria like *Acidithiobacillus ferrooxidans* (Takeuchi et al. 2005) and sulfur-oxidizing bacteria remove high concentrations of As, Cd, Cu, Co, and Zn from contaminated soils. Pesticides have also been successfully removed by bacteria. *Providencia stuartii* strain depicts potential for degradation of chlorpyrifos (Surekha Rani et al. 2008). Isolates of *Bacillus*, *Staphylococcus*, and *Stenotrophomonas* from cultivated and uncultivated soil are able to degrade dichlorodiphenyltrichloroethane (DDT) (Kanade et al. 2012). Bacterial strains are able to degrade azo dyes under aerobic and anaerobic conditions (Dos Santos et al. 2007).

Table 5.1 Contaminants removed by bacterial species

Contaminants	Bacterial species
PCB	*Rhodococcus*, *Luteibacter*, *Williamsia*
Malathion	*Azospirillum lipoferum*
PAH	*Lysinibacillus*
Hydrocarbon	*Bacillus*, *Corynebacterium*, *Staphylococcus*, *Streptococcus*, *Shigella*, *Alcaligenes*, *Acinetobacter*, *Escherichia, Klebsiella*, *Enterobacter*
Aromatic hydrocarbon	*Mycobacterium*, *Corynebacterium*, *Aeromonas*, *Rhodococcus*, *Bacillus*
PCB	*Pseudomonas*, *Burkholderia*, *Ralstonia*, *Achromobacter*, *Sphingomonas*, *Rhodococcus*, *Janibacter*, *Bacillus*, *Paenibacillus*, *Microbacterium*
Pesticides (chlorpyrifos, DDT)	*Bacillus*, *Staphylococcus*, *Stenotrophomonas*
Dyes	*Proteus* sp., *Pseudomonas* sp., *Enterococcus* sp., *Shewanella decolorationis*
Metals (*Hg*)	*Alcaligenes faecalis*, *Bacillus pumilus*, *P. aeruginosa*, *Brevibacterium iodinum*

Table 5.2 Fungal species with the potential for removing various contaminants

Contaminants	Microbial species	References
Fungi		
Oil hydrocarbons	*Aspergillus*, *Cephalosporium*, *Penicillium*	Singh (2006)
Aliphatic hydrocarbons	*Cladosporium*, *Aspergillus*	Singh (2006)
Uranium (U), thorium (Th)	*Rhizopus arrhizus*	Treen-Sears et al. (1998)
Yeasts		
Alkane	*Candida lipolytica*, *C. tropicalis*, *Rhodotorula rubra*, *Aureobasidium (Trichosporon) pullulans*	De Cássia Miranda et al. (2007)
Diesel oil	*Rhodotorula aurantiaca*, *C. ernobii*	De Cássia Miranda et al. (2007)
Aniline azo dye	*C. methanosorbosa* BP-6	Mucha et al. (2010)

Mycoremediation is a form of bioremediation in which fungi especially white rot fungus such as *Phanerochaete chrysosporium* degrade diverse range of persistent or toxic environmental contaminants (Singh 2006) (Table 5.2). The fungal mycelium secretes extracellular enzymes and acids that break down lignin and cellulose (Eaton 1985). Microfungi transform aromatic organopollutants cometabolically including polycyclic aromatic hydrocarbons (PAHs) and biphenyls, dibenzofurans,

nitroaromatics, and various pesticides (Fritsche and Hofrichter 2008). Plant growth-promoting rhizobacteria (PGPR), endophytic bacteria and other rhizospheric bacteria have been shown to potentially degrade toxic organic compounds in contaminated soil (Sylvestre et al. 2009). *Pseudomonas* sp. specifically has shown potential for hydrocarbon-degrading capacity. Yeast species such as *Trichosporon cutaneum* also utilize aromatic compounds as growth substrates.

5.4 Mechanisms of Removal of Contaminants by Microbes

The inorganic contaminants removed by bacteria mainly include heavy metals and radionuclides. Heavy metals are removed via biosorption (metal sorption to cell surface by physicochemical mechanisms), bioleaching (heavy metal mobilization through the excretion of organic acids or methylation reactions), biomineralization (heavy metal immobilization through the formation of insoluble sulfides or polymeric complexes), intracellular accumulation and enzyme-catalyzed transformation (redox reactions) mechanisms (Lloyd and Lovley 2001). The resistance to heavy metal toxicity occurs by adsorption, uptake, methylation, oxidation, and reduction mechanism. Metals are also precipitated as insoluble sulfides via metabolic activity of sulfate-reducing bacteria. Heavy metal ions can be entrapped in the cellular structure and subsequently biosorbed onto the binding sites present in the cellular structure. They pass into the cell across the cell membrane through the cell metabolic cycle. Toxic radionuclides such as U and Th from nuclear waste streams are removed by similar mechanisms (PinakiSar et al. 2004).

Both anaerobic and aerobic bacteria are capable of metabolizing organic pollutants. The initial intracellular attack of organic pollutants is an oxidative process, and the enzymatic key reaction is catalyzed by oxygenases and peroxidases. Peripheral degradation pathways convert organic pollutants step by step into intermediates of the central intermediary metabolism. Cytochrome P450 alkane hydroxylases play an important role in the microbial degradation of oil, chlorinated hydrocarbons, fuel additives, and many other compounds. The degradation of hydrocarbons is carried out under aerobic condition and is mediated by specific enzyme system. Enzymes involved in degradation of xenobiotics mainly include oxygenases. Higher chlorinated PCBs are reduced by anaerobic microorganisms, while lower chlorinated biphenyls are oxidized by aerobic bacteria (Seeger et al. 2001). Aerobic catabolic pathway for PCB degradation involves steps catalyzed by enzymes, biphenyl dioxygenase (BphA), dihydrodiol dehydrogenase (BphB), 2,3-dihydroxybiphenyl dioxygenase (DHBD) (BphC) and hydrolase (BphD) (Taguchi et al. 2001).

Fungi are an important part of degrading microbiota because, like bacteria, they metabolize dissolved organic matter. Extracellular multienzyme complexes of fungi are efficient in breaking down the natural polymeric compounds. By means of their hyphal systems, they are also able to colonize and penetrate substrates rapidly and transport and redistribute nutrients within their mycelium (Fritsche and Hofrichter 2005). Hyphal penetration provides a mechanical adjunct to the

chemical breakdown affected by the secreted enzymes. The high surface-to-cell ratio characteristic of filaments maximizes both mechanical and enzymatic contact with the environment. Second, the extracellular nature of the degradative enzymes enables fungi to tolerate higher concentrations of toxic chemicals. Among the filamentous fungi, the ligninolytic ones have been specifically investigated because of their extracellular, specific oxidoreductive enzymes that have been already successfully exploited in the degradation of many aromatic pollutants. Studies with *Aspergillus niger* AB10 Cd and *Rhizopus arrhizus* M1 have indicated Pb binding occurs via the functional groups on the cell surface. The functional groups act as ligands for metal sequestration (Pal et al. 2010). The proteins in the cell walls of AMF appear to have similar ability to sorb potentially toxic elements by sequestering them. Filamentous fungi may degrade pesticides using two types of enzymatic systems: intracellular (cytochromes P450) and exocellular (lignin-degrading system mainly consisting in peroxidases and lactases) (Chaplain et al. 2011). Yeast species use n-alkanes and other aliphatic hydrocarbons as a sole source of carbon, and energy is mediated by the existence of multiple microsomal cytochrome P450 forms.

5.5 Phytoremediation

The capacity of plants for removing and degrading various inorganic and organic contaminants from different components of the environment is referred as phytoremediation (Salt et al. 1998; Meagher 2000; Pilon-Smits 2005). It is a cost-effective, nonintrusive, aesthetically pleasing technology that removes contaminants via processes such as degradation, sequestration, or transformation mechanisms (Raskin and Ensley 2000; Garbisu et al. 2002; McCutcheon and Schnoor 2003). The major advantage of using this technology is that treatment can be done under in situ. The plants have been successfully used in removing contaminants such as explosives (trinitrotoluene), herbicides, pesticides and metals from different areas such as military areas, agricultural fields, industrial areas, mine tailings, sewage, municipal wastewater, drainage water and landfill leachate. The plants species with an effective remediation potential include mustard, alpine pennycress, hemp, and pigweed. The major concern about phytoremediation technology is that it is a time-consuming process and depends on the plant's ability to grow and thrive in contaminated environment.

Potential of both terrestrial and aquatic plant species has been exploited for removing contaminants from the environment. Efficacy of phytoremediation varies according to varieties, cultivars, genotypes and type of pollutant (Dipu et al. 2011). The selection of the plant species is very crucial for the success of this technology. Plants with less maintenance and acclimatization in native climate conditions are favored. Each plant species depicts a variation in its ability to remove contaminants from the environment. The selection of plant species depends upon factors such as:

- Tolerance to the environment
- Uptake, translocation and accumulation ability of the plant
- High growth rates and biomass production
- Tolerance to environmental conditions such as drought, salinity, etc.
- Availability of the species (annual/perennial)

Among terrestrial plants, trees and grass species with the characteristics such as deep roots, high biomass production, and fast growth are commonly preferred for remediation (EPA 1998; Schnoor 2000). Trees stabilize a pollutant and minimize spread of contaminant. Strong and dense root system (around 3 meters deep) in grasses assists in higher uptake of contaminants. The tolerance to extreme climatic variations such as drought, flood, submergence, fire, and heat and wide range of soil acidity, alkalinity, salinity and sodicity establish plants as ideal candidates for phytoremediation. *Populus deltoides* (hybrid poplar), *Brassica juncea* (Indian mustard), *Helianthus annuus* (sunflower), *Thlaspi* sp. including *T. caerulescens* and *T. rotundifolium*, *Vetiveria zizanioides*, and *Paspalum conjugatum* are some of the plant species with high capacity for removal of contaminants.

Among aquatic plant species, free-floating, submerged, and emergent forms exhibit exorbitant capacity for removal of various contaminants including heavy metals, radioactive wastes, nutrients, explosives, organic xenobiotics, and herbicides/pesticides from municipal and industrial wastewater. Features such as easy cultivation, high biomass production, faster growth rate, surplus availability and high tolerance to survive adverse environmental conditions assist in removal of contaminants and make them an ideal and most suitable candidate for use in phytoremediation technology. Aquatic plant species with high contaminant removal ability include *Eichhornia crassipes* (water hyacinth), *Salvinia herzogii*, *Salvinia minima* (water ferns), *Pistia stratiotes* (water lettuce), *Nasturtium officinale* (watercress), *Spirodela intermedia*, *Lemna minor* (duckweeds), *Azolla pinnata* (water velvet), *Potamogeton pectinatus* (American pondweed), *Ceratophyllum demersum* (coontail or hornwort), *Myriophyllum spicatum* (parrot feather), *Typha latifolia* (cattail), *Phragmites* (common reed) and *Scirpus* spp. (bulrush) (Dhir et al. 2009; Dhir 2013). Aquatic plants form an important component of constructed wetlands that remove many inorganic contaminants including metals, nitrates, phosphates, cyanides, as well as organic contaminants such as explosives and herbicides (Horne 2000; Jacobson et al. 2003; Dhir 2013).

5.5.1 Types of Phytoremediation

Plants remove contaminants by different processes such as phytoextraction/phytoaccumulation, phytodegradation/phytotransformation, phytovolatilization, rhizofiltration/phytofiltration and phytostabilization (Cunningham et al. 1995; Raskin et al. 1997; Salt et al. 1995a, 1998). Inorganic contaminants are removed by phytoextraction and/or phytostabilization processes, while organic contaminants are most commonly treated by phytodegradation and phytostimulation mechanisms.

In phytoextraction/phytoaccumulation process, contaminants are taken up by plants via roots followed by translocation to aboveground plant tissues, which are subsequently harvested (Salt et al. 1995a, b). It is used for removal of contaminants such as metals which cannot be degraded (Cd, Pb, Zn, Ni, Cr, Co, metalloids such as As, Se) and radionuclides (such as ^{90}Sr, ^{137}Cs, ^{238}U). It is also referred as phytoaccumulation, phytoabsorption, phytosequestration, phytomining, or biomining.

In phytodegradation/phytotransformation process, the metabolization and degradation of contaminants takes place within the plant with the help of enzymes produced and released by them. Phytodegradation is most suited for moderately hydrophobic organic chemicals (octanol-water partition coefficients, log Kow = 0.5 ~ 3.0). Plant enzymes such as dehalogenase, peroxidase, nitroreductase, laccase and nitrilase assist in degradation of organic pollutants, such as 2,4,6-trinitrotoluene (TNT) and polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), herbicides, pesticides, BTEX (benzene, toluene, ethylbenzene, and xylene), chlorinated solvents such as trichloroethylene (TCE) and short-chain aliphatic chemicals, explosives such as 2,4,6-trinitrotoluene (TNT), and inorganic nutrients.

In phytovolatilization process, plants take up contaminants through roots followed by their release as volatile chemicals by shoot or leaf surfaces. Biomethylated forms of metals such as Se, As and Hg form volatile molecules (less toxic), which are lost to atmosphere. Selenium is converted to methyl selenate, and the volatile form is released in the atmosphere (Meagher 2000).

In rhizofiltration/phytofiltration process, plant roots absorb, precipitate, and remove contaminants from water in either a hydroponic or a constructed wetland. This process is applicable for removal of inorganic contaminants such as metals (Pb, Cd, Cu, Fe, Ni, Mn, Zn, Cr), nutrients and radionuclide (^{90}Sr, ^{137}Cs, ^{238}U, ^{236}U) present in groundwater, surface water and wastewater (Dushenkov et al. 1995, 1997a, b).

In phytostabilization process, plants immobilize or stabilize contaminants in the soil through accumulation by plant roots or precipitation in the soil by root exudates, thereby reducing the bioavailability of contaminants in the environment. The contaminants are sequestered from the soil and the process is efficient in removing inorganic and organic contaminants from the soils, sediments, and sludges. Contaminants also bind to humic (organic) matter through the process of humification. Phytostabilization of organic contaminants or metabolic by-products is also achieved by attaching to plant components such as lignin which is referred to as "phytolignification" (Cunningham et al. 1995).

5.5.2 Mechanism of Removal of Contaminants

5.5.2.1 Inorganic

Metals and radionuclides are captured by root cells and subsequently translocated to plant parts (symplastic). Metal uptake in plants also involves cation exchange by

cell walls (apoplastic) (Williams et al. 2000; Pollard et al. 2000), or transport via symplastic pathway involves the role of membrane transport proteins (Blaylock and Huang 2000; Pollard et al. 2000). Intracellular high-affinity binding sites facilitate metal uptake across the plasma membrane (Dhankher et al. 2002; Hall 2002; Yang et al. 2005a, b). The natural resistance-associated macrophage (Nramp) family of proteins, cation diffusion facilitator (CDF) family proteins and zinc-iron permease (ZIP) family proteins (Williams et al. 2000) assist in metal transportation across the membranes. Metal chelate complexes are transported across the plasma membrane via specialized carriers. Cadmium is actively transported across the tonoplast of roots via a Cd/[H^+] antiport. CPx-type heavy metal ATPase transport proteins use ATP to pump variety of charged substrates along Cu and/or Cd across cell membranes (Williams et al. 2000). ZIP proteins mainly transport potentially toxic metals (Zn) as well as nutrients (Fe). These include the iron transporter 1 (*ITR1*) gene of *Arabidopsis* which is an iron (Fe [II]) transporter. Subsequent to uptake and translocation, heavy metals are stored in vacuole. Final sequestration of metal ions in chelated form or phytochelatins takes place in vacuole (Kramer et al. 1996). Metals are sequestered by bonding with organic sulfur (R-SH) on the cysteine residues by formation of metallothioneins (MTs) and phytochelatins (PCs). Organic acids, viz., citrate and phytosiderophores such as mugenic and avenic acid chelate metal ions, increase the efficiency for uptake and translocation of metals.

Radionuclides are translocated to the aboveground plant parts through the vascular system via high-affinity K^+ transporters. Translocation is followed by compartmentalization and complexation with ligands present in the cell including proteins, cysteine and glutathione. Radionuclides passively bind to negatively charged groups on the cell surface followed by transport to the cell wall. In active process, metabolically dependent penetration of ions through the cell membrane, movement inside cytoplasm and the bioaccumulation of the metal ions onto the protoplasts take place.

5.5.2.2 Organic

Organic contaminants (xenobiotic compounds) are subjected to partial or complete degradation within plants (Sandermann 1994). Plants absorb xenobiotics by simple diffusion primarily through roots and leaves (Wang and Liu 2007). Uptake and metabolism of hydrophobic organic contaminants is rapid. They are bound strongly to the surface of the roots especially by hemicellulose in the cell wall and the lipid bilayer of plant membranes; hence, their translocation within the plant is slow. They are actively transported through plant membranes (Meagher 2002; Pilon-Smits 2005). Several enzymes including monooxygenases, dioxygenases, dehydrogenases, hydrolases, peroxidases, nitroreductases, nitrilases, dehalogenases, phosphatases and carboxylesterases play an important role in degradation of xenobiotics (Dietz and Schnoor 2001; Pilon-Smits 2005). The detoxification of xenobiotic is carried out in three stages, namely, transformation, conjugation and sequestration.

Xenobiotics generally undergo transformation via chemical modification (oxidation, reduction, hydrolysis), conjugation (with glutathione, sugars, amino acids

resulting in soluble, polar compounds), and sequestration or compartmentalization (conjugants are converted to other conjugates and deposited in plant vacuoles or bound to the cell wall and lignin) (Cherian and Oliveira 2005). Oxygenation increases water solubility and provides site for conjugation via glycosidic bond formation. The reaction is catalyzed by enzymes such as P450 monooxygenases, carboxylesterases, cytochrome P450 and peroxidases. Oxidation reactions are followed by reduction and/or hydrolysis reactions after which conjugation with glutathione (GSH), sugars, or organic acids takes place. Enzymes such as glutathione S-transferases, carboxylesterases, O-glucosyltransferases, O-malonyltransferases, N-glucosyltransferases and N-malonyltransferases are associated with xenobiotic metabolism.

The conjugation-sequestration involve coupling of glucose or malonyl group to the organic compound followed by the transport of the conjugate to the vacuole or the apoplast. Conjugated xenobiotics are then sequestered as part of insoluble cell wall polymers or in cellular compartments such as vacuoles and further metabolized to form CO_2 (Pilon-Smits 2005). Cell compartmentation is mediated by a wide array of glutathione S-transferases (GSTs). ATP-binding cassette (ABC) transporters play a key role in the transfer of conjugates from the cytosol to either the vacuole or the apoplast (Klein et al. 2006).

The metabolism of certain nonagricultural contaminants such as PAHs, TCE, 2,4,6-trinitrotoluene (TNT), glyceryl trinitrate (GTN), and other chlorinated compounds has been well documented in literature (Macek et al. 2000; Alkorta and Garbisu 2001). Poplar trees have shown the potential of oxidizing alkanes, alkenes and methane and their halogenated analogues via dehalogenase enzyme. Dehalogenase(s) ultimately mineralize TCE to CO_2 via an oxidative pathway.

5.6 Factors Affecting Bioremediation Process

The bioremediation processes is regulated by many factors. These mainly include metabolic capacity of the organism, availability of contaminants, and the environmental factors such as type of soil, temperature, pH and the presence of oxygen and nutrients. The compounds either serve as primary or secondary substrate to the organism (Boopathy 2000). Type of contaminants, their concentration and the physicochemical bioavailability of pollutants critically regulate the biodegradation potential. The growth and activity of microbes is affected by pH, temperature and moisture. The rate of enzymatic reactions within microorganisms is also regulated by temperature. After every 10 °C rise in temperature, the rate of biochemical reactions gets doubled due to increase in enzymatic activity. Bacteria found in soil are mesophiles and degrade petroleum hydrocarbons at an optimum temperature ranging from 25 °C to 45 °C. Soil pH is one of the important factors because it affects survival of microbial species and also affects availability of nutrients. Biodegradation of organic contaminants is optimal at a pH range of pH 6–8. Moisture influences the rate of contaminant metabolism because it influences the

kind and amount of soluble materials that are available as well as the osmotic pressure and pH of terrestrial and aquatic systems.

Aerobic or anaerobic conditions also decide the rate and extent of biodegradation process. Hydrocarbons are readily degraded under aerobic conditions, whereas chlorinate compounds are degraded only in anaerobic ones. Stimulation of microorganisms is achieved by the addition of growth substances, nutrients, terminal electron acceptor/donors, or some combination, thereby resulting in an increase in organic pollutant degradation and biotransformation. The process of bioremediation can be enhanced by supplementing microorganisms with nutrients, carbon sources, or electron donors. Establishment of such microbial consortia can be done in several ways, e.g., by promoting growth through addition of nutrients, by adding terminal electron acceptor, or by controlling moisture and temperature conditions (Agarwal 1998). Addition of supplements such as fertilizers, oxygen, etc. assists in bioremediation as they act as biostimulants. Sufficient amount of nutrient and oxygen must be available in a usable form and in proper proportions for unrestricted microbial growth to occur.

Among the biological factors, metabolic ability of microorganisms affects the microbial degradation of organic compounds. The capacity of the plants to remove contaminants varies according to varieties, cultivars or genotypes and type of pollutant (Dipu et al. 2011). The selection of the plant species is very crucial. Plants with less maintenance, acclimatization to varied climate conditions, and increased biomass production are favored. The tolerance to contaminant also regulates the extent of contaminant removal capacity of plants.

5.7 Success Stories in Bioremediation

In situ bioremediation of U-contaminated sites has been conducted successfully with *Desulfosporosinus* spp. and *Closteridium* spp. (Bruschi and Florence 2006). Consortium of SRB (sulfate-reducing bacteria) has been used successfully to remove Zn and sulfate. The metals were precipitated as sulfides. Eight months after project implementation, 80% reduction in Site COC comprised a complex mixture of halogenated organic compound (mixture of brominated and chlorinated organic compounds). A company named TMPD technologies, Lafayette, LA, treated acres of land with multiple contaminants ranging from PCBs to hydrocarbons using microbes. It also removed oil spill from Lake Charles Refinery in Lake Charles, LA, via bioremediation techniques involving biostimulation and bioaugmentation. The Microbiological Resource Centers (MIRCENs) at Cairo, Egypt, is examining the use of microbes in degrading persistent pesticides pollutants.

The companies such as Edenspace Systems Corporation of the USA have successfully used Indian mustard plant to treat the soil contaminated with radionuclide strontium ($Sr^{89/90}$) at Fort Greely in Alaska, USA and Cs^{137} from the contaminated pond waters (Singh et al. 2006). Indian mustard plant was used with sunflower (*Helianthus annuus*) to phytoremediate the Pb-contaminated soil

at industrial facility in Connecticut, USA (Singh et al. 2006). Plants remove contamination by bioaccumulation in aerial parts. The Phytotech, Florida, USA, used the Indian mustard plant to remediate Pb and Cd from contaminated soil at the Czechowice Oil Refinery, Katowice, in Poland (Singh et al. 2006). In Milwaukee, Wisconsin, USA, the Ecolotree Inc. used the hybrid poplar trees to phytoremediate soil and groundwater contamination with petroleum-related organics, PAHs, and chlorinated organic compounds. In Illinois, USA, the Ecolotree Inc. used the hybrid poplar to treat soil contaminated with chemical fertilizer and pesticides. Hybrid polar was successfully used by Phytokinetics Inc., USA, to treat groundwater contaminated with chlorinated volatile organics including dichlorobenzidines and soils contaminated by gasoline and diesel compounds at old gas filling station at Axelved, Denmark, and cyanide, PAHs, oil, and BTEX (benzene, toluene, ethylbenzene, and xylene) in Denmark.

5.8 Genetic Engineering Approach for Improving Bioremediation

The genetic engineering technology has proved useful in improving the bioremediation process (Rugh et al. 1998; Bizily et al. 1999; Joutey et al. 2014). Recombinant DNA techniques enhance the capacity of organisms for degradation and breakdown of toxicants such as hydrocarbons and pesticides. Recombinant DNA techniques help to create organism with an ability to metabolize xenobiotics by detection of genes responsible for enzymes involved in degradation. Transgenic plants show improved metal tolerance, accumulation and enhanced capacity for degradation of organic compounds (Meagher 2000; Kramer and Chardonnens 2001; Pilon-Smits 2005). The genes encoding for biodegradative enzymes are present in chromosomal and extrachromosomal DNA of microbes. Plasmid exchange results in the production of novel microbial strains with a large number of degradative capabilities.

Inorganic contaminant removal is achieved via plants engineered to improve pollutant uptake by overexpression or knockdown of specific membrane transporter proteins or enzymes, root-shoot translocation abilities, sequestration and volatilization. The expression of the introduced gene is regulated by promoters. The protein may be directed to different cellular compartments, such as the chloroplast, the vacuole, or the cell wall. Various transgenic plants were created with metal tolerance and accumulation properties, either by overexpression of membrane transporter proteins (Hirschi et al. 2000; Song et al. 2003) or by overproduction of chelator molecules (Zhu et al. 1999a, b; Dhankher et al. 2002). Transgenic plants have been raised by transfer of metal hyperaccumulator genes to high-biomass, fast-growing species (Chaney et al. 2000; LeDuc et al. 2004). Synthesis of metal chelators leading to enhanced metal uptake, translocation, and sequestration has been overexpressed in plants (Cherian and Oliveira 2005; Pilon-Smits 2005). The biosynthesis of MTs is regulated at the transcriptional level and is induced by several factors, such as hormones, cytotoxic agents, and metals, including Cd, Zn, Hg, Cu, Au, Ag, Co, Ni, and Bi (Yang et al. 2005a, b). Phytochelatins are a class of

posttranslationally synthesized (cysteine-rich metal-chelating) peptides that play a pivotal role in heavy metal tolerance in plants by chelating these substances and decreasing their free concentrations (Vatamaniuk et al. 1999). Metal-tolerant tobacco (*Nicotiana tabacum*) has been developed by expressing a yeast metallothionein gene for higher tolerance to Cd. *Brassica juncea* was genetically engineered with *E. coli gshl gene* for increased glutathione and phytochelatin production for high Cd tolerance and high concentrations of phytochelatins (Fulekar et al. 2009). Overexpression of a bacterial glutathione synthetase (GS) for higher GSH and PC concentrations and increased Cd tolerance/accumulation by *Brassica juncea* has also been noted. Overexpression of plant phytochelatin synthase (PS) in transgenic yeast increases tolerance and accumulation of Cd. Manipulation of GSH and PC concentrations increases potential for increasing the accumulation of toxic metals by plants. Abhilash et al. (2009) reported the introduction of genes for enzyme glutathione S-transferase (GST) (responsible for GSH synthesis), by introduction of a [gamma]-glutathione synthetase into *Populus trichocarpa* (Gullner et al. 2001). For heavy metals, Sriprang et al. (2003) introduced *Arabidopsis thaliana* gene for phytochelatin synthase (PCS; PCSAt) into *Mesorhizobium huakuii* subsp. rengei strain B3 and then established the symbiosis between *M. huakuii* subsp. rengei strain B3 and *Astragalus sinicus*. The gene was expressed to produce phytochelatins and accumulate Cd^{2+}, under the control of bacteroid-specific promoter, the nifH gene.

Some genes for increased heavy metal (Cd) resistance and uptake, like *AtNramps* (Thomine et al. 2000), *AtPcrs* (Song et al. 2004) and *CAD1* (Ha et al. 1999) from *Arabidopsis thaliana*; *gshI*, *gshII* (Zhu et al. 1999a), and PCS cDNA clone (Heiss et al. 2003) from *Brassica juncea*, tobacco (Goto et al. 1998) and rice (Goto et al. 1998); *ferritin* from soybean for increased Fe accumulation; and *merA* from bacteria to *A. thaliana* and tobacco for resistance to Hg with gene (Bizily et al. 1999; Eapen and D'Souza 2005), have been introduced into plants. Transgenics have also been raised for Se tolerance with a bacterial glutathione reductase in the cytoplasm and chloroplast for Indian mustard. Transgenic *A. thaliana* plants expressing SRSIp/ArsC and ACT 2p/γ-ECS with high tolerance to As than wild plants and transgenic plants expressing γ-ECS or ArsC alone have also been reported (Dhankher et al. 2002; Mello-Farias and Chaves 2008). Studies also report overexpression of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase for an enhanced metal accumulation (Eapen and D'Souza 2005).

The genes for phytovolatilization have also been introduced into plants. Introduction of bacterial mercury reductase (*MerA*) and organomercurial lyase (*MerB*) genes into plants such as *Arabidopsis thaliana* increases plants' tolerance to Hg. Toxic organic mercuric compounds are converted into volatile elemental Hg (Rugh et al. 1996; Bizily et al. 2000; Dhankher et al. 2002; Eapen and D'Souza 2005). Overexpression of two key enzymes, cystathionine gamma-synthase and selenocysteine methyltransferase, which promote the conversion of selenocysteine to volatile Se has also been reported (van Huysen et al. 2003; LeDuc et al. 2004). Transgenic plants engineered to have enhanced sulfate/selenate reduction showed fivefold higher Se accumulation in the field (Bañuelos et al. 2005). Transgenic

Arabidopsis plants which could transport oxyanion arsenate to aboveground, reduce to arsenite, and sequester it to thiol peptide complexes by transfer of *Escherichia coli C* and *γ-ECS* genes have been developed (Eapen and D'Souza 2005).

The degradation of organic pollutant can be improved by overexpressing enzymes that facilitate degradation in plant tissue or rhizosphere. The genes procured from other organisms such as bacteria or mammals are introduced in plants. The transformed organisms possess the enzymatic machinery required to achieve a complete mineralization of organic molecules. Specific proteins or peptides for binding and transporting xenobiotics and enzymes involved in biodegradation have been introduced or overexpressed in plants to achieve compete degradation. The genetically transformed plants for degrading herbicides, organomercurials, phenolic compounds, PCBs and nitroaromatics (Bizily et al. 1999; Karavangeli et al. 2005; Rylott et al. 2006; Mohammadi et al. 2007) include *Arabidopsis*, tobacco (*Nicotiana tabacum*), Indian mustard (*Brassica juncea*), hybrid poplar (*Populus* sp.), and yellow poplar (*Liriodendron* sp.). Transgenic wetland species include *Spartina* spp., reeds and *Typha* spp. (Czako et al. 2005). Abhilash et al. (2009) reported the introduction of genes and enzymes such as mammalian cytochrome p450s gene into rice plant.

The genes coding for cytochrome P450 and GST for the enhanced degradation and remediation of herbicides, explosives, PCBs etc. have been overexpressed in plants. Increased expression of extracellular enzymes laccases, peroxidases, and cytochrome P450 has been proposed as an approach for remediation of small organic compounds (Doty 2008). *Pseudomonas putida* MHF 7109 isolated from cow dung has shown ability for biodegradation of petroleum hydrocarbon compounds – benzene, toluene and o-xylene (BTX). The bacterium *Deinococcus radiodurans* (the most radioresistant organism known) has been modified to consume and digest toluene and Hg from highly radioactive nuclear waste. Transgenic poplar trees and tobacco plants overexpressing a mammalian cytochrome P450 2E1 (CYP2E1) and human cytochrome P450 2E1 were developed with the capacity for metabolizing trichloroethylene (TCE). Rabbit cytochrome P450 has been introduced in *Atropa belladonna* to facilitate faster metabolism of TCE. Transgenic plants removed organic compounds as high as 79% of TCE, 49% of vinyl chloride, and 40% of benzene in comparison to 10–30% controls. Bacterial genes dhlAB from *Xanthobacter* improved removal and degradation of 1,2-dichlorethane in plants. Higher expression of genes responsible for root development has been targeted for effective remediation of atrazine and alachlor. The expression of bacterial genes atrazine chlorohydrolase (AtzA) and 1-aminocyclopropane-l-carboxylate deaminase has shown promising role in remediation of atrazine and alachlor (Wang et al. 2008). Hydrophilic organics cannot pass the hydrophobic interior of membranes passively as there is no suitable transporter available in the plant. Hydrophobic organic contaminants stick to soil particles, thereby reducing their bioavailability, or become stuck inside root membranes preventing their movement into the cell's interior. Rhizoremediation utilizes the capacity of plant-associated microbes that have been proposed for remediation of PCBs (Doty 2008; Rylott and Bruce 2009). The degradation of PCBs takes place in two steps. In the

first step, PCB degradation takes place by expressing the genes of first multicomponent enzyme biphenyl 2,3-dioxygenase in degradation pathway. The released intermediate compounds undergo further transformation by rhizospheric bacteria. In the second step, expression of 2,3-dihydroxybiphenyl dioxygenase enzyme harbors bphC and avoids plants' inability to cleave toxic dihydroxybiphenyls. These transgenic plants are more resistant to PCBs than wild type indicating the potential utility of plants for effective rhizoremediation of PCBs.

Shiota et al. (1994) made transgenic tobacco plants by fusing rat P450 1A1 to yeast NADPH P450 oxidoreductase for metabolizing the herbicide chlortoluron. *Helianthus tuberosus* CYP76B1 and *Glycine max* CYP71A10 were the first transgenic plant with enzymes to actively metabolize organic contaminants (Siminszky et al. 1999). Human P450s have been shown to significantly enhance herbicide tolerance in transgenic potato (*Solanum tuberosum* L.) (Inui et al. 2001), rice (*Oryza sativa* L.) (Kawahigashi et al. 2007), *Arabidopsis* and tobacco (*Nicotiana tabacum* L.) (Didierjean et al. 2002).

Transgenic plants have been developed by introducing genes that are able to degrade explosive nitrate esters and NACs by introducing the bacterial enzyme pentaerythritol tetranitrate reductase (French et al. 1999). Van Aken (2008) reported the development of transgenic plants for remediation of 2,4,6-trinitrotoluene, hexahydro-l,3,5-trinitro-l,3,5-triazine and glyceroltrinitrate by introducing and expressing bacterial nitroreductases and cytochrome P450s. Plants expressing these genes show significantly increased tolerance, uptake and detoxification of the targeted explosives. The introduction of the pnrA gene encoding for nitroreductase from *Pseudomonas putida* into a fast-growing tree aspen has shown promising results for remediation of explosives in contaminated field conditions (Rylott and Bruce 2009; James and Strand 2009). Transgenic approaches increased the ability of tobacco to degrade explosives such as GTN and TNT by overexpressing a bacterial NADPH-dependent nitroreductase (French et al. 1999). The genes encoding a nitroreductase from a bacterium have been inserted in tobacco, and the transformed species showed faster removal of TNT and enhanced resistance to the toxic effects of TNT.

Genetically engineered microorganisms (GEMs) have enhanced degrading capabilities of a wide range of chemical contaminants. The principles involved in the development of GEM plants include (1) modification of enzyme specificity and affinity; (2) pathway construction and regulation; (3) bioprocess development, monitoring, and control; and (4) bioaffinity bioreporter sensor applications for chemical sensing, toxicity reduction and end point analysis. Genes responsible for degradation of environmental pollutants, for example, toluene, chlorobenzene acids and other halogenated pesticides and toxic wastes, have been identified. For every compound, one separate plasmid is required. It is not like that one plasmid can degrade all the toxic compounds of different groups. The plasmids are grouped into four categories: (1) OCT plasmid which degrades octane, hexane and decane, (2) XYL plasmid which degrades xylene and toluenes, (3) CAM plasmid that decomposes camphor, and (4) NAH plasmid which degrades naphthalene. The potential for creating, through genetic manipulation, microbial strains able to

degrade a variety of different types of hydrocarbons has been demonstrated. They successfully produced a multiplasmid-containing *Pseudomonas* strain capable of oxidizing aliphatic, aromatic, terpenic, and polyaromatic hydrocarbons. *Pseudomonas putida* that contained the XYL and NAH plasmid as well as a hybrid plasmid derived by recombinating parts of CAM and OCT developed by conjugation could degrade camphor, octane, salicylate, and naphthalene and could grow rapidly on crude oil because it was capable of metabolizing hydrocarbons more efficiently than any other single plasmid. This product of genetic engineering was called as superbug (oil eating bug). The plasmids of *P. putida* degrading various chemical compounds are TOL (for toluene and xylene), RA500 (for 3,5-xylene), pAC 25 (for 3-cne chlorobenxoate), and pKF439 (for salicylate toluene). Plasmid WWO of *P. putida* is one member of a set of plasmids now termed as TOL plasmid. *Alcaligenes eutrophus* AE104 (pEBZ141) was used for chromium removal from industrial wastewater, and the recombinant photosynthetic bacterium, *Rhodopseudomonas palustris*, was constructed to simultaneously express mercury transport system and metallothionein for Hg^{2+} removal from heavy metal wastewater. For polychlorinated biphenyl degradation, chromosomally located PCB catabolic genes of *R. eutropha* A5, *Achromobacter* sp. LBS1C1, and *A. denitrificans* JB1 were transferred into a heavy metal-resistant strain *R. eutropha* CH_34 through natural conjugation.

5.9 Conclusions

Bioremediation is a natural process utilizing bacteria and fungi or plants to degrade or detoxify substances hazardous to human health and/or the environment. The microorganisms indigenous to a contaminated area or site aid in removal of contaminants. Biotechnology utilizes the application of genetic engineering to improve the efficiency of microorganisms to reduce the toxic substances. Bioremediation must be tailored to the site-specific conditions. More research is needed to develop and engineer bioremediation technologies that are appropriate for sites with complex mixtures of contaminants and are not evenly dispersed in the environment. This technology can be applied both in situ and ex situ for removing broad range of environmental contaminants, viz., organic and inorganic. Environmental conditions regulate the growth and degradation ability of organism. Resistance to degradation is some of the major concerns for bioremediation technology. A comprehensive understanding of the transport and sequestration mechanisms in plant cells is essential for formulating effective strategies to develop genetically engineered plants with higher phytoremediation efficiency. Genetic engineering of endophytic and rhizospheric bacteria can be used in plant-associated degradation of toxic compounds in soil and is considered one of the most promising new technologies for remediation of contaminated environmental sites.

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