MICROBIOLOGY COURSE MATERIAL

Semester – IV (CC 9)

S-IV/SEM-IV/U-5

By

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B.Sc (HONOURS) MICROBIOLOGY (CBCS STRUCTURE)

SEMESTER – IV
CC-9: ENVIRONMENTAL MICROBIOLOGY (THEORY)
UNIT 5: MICROBIAL BIOREMEDIATION

❖ Inorganic (Metals) Matter:

Pollution of the environment keeps on increasing at an alarming rate due to the activities of man such as urbanization, technological advancement, unsafe agricultural practices and rapid industrialization which degrades the environment. Heavy metals released into the environment are persistent due to their toxicity which poses a severe threat to organisms exposed to high levels of such pollutants. Metals are essential to the biological functions of plants and animals but at elevated levels, they interfere with metabolic reactions in systems of organisms. Toxic heavy metals such as lead (Pb), cadmium (Cd), mercury (Hg), chromium (Cr), zinc (Zn), uranium (Ur), selenium (Se), silver (Ag), gold (Au), nickel (Ni) and arsenic (As) which are not useful to plants, are capable of reducing plant growth due to reduced photosynthetic activities, plant mineral nutrition, and reduced activity of essential enzymes. Heavy metals are cytotoxic at low concentrations and could lead to cancer in humans. These toxic metals could accumulate in the body when consumed in contaminated food through the food chain and become health risks to living organisms. This causes oxidative stress, an unevenness involving the production of free radicals and the capacity of cells to eradicate them or repair the damage. This leads to base damage through formation of reactive oxygen species (ROS) which includes oxygen radicals (superoxide and hydroxyl) and non-radical derivatives of molecular oxygen (O_2) such as hydrogen peroxide (H_2O_2), as well as breakage of the DNA molecule. Heavy metal toxicity increases the production of ROS thereby decreasing the antioxidant systems (glutathione, superoxide dismutase, etc.) which protect cells. If this condition continues, the normal functioning of the organism is affected and may invariably lead to cell death.

Bioremediation is a technique used to remove environmental contaminants from the ecosystem. It utilizes the biological mechanisms inherent in microbes and plants to eradicate hazardous pollutants and restore the ecosystem to its original condition. The basic principles of bioremediation involve reducing the solubility of these environmental contaminants by changing pH, the redox reactions and adsorption of contaminants from polluted environment. Bioremediation technologies are based on redox processes which focus on modifying the chemistry and microbiology of water by injecting selected reagents into contaminated water to enhance the degradation and extraction of various contaminants by in situ chemical oxidation/reduction reactions. Redox reactions involve chemically transforming harmful contaminants into innocuous or less toxic compounds that are more stable, less mobile or inert. It plays a vital role in the transformation of toxic heavy metals, especially As, Cr, Hg and Se in soils and sediments into less toxic or innocuous forms. Redox reactions in contaminated soil sediments and groundwater are often affected by the physicochemical properties of the medium, but this can be manipulated by addition of organic and inorganic amendments such as composts and biochar. The application of organic amendments such as compost in metal-contaminated soils could cause differences in the soil microbial population by changing pH, decreasing the solubility of heavy metals and increasing allochthonous microbial biomass and available nutrients. **Biochar** is a product of pyrolysis of biomass obtained from sources such as crop residue, manure and solid wastes which can be used to stimulate microorganisms for bioremediation by making the environment more favorable. It is an effective agent in immobilization of metals and organic pollutants. Biochar has the ability to donate, accept or transfer electrons within their environments abiotically or through biological pathways. Biochar acts by increasing the pH of contaminated soils thereby affecting the bioavailability of heavy metals for plant uptake. The mobility and toxicity of many elements, such as chromium, selenium, lead, arsenic, nickel and copper, rely basically on their oxidation states which, in turn, are controlled by the redox reactions.

The effectiveness of bioremediation depends on several factors such as the nature of the organisms utilized, the prevailing environmental factors at the contaminated site, as well as the degree of the pollutants in that environment. Bioremediation can be achieved with the use of microorganisms (microbial bioremediation) which depends on the metabolic potential of the microorganisms to degrade environmental pollutants and change them to innocuous forms through redox processes. It can also be carried out by plants which bind, extract and remediate pollutants from the environment (phytoremediation). The level of contaminated soil, the bioavailability of the metal contaminant, as well as the accumulation of metals as biomass by the plant, are critical to the success of phytoremediation as a means of eradicating heavy metals from contaminated sites using plants. Bioremediation could be in-situ or ex-situ. **In-situ** bioremediation is an onsite cleanup process of contaminated environments which involves supplementing contaminated soils with nutrients to stimulate microorganisms in their ability to degrade contaminants, as well as add new microorganisms to the environment or improve the indigenous microorganisms to degrade specific contaminants using genetic engineering. Utilization of natural microorganisms in the environment for in situ bioremediation is affected by the non-availability of suitable nutrient levels and/or environmental setting at the polluted location. **Ex-situ** bioremediation involves taking the contaminated media from its original site to a different location for treatment based on the cost of treatment, deepness of contamination, pollutant type and the extent of pollution, geographical locality and geology of the contaminated site.

❖ Effects of Heavy Metals on the Environment

The non-biodegradability of heavy metals makes it hard to remove them from contaminated biological tissues and this is a major concern for global health because of their lethal nature. Heavy metals such as cobalt (Co), copper (Cu), iron (Fe), manganese (Mn) and molybdenum (Mo) are required in small quantities for the survival of living organisms, but at higher concentrations, they could become detrimental. The heavy metals Hg, Cr, As, Zn, Cd, Ur, Se, Ag, Au and Ni are hazardous heavy metals that contaminate the environment and adversely affect the quality of the soil, crop production as well as public health, if their concentration exceeds the maximum permissible concentration in water. These pollutants are major sources of life-threatening degenerative diseases affecting humans such as cancer, Alzheimer's disease, atherosclerosis, Parkinson's disease, etc. The degree of toxicity of each metal is determined by the duration of exposure as well as the absorbed dosage by the organisms. Among organisms greatly affected by heavy metal toxicity are plants as their normal physiological activities are severely hampered. For example, the processes of respiration, photosynthesis, electron transport chain and cell division are negatively affected by elevated levels of heavy metals as documented by laboratory experiments. Moreover, high metal toxicity inhibits cytoplasmic enzymes in plant cells and causes damage to cell structures due to oxidative stress which consequently affects plant growth and metabolism. Exposure of the body to high levels of Pb could cause serious health implications such as lack of coordination and paralysis, while severe exposure to Cd damages internal organs of the body such as the kidney, liver and cardiac tissues. Arsenic is the most common cause of acute heavy metal poisoning in adults and children and could result in respiratory diseases such as reduced pulmonary function or lung cancer. The central nervous system is affected by Hg, a neurotoxin which impairs speech and hearing, and causes weakness of the muscles. It accumulates in the cells of microbes in aquatic bodies where it gets converted to methyl mercury in the microbes and becomes detrimental to aquatic lives. Consumption of fish and other aquatic animals by man can cause the transfer of toxic methyl mercury to man. Due to the detrimental effects of these heavy metals, concerted efforts need to be made to effectively eradicate them from the environment and stabilize the ecosystem.

❖ Mechanism of Heavy Metal Remediation by Microorganisms

Heavy metals are known to dislodge important components in biological molecules, hindering the functions of the molecules and changing enzyme, protein or membrane transporter structure or function thereby becoming toxic to plants. The major treatment regimes used for heavy metal degradation include methods such as coagulation, chemical precipitation, electrodialysis, evaporative recovery, floatation, flocculation, ion exchange, nanofilteration, reverse osmosis, ultrafiltration, etc., as well as physico-chemical methods such as extraction, stabilization, immobilization, soil washing, etc. These methods, even if effective, are generally expensive as a result of high energy and chemical reagent requirements, apart from production of secondary noxious end-products. An efficient way of removing toxic metal contaminants from the environment and stabilizing the ecosystem is to make use of indigenous microorganism with mechanisms capable of degrading such heavy metals, or genetically engineered microorganisms to treat polluted environments by converting toxic heavy metals into non-hazardous forms. However, the bioremediation process will only be successful if only microorganisms with proven ability to remediate and tolerate heavy toxicity are utilized.
The following mechanisms are used for microbial bioremediation:

(1) Sequestration of toxic metals by cell wall components or by intracellular metal binding proteins and peptides such as metallothioneins (MT) and phytochelatins along with compounds such as bacterial

siderophores which are mostly catecholates, compared to fungi that produce hydroxamate siderophores.

(2) Alteration of biochemical pathways to block metal uptake.

(3) Conversion of metals to innocuous forms by enzymes.

(4) Reduction of intracellular concentration of metals using precise efflux systems.

The mechanisms used in remediation of heavy metals from contaminated soils are presented in Figure 1.

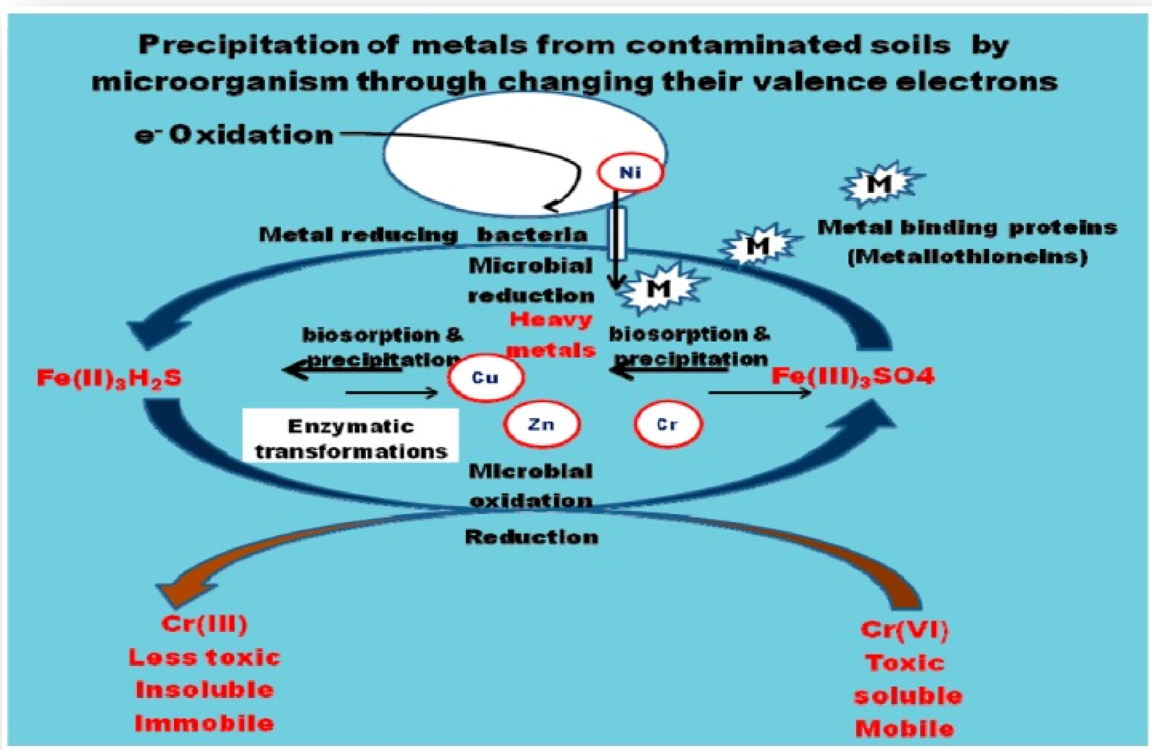

Figure 1: *Mechanisms of removal of heavy metals from contaminated soils by microorganims through the processes of precipitation, biosorption via sequestration by intracellular metal binding proteins (metallothioneins) and conversion of metals to innocous forms by enzymes (enzymatic transformation).*

Bacteria produce iron-chelating substances called siderophores which enhances mobility and reduces bioavailability of metals and its subsequent removal from soil. Sulphate-reducing bacteria such as ***Desulfovibrio desulfuricans*** have the ability to convert sulphate to hydrogen sulphate which then reacts with heavy metals such as Cd and Zn to form insoluble forms of these metal sulphides. Biosorption is the removal of heavy metals, compounds and particulates from a solution by low cost biological materials such as dead mass or natural materials with greater degradative ability. The mechanisms involved in biosorption could either be dependent on the cell's metabolism or the area of metal removal which is an independent metabolism. This could be extracellular accumulation/precipitation, cell surface sorption/precipitation and intracellular accumulation as presented in Figure 2.

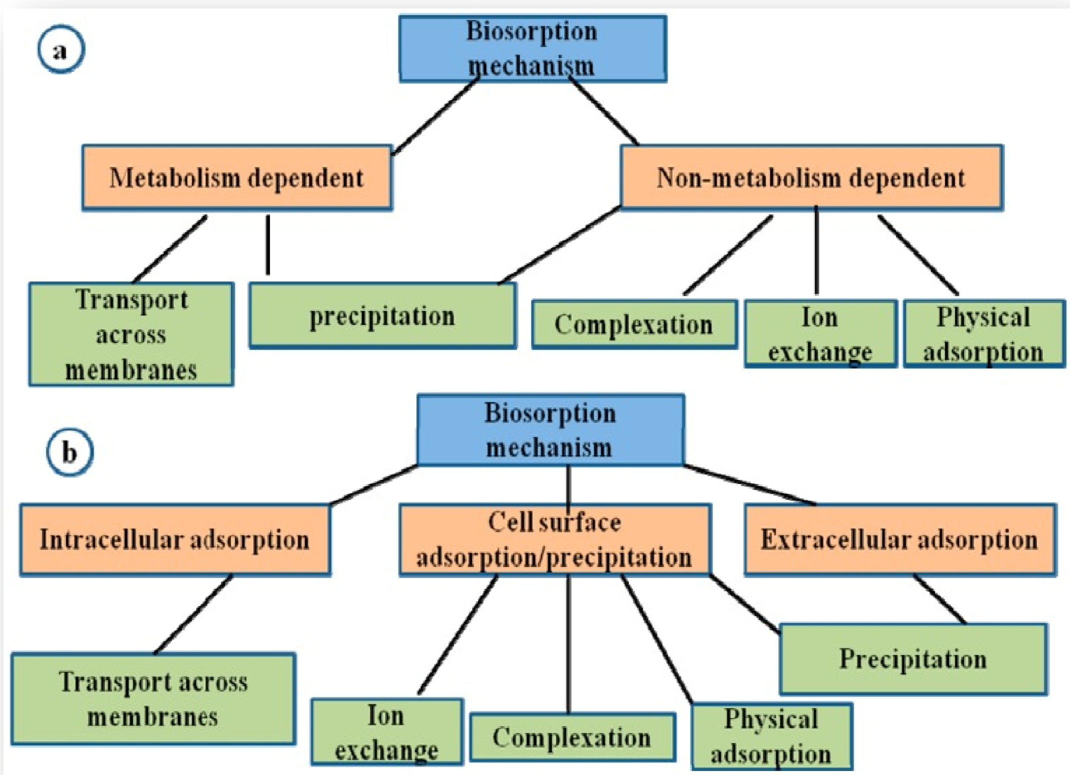

Figure 2: *Mechanisms of biosorption based on (**a**) dependence on cell metabolism; (**b**) location within the cell where the metal is removed*

Biosorptive abilities of microbial biomass vary within groups but the biosorption competence of each biosorbent is affected by prehistory and pretreatment, as well as the experimental conditions. The negatively charged functional groups present in biomolecules of microbial cell wall surfaces such as hydroxyl groups, phosphate groups, carbonyl groups, etc. bind readily to heavy metal ions. Bacterial functional groups, such as uronic acid of carboxyl groups ($RCOO^-$) and sulfate groups (SO_4^{2-}) are also capable of carrying out ion exchange. Gram-positive bacteria cell walls consist of peptidoglycan layers that contain the amino acids alanine and glutamic acid as well as meso-di-aminopimelic acid and teichoic acid, while enzymes, glycoproteins, lipopolysaccharides, lipoproteins and phospholipids are present in Gram-negative bacteria cell walls. These components of the cell wall are the active sites for binding processes in bacteria as they act as ligands for binding metal ions, resulting in their ultimate remediation from contaminated environments. Bacteria are essential biosorbents for the treatment of polluted environments because they are able to grow under controlled conditions and can withstand intense environmental conditions. They act as good biosorbents for heavy metals from polluted environments. Likewise, fungi are able to withstand and detoxify metal ions by active accumulation, intracellular and extracellular precipitation, and valence transformation, hence they are potential biocatalysts for the bioremediation of heavy metals as they are able to absorb heavy metals into their mycelium and spores. Yeast (***Sacharomyces cerevisiae***) is also used as efficient agents of bioremediation because they have the ability to remediate toxic metals from contaminated wastewaters by biosorption through the mechanism of ion exchange. Algae turns out large biomass which gives them a high sorption capacity compared to other microbial biosorbents. A summary of remediation of heavy metal contaminants using microorganisms is presented in Table 1.

Table 1: *Microorganisms used in heavy metal remediation of contaminated sites.*

Class of Microorganisms	Heavy Metal Removed
1. Bacteria	
Bacillus cereus strain **XMCr-6**	Cr (VI)
Kocuria flava	Cu
Bacillus cereus	Cr (VI)
Sporosarcina ginsengisoli	As (III)
Pseudomonas veronii	Cd, Zn, Cu
Pseudomonas putida	Cr (VI)
Enterobacter cloacae **B2-DHA**	Cr (VI)
Bacillus subtilis	Cr (VI)
2. Fungi	
Aspergillus versicolor	Ni, Cu
Aspergillus fumigatus	Pb
Gloeophyllum sepiarium	Cr (VI)
Rhizopus oryzae **(MPRO)**	Cr (VI)
3. Yeast	
Sacharomyces cerevisiae	Pb, Cd
4. Algae	
Spirogyra **spp. and** *Cladophora* **spp.**	Pb (II), Cu (II)
Spirogyra **spp. and** *Spirullina* **spp.**	Cr Cu, Fe, Mn, Zn
Hydrodictylon, *Oedogonium* **and** *Rhizoclonium* **spp.**	As

Microbes make use of heavy metals and trace elements as terminal electron acceptors from which they acquire the needed energy to detoxify metals via enzymatic and non-enzymatic processes. Bacterial cells are also capable of bioaccumulation which is the ability to build up heavy metal ions in both particulate as well as insoluble forms and their by-products. The most essential constituent in such bacterial cells having ion sequestration capability is exopolysaccharide (EPS). Exopolysaccharide is mainly composed of complex high molecular weight organic macromolecules like polysaccharide along with smaller proportions of protein and uronic acid. Exopolysaccharide protects the bacteria against environmental stresses such as heavy metal toxicity, drought, salinity, etc. The strategies for achieving heavy metal remediation through bacterial EPS has to be focused on utilizing the non neutral, negatively charged EPS (EPS packed with abundant anionic functional groups) to be incorporated as a suitable biosorbent. Some commercial bacterial EPS with the required anionicity includes; alginate (***Pseudomonas aeruginosa***, ***Azotobacter vinelandii***), gellan (***Sphingomonas paucimobilis***), hyaluronan (***Pseudomonas aeruginosa***, ***Pasteurella multocida***, ***Streptococci*** attenuated strains), xanthan (***Xanthomonas campestris***), galactopol (***Pseudomonas oleovorans***) and fucopol (***Enterobacter*** A47). Exopolysaccharide production is associated with processes such as biofilm production which is essential in the biosorption and biomineralization of metal ions.

Biofilms constitute a consortia of microorganisms enclosed in an extracellular matrix of polysaccharides made by a single bacterial species. Exopolysaccharide can be modified chemically by acetylation, carboxymethylation, methylation, phosphorylation and sulphonylation which modifies the biological activities of EPS, thereby enhancing the applicability of the polymer.

❖ Phytoremediation:

Phytoremediation deals with the cleanup of organic pollutants and heavy metal contaminants using plants and rhizospheric microorganisms. It is inexpensive, eco-friendly and an efficient means of restoration of polluted environments especially those that of heavy metals. Nonetheless, the level of soil contamination, the quantity of metal contaminant in the soil, as well as the ability of plants to aggressively take up metals from the soil, determine the success of phytoremediation at any polluted site. Plants utilized in phytoremediation are the hyperaccumulators with very high heavy metal accumulation potential and little biomass efficiency, and non-hyperaccumulators which possess lesser extraction capacity than hyperaccumulators, but whose total biomass yield is substantially higher and are fast-growing species. Several processes are used to remove heavy metals from contaminted soils by some plants as illustrated in Figure 3.

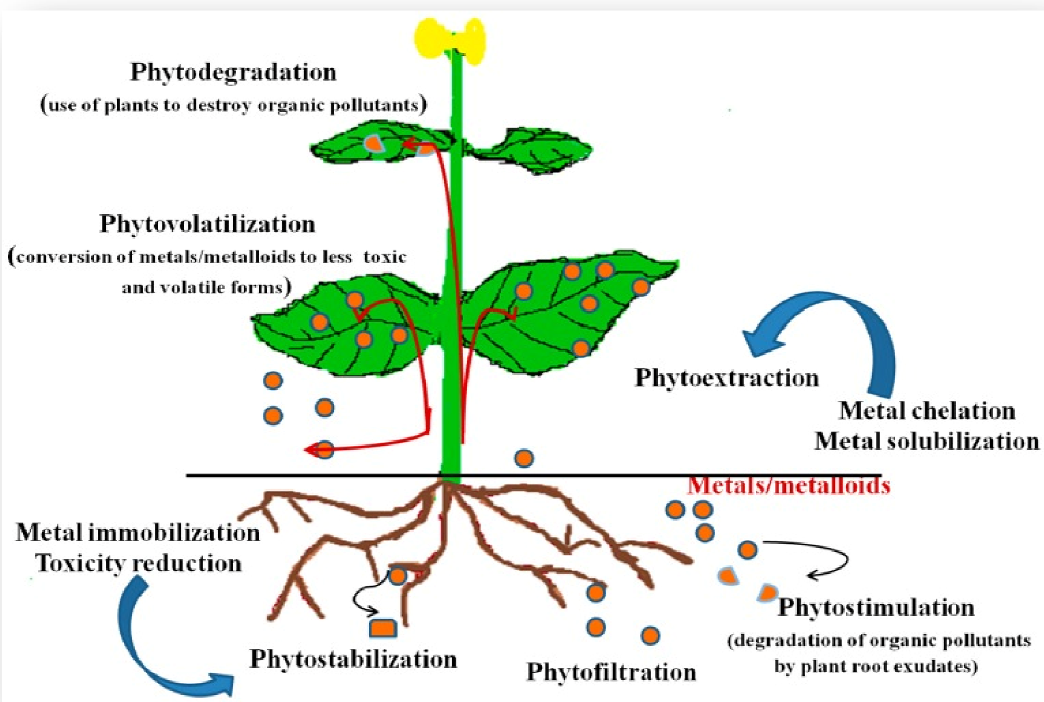

Figure 3: *Processes used in phytoremediation of heavy metals*

❖ Phytoextraction/Phytoaccumulation:

Phytoextraction involves the uptake and movement of metal pollutants in the soil through plant roots into above-ground components of the plants, based on the mechanism of hyperaccumulation. Hyperaccumulator plants take up metals in large quantities from contaminated soils, then transport and accumulate them in organs above the ground at concentrations from 100 to 1000 times higher than those found in non-hyperaccumulating species without suffering any apparent phytotoxic effect, hence they are very suitable for phytoremediation. These plants are usually found growing in areas with long-lasting metal contamination in soil over time and produce abundant biomass that can be easily harvested. The popular ones are representatives of the following families: ***Brassicaceae***, ***Caryophylaceae***, ***Violaceae***, ***Fabaceae***, ***Euphorbiaceae***, ***Lamiaceae***, ***Asteraceae***, ***Cyperaceae***, ***Poaceae***, ***Cunouniaceae*** and ***Flacourtiacea***, as indicated in Table 2. These plants are unique because of the following characteristics:

(1) a much greater capacity to take up heavy metals from the soil;
(2) enhanced root-to-shoot translocation of metal ions;
(3) a much greater ability to detoxify and sequester extremely large amounts of heavy metals in the shoots;
(4) ability to grow fast; and
(5) a profuse root system.

Despite the benefits of phytoextraction, its effectiveness can be hampered if the heavy metal conentration is very high, few biomass is produced by the plant or its growth rate is slow, which will hinder metal uptake. In such cases the phytoextraction process can be enhanced by using chelators such as citric acid and EDTA which increase mobility of soil heavy metals, or the use of organic supplements such as chicken manure which has been proven to increase growth of the species ***Rorippa globosa*** and decreased soil-extractable Cd and concentrations of Cd. The type of soil present at a polluted site and the degree of metal contamination determines the rate at which hyperaccumulating plants can remediate that site. The efficiency of phytoextraction is based on several factors which include:

(a) the choice of plant used,

(b) the degree of plant tolerance to higher concentrations of heavy metals and (c) the capacity of plants to drastically take up heavy metals and move them from the roots to exposed surfaces which are essential for the phytoextraction process.

Table 2: *Some hyperaccumulator plants used in phytoextraction of heavy metals*

Family	Species	Heavy Metals
Asteraceae	***Berkheya coddii***	Ni
Asteraceae	***Helianthus annuus***	Pb, Cd, Zn
Brassicaceae	***Alyssum bertolonii***	Ni
Brassicaceae	***Alyssum murale***	Ni
Brassicaceae	***Arabidopsis halleri***	Zn, Cd
Brassicaceae	***Arabidopsis halleri***	Cd Cd
Caryophyllaceae	***Minuartia verna***	Zn, Cd, Pb
Crassulaceae	***Sedum alfredii***	Pb
Euphorbiaceae	***Euphorbia cheiradenia***	Cu, Fe, Pb, Zn
Fabaceae	***Astragalus racemosus***	Se
Fabaceae	***Medicago sativa***	Pb
Poaceae	***Spartina argentinensis***	Cr
Pteridaceae	***Pteris vittata***	As
Pteridaceae	***Pteris vittata***	Hg
Violaceae	***Viola boashanensis***	Pb, Zn, Cd

Phytoextraction can be commercially viable; besides removal of heavy metals from the soil, it also produces biomass with added value. Phytoextraction is the most preferred method used by plants for remediation of polluted environments as it is enhanced by plant growth promoting rhizobacteria (PGPR) associated with the plant roots.

- **Phytofiltration:**

Phytofiltration could be in any of the three forms of rhizofiltration (the use of plant roots), blastofiltration (the use of seedlings) and caulofiltration (the use of excised plant shoots). It is the cleanup of polluted environments using plant roots or seedlings from aqueous wastes. For the effective use of phytofiltration as a phytoremediation technique, more studies have to be carried out to identify the parts of the plant that would be more efficient in accumulating the metal contaminants. This is essential for the use of this technique in bioremediation.

- **Phytostimulation:**

Phytostimulation is the enhancement of microbial activity to degrade organic contaminants by exudates from plant roots. Ethylene at low concentration stimulates root elongation but at high levels inhibits cell division and DNA synthesis. Nevertheless, this can be prevented by reducing ethylene concentration in plants using the enzyme 1-aminocyclopropane-1-carboxylase deaminase which reduces abiotic stress in plants by balancing plant ethylene-level production. This enzyme is made by PGPR linked with plant roots using exudates released by plants as carbon and energy sources to degrade metal contaminants.

- **Phytostabilization:**

This involves the use of plant roots to absorb pollutants from the soil and retain them within the rhizosphere, and get separated and stabilized, rendering them harmless and preventing the pollutants from spreading in the environment. The accessibility or mobility of heavy metals in the environment is reduced by precipitation in the region around plant roots, root sorption, metal valence reduction and complexation. The amount of metal in rhizosphere soil available for uptake determines how efficient metals are moved within the plant and the success of phytostabilization process. Plants used in phytostabilization should have a broad root system and low mobility of metals from roots to shoots. The phytostabilization ability of a plant could be enhanced by changing the pH and organic matter content by addition of biochar or compost which will increase plant yield and immobilize the metals. Phytostabilization is a better alternative of capturing metals in situ because the pollutants are not taken up into tissues of the plants and do not spread into the environment. It focuses primarily on heavy metal sequestration only within the rhizosphere.

- **Phytovolatilization:**

This deals with the removal of soil contaminants by plants which are readily changed into vapour and consequently released into the atmosphere. Tobacco plants have the ability to accumulate highly toxic methyl mercury from Hg-contaminated sites and transform it to the less toxic elemental Hg in a volatile form that escapes through the leaves to the atmosphere. The conversion of contaminants into volatile forms released during phytovolatilization is due to the metabolic potentials of the plants in union with microorganisms residing inside the rhizosphere.

- **Phytodegradation:**

Phytodegradation is the breakdown of organic contaminants into non-hazardous forms by plant enzymes. Specific enzymes such as nitroreductases and dehalogenases are used by plants to degrade organic contaminants. These enzymes must be used at optimal conditions of temperature and pH for efficient degradation of contaminants. The degradation of organic pollutants in the soil could also be enhanced by rhizospheric microorganisms through the process of rhizodegradation. This is made possible because the rhizospheric region of the plant contains elevated levels of nutrients released from the roots that draw more bacteria to aid degradation of the contaminants compared to bulk soil which has less organic compounds and, hence, would contain less microbes. This process is, however, restricted only to removal of organic pollutants since heavy metals are nonbiodegradable.

- **Rhizofiltration:**

Rhizofiltration involves the elimination of toxic substances or pollutants from ground water through filtration by the roots of plants. The process of rhizofiltration is based on the mechanism of rhizospheric accumulation by plants. Terrestrial plants are more efficient for rhizofiltration compared to aquatic plants because they employ natural solar driven pumps to take up particular elements from the environment. Plants that have the ability to take up and resist high concentrations of toxic metals such as hyperaccumulators are best suited for rhizofiltration. Introduction of PGPR to a contaminated site decreases metal toxicity in plants as bioavailability of such metals reduces, thereby increasing the capacity of plants to get rid of heavy metal contaminants and get protected from environmental stress. Nevertheless, phytoremediation technology has some limitations which include: decrease in the rate at which remediation occurs which frequently becomes insufficient when there are many pollutants at the contaminated site, and the accumulation and storage of pollutants in the plant materials.

Bioremediation is gradually being accepted as the standard practice for the restoration of heavy-metal-contaminated soils since it is more eco-friendly and cost effective compared to the conventional chemical and physical methods, which are often very expensive and ineffective when metal concentrations are low, in addition to producing significant amounts of toxic sludge. The ability of microorganisms to degrade pollutants depends on the suitability of environmental conditions for their growth and metabolism which include suitable temperature, pH, and moisture.

❖ Biosurfactants

Biosurfactants can be defined as the surface-active biomolecules produced by microorganisms with wide-range of applications. In recent years, due to their unique properties like specificity, low toxicity and relative ease of preparation, these surface-active biomolecules have attracted wide interest. Due to their unique functional properties, biosurfactants were used in several industries including organic chemicals, petroleum, petrochemicals, mining, metallurgy (mainly bioleaching), agrochemicals, fertilizers, foods, beverages, cosmetics, pharmaceuticals and many others. They can be used as emulsifiers as well as demulsifiers, wetting agents, foaming agents, spreading agents, functional food ingredients and detergents. The interfacial surface tension reducing ability of biosurfactants made them to play important role in oil recovery and bioremediation of heavy crude oil. The three major functions played by biosurfactants are:

1. They were used to increase the surface area of hydrophobic substrates.
2. Biosurfactants are also used to increase the bioavailability of hydrophobic substrates through solubilization/desorption.
3. They also regulate the attachment and removal of microorganisms from the surfaces.

Biosurfactants possess both hydrophilic and hydrophobic regions causing them to aggregate at interfaces between fluids with different polarities such as hydrocarbons and water, hence it decreases interfacial surface tension. They also found to be enhancing the nutrient transport across membranes and affect in various host-microbe interactions.

When compared to chemical or synthetic surfactants, biosurfactants gained several advantages including their biodegradability, biocompatability and digestibility. The biosurfactants can be used in environmental cleanup by biodegradation, detoxification of industrial effluents and in bioremediation of contaminated soil. Their specificity and availability of raw materials also made them most preferred surfactants.

- **Properties:**

The unique and distinct properties of biosurfactants when compared to their chemically synthesized counterparts and broad substrate availability made them suitable for commercial applications. The distinctive features of microbial surfactants are related to their surface activity, tolerance to pH, temperature and ionic strength, biodegradability, low toxicity, emulsifying and demulsifying ability and antimicrobial activity. The major distinctive features of each property of biosurfactant are discussed below.

1. **Surface and interface activity:** Surfactant helps in reducing surface tension and the interfacial tension. Surfactin produced by *B. subtilis* can reduce surface tension of water to 25 mN m^{-1} and interfacial tension water/hexadecane to less than 1 mN m^{-1}. The rhamnolipids produced by *P. aeruginosa* decreased surface tension of water to 26 mN m^{-1} and interfacial tension of water/hexadecane to value less than 1 mN m^{-1}. In general, biosurfactants are more effective and efficient and their Critical Micelle Concentration (CMC) which is about several times lower than chemical surfactants, i.e., for maximal decrease on surface tension, less surfactant is necessary.

2. **Temperature and pH tolerance:** The biosurfactant production from extremophiles has gained attention in last decades for their considerable commercial interest. Most of the biosurfactants and their

surface activity are resistant towards environmental factors such as temperature and pH. *Bacillus licheniformis* was found to be resistant to temperature up to 50°C, pH between 4.5 and 9.0 and NaCl and Ca concentrations up to 50 and 25 g L^{-1}, respectively. Another biosurfactant produced by *Arthrobacter protophormiae* was found to be both thermostable (30-100°C) and pH (2 to 12) stable. Since, industrial processes involve exposure to extremes of temperature, pH and pressure, it is necessary to isolate novel microbial products that able to function under these conditions.

3. **Biodegradability:** Microbially derived compounds can be easily degraded when compared to synthetic surfactants and are suitable for environmental applications such as bioremediation/biosorption. Synthetic chemical surfactants impose environmental problems and hence, biodegradable biosurfactants from marine microorganisms were concerned for the biosorption of poorly soluble polycyclic aromatic hydrocarbon, phenanthrene contaminated in aquatic surfaces controlled the blooms of marine algae, *Cochlodinium* using the biodegradable biosurfactant sophorolipid with the removal efficiency of 90% in 30 min treatment.

4. **Low toxicity:** biosurfactants are generally considered low or non-toxic products and are appropriate for pharmaceutical, cosmetic and food uses. The higher toxicity of the chemical-derived surfactant (Corexit) which displayed a LC50 against *Photobacterium phosphoreum* and was found to be 10 times lower than that of rhamnolipids. The comparison between the toxicity and mutagenicity profile of biosurfactant from *Pseudomonas aeruginosa* and chemically derived surfactants indicated that biosurfactant are non-toxic and non-mutagenic. The low

toxicity profile of biosurfactant, sophorolipids from *Candida bombicola* made them useful in food industries.

5. **Emulsion forming and emulsion breaking:** Biosurfactants may act as emulsifiers or de-emulsifiers. An emulsion can be described as a heterogeneous system, consisting of one immiscible liquid dispersed in another in the form of droplets, whose diameter in general exceeds 0.1 mm. Emulsions are generally two types: oil-in-water (o/w) or water-in-oil (w/o) emulsions. They possess a minimal stability which may be stabilized by additives such as biosurfactants and can be maintained as stable emulsions for months to years. Liposan is a water-soluble emulsifier synthesized by *Candida lipolytica* which have been used to emulsify edible oils by coating droplets of oil, thus forming stable emulsions. These liposans were commonly used in cosmetics and food industries for making oil/water emulsions for making stable emulsions.

6. **Antiadhesive agents:** A biofilm can be described as a group of bacteria/other organic matter that have colonized/accumulated on any surface. The first step on biofilm establishment is bacterial adherence over the surface was affected by various factors including type of microorganism, hydrophobicity and electrical charges of surface, environmental conditions and ability of microorganisms to produce extracellular polymers that help cells to anchor to surfaces. The biosurfactants can be used in altering the hydrophobicity of the surface which in turn affects the adhesion of microbes over the surface. A surfactant from *Streptococcus thermophilus* slows down the colonization of other thermophilic strains of *Streptococcus* over the steel which are responsible for fouling. Similarly, a biosurfactant from *Pseudomonas fluorescens* inhibited the attachment of *Listeria monocytogenes* onto steel surface.

❖ Types of biosurfactants:

The chemically synthesized surfactants are usually classified according to their polarity, whereas, biosurfactants are generally categorized by their microbial origin and chemical composition as follows:

1. **Glycolipid:** They are carbohydrates linked to long-chain aliphatic acids or hydroxyaliphatic acids by an ester group. Biosurfactants are majorly glycolipids. Among the glycolipids, the best known are rhamnolipids, trehalolipids and sophorolipids. The sources and properties of the different glycolipids were discussed below:

Rhamnolipids: Rhamnolipids are glycolipids, in which, one or two molecules of rhamnose are linked to one or two molecules of hydroxydecanoic acid. It is a widely studied biosurfactant which are the principal glycolipids produced by *P. aeruginosa.*

Trehalolipids: These are associated with most species of *Mycobacterium, Nocardia* and *Corynebacterium.* Trehalose lipids from *Rhodococcus erythropolis* and *Arthrobacter* spp. lowered the surface and interfacial tension in culture broth from 25-40 and 1-5 mNm, respectively.

Sophorolipids: These are glycolipids which are produced by yeasts and consist of a dimeric carbohydrate sophorose linked to a long-chain hydroxyl fatty acid by glycosidic linkage. Sophorolipids, generally is a mixture of at least six to nine different hydrophobic sophorolipids and lactone form of the sophorolipid is preferable for many applications.

2. **Lipopeptides and lipoproteins:** These consist of a lipid attached to a polypeptide chain. Several biosurfactants have shown antimicrobial action against various bacteria, algae, fungi and viruses. the antifungal and antibacterial property of the lipopeptide, iturin which was produced by *Bacillus subtilis* was found to be active even after autoclaving, pH 5-11 and with a shelf life of 6 months at -18°C.

3. **Surfactin:** The cyclic lipopeptide surfactin are one of the most powerful biosurfactants composed of a seven amino-acid ring structure coupled to a fatty-acid chain via lactone linkage. Previously some study reported that various physic-chemical properties of surfactin from *B. subtilis* are able to reduce the surface tension and interfacial tension of water. The inactivation of herpes and retrovirus was also observed with surfactin.

4. **Lichenysin:** *Bacillus licheniformis* produces several biosurfacants which exhibit excellent stability under extreme temperature, pH and salt conditions which are similar to surfactin. They are able to reduce the surface tension and interfacial tension of water to 27 and 0.36 mN m^{-1}, respectively.

5. **Fatty acids, phospholipids and neutral lipids:** Several bacteria and yeast produce large quantities of fatty acids and phospholipid surfactants during growth on n-alkanes. In *Acinetobacter* spp. 1-N, phosphatidyl ethanolamine-rich vesicles are produced which form optically clear micro-emulsions of alkanes in water. These biosurfactant are essential for medical applications. The deficiency of phospholipid protein complex is found to be the major cause for the respiration failure in the prematurely born children. They have also

suggested that the isolation and cloning of the genes responsible for such surfactant can be employed in their fermentative production.

6. **Polymeric biosurfactants:** These are the best-studied polymeric biosurfactants including emulsan, liposan, alasan, lipomanan and other polysaccharide-protein complexes. Emulsan is an effective emisifying agent for hydrocarbons in water, even at a concentration as low as 0.001-0.01%. Liposan is an extracellular water-soluble emulsifier synthesized by *Candida lipolytica* and is composed of 83% carbohydrate and17% protein. The application of such polymeric biosurfactant, liposan, as emulsifier in food and cosmetic industries.

7. **Particulate biosurfactants:** These form the extracellular membrane vesicles partition to form a microemulsion which plays an important role in alkane uptake by microbial cells. Vesicles of *Acinetobacter* spp. strain HO1-N with a diameter of 20-50 nm and a buoyant density of 1.158 cubic gcm are composed of protein, phospholipids and lipo-polysaccharide.

❖ Sources of Biosurfactants:

Many of the biosurfactant producing microorganisms are found to be hydrocarbon degraders. However in the past decades, many studies have showed the effects of microbially produced surfactants not only on bioremediation but also on enhanced oil recovery.

1. **Bacterial biosurfactants:** Microorganisms make use of a wide range of organic compounds as a source of carbon and energy for their growth. When the carbon source is in an insoluble form like a hydrocarbon, microorganisms make possible their diffusion into the cell by producing a variety of substances, the biosurfactants. Some of the

bacteria and yeasts excrete ionic surfactants which emulsify the CxHy substance in the growth medium. A few examples of this group of biosurfactant are rhamnolipids that are produced by different *Pseudomonas* spp. or sophorolipids that are produced by several *Torulopsis* spp. Some other microorganisms are able to change the structure of their cell wall which are achieved by them by producing nonionic or lipopolysaccharides surfactants in their cell wall. Some examples of this group are: *Rhodococcus erythropolis* and various *Mycobacterium* spp. and *Arthrobacter* spp. which produce nonionic trehalose corynomycolates. There are lipopolysaccharides, such as emulsan, produced by *Acinetobacter* spp. and lipoproteins such as surfactin and subtilisin, that are produced by *Bacillus subtilis*.

2. **Fungal biosurfactants:** Where the field of production of biosurfactants by bacterial species is well explored, relatively fewer fungi are known to produce biosurfactants. Among fungi, *Candida bombicola*, *Candida lipolytica*, *Candida ishiwadae*, *Candida batistae*, *Aspergillus ustus* and *Trichosporon ashii* are the explored ones. Many of these are known to produce biosurfactant on low cost raw materials. The major type of biosurfactants produced by these strains is sophorolipids (glycolipids). *Candida lipolytica* produces cell wall-bound lipopolysaccharides when it is growing on n-alkanes.

- **Applications:**

1. Food industries: The surfactants can have various other functions in food industries, apart from their obvious role as agents that decrease surface and interfacial tension, thus facilitating the formation and stabilization of emulsions. For example, to control the aggregation of fat globules, stabilization of aerated systems, improvement of texture and shelf-life of products containing starch, modification of rheological properties of wheat dough and improvement of constancy and texture of fat-based products. In bakery and ice-cream formulations, biosurfactants act by controlling the consistency, slowing staling and solubilizing the flavour oils; they are agents during cooking of fats and oil. Improvement in the stability of dough, volume, texture and conservation of bakery products is obtained by the addition of rhamnolipid surfactants. The study also suggested the use of rhamnolipids to improve the properties of butter cream and frozen confectionery products. L-Rhamnose has substantial potential as a forerunner for flavouring.

2. **Removal of oil and petroleum contamination:** Hydrocarbon culture media stimulated the growth of a rhamnolipid producing strain of *P. aeruginosa*. Recent research findings confirmed the effects of biosurfactant on hydrocarbon biodegradation by increasing microbial accessibility to insoluble substrates and thus enhance their biodegradation. Biosurfactants increase the apparent solubility of these organic compounds at concentrations above the Critical Micelle Concentration (CMC) which enhance their availability for microbial uptake. For these reasons, inclusion of biosurfactants in a bioremediation treatment of a hydrocarbon polluted environment could be really promising, facilitating their assimilation by microorganisms.

   Most of biosurfactants involve their efficiency in bioremediation, dispersion of oil spills and enhanced oil recovery.

   - *Alcanivorax* and *Cycloclasticus* genera are highly specialized hydrocarbon degraders in marine environments.

- *Alcanivorax borkumensis* utilizes aliphatic hydrocarbons as its main carbon source for growth and produces an anionic glucose lipid biosurfactant.
- Several species of *P. aeruginosa* and *B. subtilis* produce rhamnolipid, a commonly isolated glycolipid biosurfactant and surfactin, a lipoprotein type biosurfactant, respectively to increase solubility and bioavailability of a petrochemical mixture and also stimulate indigenous microorganisms for enhanced biodegradation of diesel contaminated soil.
- *Gordonia* species BS29 grows on aliphatic hydrocarbons as sole carbon source has been found to produce bioemulsan which effectively degrade crude oil, Polycyclic Aromatic Hydrocarbons (PAH) and other recalcitrant branched hydrocarbons from contaminated soils. The rate of biodegradation is dependent on the physicochemical properties of the biosurfactants and not by the effects on microbial metabolism.

- **Bioremediation of toxic pollutants:**

Bioremediation involves the acceleration of natural biodegradative processes in contaminated environments by improving the availability of materials (e.g. nutrients and oxygen), conditions (e.g., pH and moisture content) and prevailing microorganisms. Thus, bioremediation usually consists of the application of nitrogenous and phosphorous fertilizers, adjusting the pH and water content, if necessary, supplying air and often adding bacteria. The addition of emulsifiers is advantageous when bacterial growth is slow e.g. at cold temperatures or in the presence of high concentrations of pollutants or when the pollutants consist of compounds that are difficult to degrade, such as PAHs. Bioemulsifiers can be applied as an additive to stimulate the bioremediation process, however with advanced genetic technologies it is expected that the increase in bioemulsifier concentration during bioremediation would be achieved by the addition of bacteria that overproduce bioemulsifiers. This approach has been recently used successfully in the cleaning of oil pipes. Cultures of *A. radioresistens* which produce the bioemulsifier alasan but are unable to use hydrocarbons as a carbon source, were added to a mixture of oil-degrading bacteria to enhance oil bioremediation.

Persistent organic pollutants found in oil containing wastewater and sediments, such as PAHs (phenanthrene, crysene) are also hydrophobic in nature and thus water solubility of PAHs normally decrease with the increasing number of rings in molecular structure. This property induces the low **bioavailability** of these organic compounds that is a crucial factor in the biodegradation of PAHs. The water solubility of some PAHs can be improved by addition of biosurfactants owing to their amphipathic structure by several folds. In addition, most hydrocarbons exist in strongly adsorbed forms when they are introduced into soils. Thus, their removal efficiency can be limited in low mass transfer phases. However, additions of solubilization agents, such as biosurfactants to the system enhance the **bioavailability** of low solubility and highly sorptive compounds.

❖ Mechanism behind Bioremediation:

There are at least two ways in which biosurfactants are involved in bioremediation:

1. increasing the surface area of hydrophobic water-insoluble substrates and
2. increasing the bioavailability of hydrophobic compounds.

- **Increasing the surface area of hydrophobic water insoluble substrates:**

For bacteria growing on hydrocarbons, the growth rate can be limited by the interfacial surface area between water and oil. When the surface area becomes limiting, biomass increases arithmetically rather than exponentially. The evidence that emulsification is a natural process brought about by extracellular agents is indirect and there are certain conceptual difficulties in understanding how emulsification can provide an (evolutionary) advantage for the microorganism producing the emulsifier. Stated briefly, emulsification is a cell-density-dependent phenomenon: that is, the greater the number of cells, the higher the concentration of extracellular product. The concentration of cells in an open system, such as an oil-polluted body of water, never reaches a high enough value to effectively emulsify oil. Furthermore, any emulsified oil would disperse in the water and not be more available to the emulsifier-producing strain than to competing microorganisms.

One way to reconcile the existing data with these theoretical considerations is to suggest that the emulsifying agents do play a natural role in oil degradation but not in producing macroscopic emulsions in the bulk liquid. If emulsion occurs at, or very close to, the cell surface and no mixing occurs at the microscopic level, then each cluster of cells creates its own microenvironment and no overall cell-density dependence would be expected.

- **Increasing the bioavailability of hydrophobic water-insoluble substrates:**

The low water solubility of many hydrocarbons, especially the Polycyclic Aromatic Hydrocarbons (PAHs), is believed to limit their availability to microorganisms which is a potential problem for bioremediation of contaminated sites. It has been assumed that surfactants would enhance the bioavailability of hydrophobic compounds. Several non-biological surfactants have been studied and both negative and positive effects of the surfactants on biodegradation were observed. For example, the addition of the surfactant Tergitol NP-10 increased the dissolution rate of solid-phase phenanthrene and resulted in an overall increase in the growth of a strain of *Pseudomonas stutzeri*. A similar effect was obtained by the addition of Tween 80 to two *Sphingomonas* strains, the rate of fluoranthene mineralization was almost doubled. By contrast, the same surfactant inhibited the rate of fluoranthene mineralization by two strains of *Mycobacterium* and no stimulation was observed in other studies using several surfactants.

❖ Conclusion

Biosurfactants show several properties which could be useful in many fields of food industry; recently, their antiadhesive activity has attracted attention as a new tool to inhibit and disrupt the biofilms formed in food contact surfaces. The combination of particular characteristics such as emulsifying, antiadhesive and antimicrobial activities presented by biosurfactants suggests potential application as multipurpose ingredients or additives. Scant information regarding toxicity, combined with high production costs seems to be the major cause for the limited uses of biosurfactants in food area. However, the use of agroindustrial wastes can reduce the biosurfactants production costs as well as the waste treatment expends and also renders a new alternative for food and food-related industries not only for valorizing their wastes but also to becoming microbial surfactant producers. Biosurfactants obtained from Generally Regarded As Safe (GRAS) microorganisms like lactobacilli and yeasts are of great promise for food and medicine applications though, much more research is already required on this field. The prospect of new types of surface-active compounds from microorganisms can contribute for the detection of different molecules in terms of structure and properties but the toxicological aspects of new and current biosurfactants should be emphasized in order to certify the safety of these compounds for food utilization.

❖ Future trends:

A promising approach seems to be the application of inoculants of biosurfactant producing bacteria in phytoremediation of hydrocarbon polluted soil to improve the efficiency of this technology. Application of the biosurfactants in phytoremediation on a large scale requires studies to identify their potential toxic effect on plants. Although the biosurfactants are thought to be ecofriendly, some experiments indicated that under certain circumstances they can be toxic to the environment. Nevertheless, careful and controlled use of these interesting surface active molecules will surely help in the enhanced cleanup of the toxic environmental pollutants and provide us with a clean environment.