Chapter II

REVIEW OF LITERATURE
Nowadays, with growth of industrialization and extraction of natural resources, there has been a considerable increase in the discharge of industrial waste to the environment, mainly soil and water, which has led to accumulate the ions of heavy metals. Consequently, contamination of soils, groundwater, sediments, surface water, and air with hazardous heavy metals and toxic chemicals is one of the major threats facing the world, as they cannot be broken down to non-toxic forms and therefore have long-lasting effects on the ecosystem. According to recent study by Asha et al., (2013), the need to remediate these natural resources has led to the development of new technologies that emphasize the destruction of the pollutants rather than the conventional approach of disposal because of their potential to enter the food chain. Pollution of the natural environment by heavy metals is a worldwide problem as these metals are indestructible and have toxic effects on living organisms when they exceed a certain concentration limit (MacFarlane and Burchett, 2000).

The aquatic system extends over very densely populated areas and is subjected to intensive exploitation. Heavy metals in environment mostly come from lithogenic and anthropogenic sources. Chemical leaching of bedrocks, water drainage basins and runoff from banks are the primary sources for the lithogenic contribution of heavy metals. Discharge of urban and industrial waste water, combustion of fossil fuels, mining and smelting operations, processing and manufacturing industries, waste disposal including dumping, etc., are primary anthropogenic sources of pollution (Klavins et al., 2000). In the past two decades, this increase in urbanization and industrialization leads to an increase of marine discharges and, therefore, the total load of pollutants being delivered to the sea (McGlashan, 1989). These discharges may contain heavy metals among other pollutants. Through the natural process of biomagnifications, minute quantities of metals become part of the various food chains and concentrations become elevated to levels which can be proved to be toxic for both human and other living organisms (Bryan, 1971).
Because of human activities like metalliferous mining and smelting, agriculture, waste disposal or industry discharge a variety of metals such as Silver (Ag), Arsenic (As), Gold (Au), Cadmium (Cd), Cobalt (Co), Chromium (Cr), copper (Cu), mercury (Hg), Nickel (Ni), Lead (Pb), selenium (Se), and zinc (Zn), which can produce harmful effects on human health when they are taken up in amounts that cannot be processed by the organism. Some metals are required by plants in very small amounts for their growth and optimum performance. However, the increasing concentration of several metals in soil and waters due to industrial revolution has created an alarming situation for human life and aquatic biota. The indiscriminate release of heavy metals into the soil and waters is a major health concern worldwide, as they cannot be broken down to non-toxic forms and therefore have long-lasting effects on the ecosystem. Many of them are toxic even at very low concentrations; arsenic, cadmium, chromium, copper, lead, mercury, nickel, selenium, silver, zinc etc. are not only cytotoxic but also carcinogenic and mutagenic in nature (Salem et al., 2000).

Some metals are required by plants in very small amounts for their growth and optimum performance. However, the increasing concentration of several metals in soil and waters due to industrial revolution has created an alarming situation for human life and aquatic biota. This is evident from various reports citing harmful effects of heavy metals on human health (Table 2.1). In order to make the environment healthier for human beings, contaminated water bodies and land need to be rectified to make them free from heavy metals and trace elements. There are several techniques to remove these heavy metals, including chemical precipitation, oxidation or reduction, filtration, ion-exchange, reverse osmosis, membrane technology, evaporation and electrochemical treatment. But most of these techniques become ineffective when the concentrations of heavy metals are less than 100 mg/L (Ahlulwalia and Goyal, 2007). Most heavy metal salts are water-soluble and get dissolved in wastewater, which means they cannot be separated by physical separation methods (Hussein and Farag, 2004). Additionally, physico-chemical methods are ineffective or expensive when the concentration of heavy metals is very low. Alternately, biological methods like biosorption and/or
bioaccumulation for removal of heavy metals may be an attractive alternative to physico-chemical methods (Kapoor and Viraraghvan, 1995).

Conventional methods to remediate heavy metals contaminated site are excavation and solidification/ stabilization, these technologies are suitable to control contamination but not permanently remove heavy metals (Bahi et al., 2012). However, they have some disadvantages, among them cost-effectiveness limitations, generation of hazardous by-products or inefficiency. On the other hand, biological methods potentially solve these drawbacks since they are easy to operate, do not produce secondary pollution. Heavy metals having relatively high density are toxic at low concentration (Iram et al., 2013).

Use of microorganisms and plants for remediation purposes is thus a possible solution for heavy metal pollution since it includes sustainable remediation technologies to rectify and re-establish the natural condition of soil. However, introduction of heavy metals into the soil causes considerable modification of the microbial community, despite their vital importance for the growth of microorganisms at relatively low concentrations (Doelman, 1994). The modification of the microbial make up is mainly brought about by exerting an inhibitory action through blockage of essential functional groups, displacement of essential metal ions or modification of active conformations of biological molecules (Wood and Wang, 1983; Li and Tan, 1994). The response of microbial communities to heavy metals depends on the concentration and availability of heavy metals and is a complex process which is controlled by multiple factors, such as type of metal, the nature of the medium, and microbial species (Goblenz et al., 1994).

Microorganisms and plants are usually used for the removal of heavy metals. Process of involvement of microorganisms to reduce pollutant concentration is known as bioremediation which is a natural process and its importance of biodiversity (above or below the ground) is increasingly considered for clean-up of metal contaminated and polluted ecosystem. All the metals are toxic, but some of these are useful in low concentration. These metal toxicity cause serious morbidity and mortality (Surajana et
Furthermore, Jin et al., (2011) reported that the bioavailability can be improved by addition of organic nutrients to the soil such as manure, compost, biosolids, which condition the soil and increases the fertility of soil. In order to make the environment healthier for human beings, contaminated water bodies and land need to be rectified to make them free from heavy metals; they are trace elements. There are several techniques to remove these heavy metals, chemical precipitation, oxidation or reduction, filtration, ion-exchange, reverse osmosis, membrane technology, evaporation and electrochemical treatment. Moreover, most heavy metal salts are water soluble and get dissolved in wastewater, which means they cannot be separated by physical separation methods (Hussein et al., 2004).

Additionally, physico-chemical methods are ineffective or expensive when the concentration of heavy metals is very low. Alternately, biological methods like biosorption and/or bioaccumulation for removal of heavy metals may be an attractive alternative to physico-chemical methods (Kapoor et al., 1995). Use of microorganisms and plants for remediation purposes is thus a possible solution for heavy metal pollution since it includes sustainable remediation technologies to rectify and re-establish the natural condition of soil. However, introduction of heavy metals into the soil causes considerable modification of the microbial community, despite their vital importance for the growth of microorganisms at relatively low concentrations (Doelman et al., 1994). Moreover, according to report by Wood and Wang, (1983) and Li et al., (1994) the modification of the microbial make up is mainly brought about by exerting an inhibitory action through blockage of essential functional groups, displacement of essential metal ions or modification of active conformations of biological molecules. Moreover, the response of microbial communities to heavy metals depends on the concentration and availability of heavy metals and is a complex process which is controlled by multiple factors, such as type of metal, the nature of the medium and microbial species (Goblenz et al., 1994).
Sources of heavy metal in the environment

Heavy metals occur naturally in the environment from pedogenetic processes of weathering of parent materials and also through anthropogenic sources (Figure 2.1). The most significant natural sources are weathering of minerals, erosion and volcanic activity, while the anthropogenic sources depend upon human activities such as mining, smelting, electroplating, use of pesticides and phosphate fertilizer discharge, as well as biosolids (e.g., livestock manures, composts, and municipal sewage sludge), atmospheric deposition, etc. (Salem et al., 2000; Ahluwalia and Goyal, 2007; Hussein and Farag, 2004; Kapoor and Viraraghvan, 1995; Doelman, 1994; Wood and Wang, 1983; Li and Tan, 1994). The disturbance of nature’s slowly occurring geochemical cycle of metals by man results in accumulation of one or more of heavy metals in the soil and waters, and above defined levels, this is enough to cause risk to human health, plants, animals and aquatic biota (D’Amore et al., 2005). The heavy metals essentially become contaminants in the soil and water environment because of their excess generation by natural and man-made activities, transfer from mines to other locations where higher exposure to humans occurs, discharge of high concentration of metal waste through industries, and greater bioavailability.

Bioremediation

Bioremediation is an innovative and promising technology available for removal of heavy metals and recovery of the heavy metals in polluted water and lands. Since microorganisms have developed various strategies for their survival in heavy metal-polluted habitats, these organisms are known to develop and adopt different detoxifying mechanisms such as biosorption, bioaccumulation, biotransformation and biomineralization, which can be exploited for bioremediation either ex situ or in situ (Lin and Lin, 2005) (Figure 2.2). A global survey to examine the use of bioremediation technologies for addressing the environmental problems was carried out by Elekwachi and his co-workers (2014). They found that despite aspirations from respondents to apply bioremediation techniques, it should not become the current practice. Developed economies made higher use of low-cost in situ bioremediation technologies such as
monitored natural attenuation, while their developing counterparts appeared to focus on occasionally more expensive ex situ technologies.

Despite significant investment and widespread availability of online resources, their limited use underlines the need to explore improved training and development of more user-friendly resources. There are many reports about biodegradation and bioremediation strategies being utilized by bacteria or plant species (Salem et al., 2000; Ahluwalia and Goyal, 2007; Hussein and Farag, 2004; Kapoor and Viraraghvan, 1995), but so far none of these investigations suggest possible drivers in the global use of the said techniques (Kapoor and Viraraghvan, 1995). Among the preferred methods for treatment of contaminated areas, 51% of the respondents preferred environment friendly approaches, including microbial remediation (35%) and phytoremediation (16%) (Environmental Agency, 2015).

The concept of bioremediation

The quality of life on Earth is linked to the overall quality of the environment. The problems associated with contaminated sites now assume increasing prominence in many countries. Enormous quantities of organic and inorganic compounds are released into the environment each year as a result of human activities. Contaminated lands generally result from industrial activities, use and disposal of hazardous substances, and the like. It is now widely recognized that contaminated land is a potential threat to human health, and its continual discovery over recent years has led to international efforts to remedy many of the sites, either as a response to the risk of adverse health or environmental effects caused by contamination or to enable the site to be redeveloped for use (Damodaran and Suresh, 2011).

Bioremediation is an innovative and promising technology available for removal of heavy metals and recovery of the heavy metals in polluted water and lands. Since microorganisms have developed various strategies for their survival in heavy metal-polluted habitats, these organisms are known to develop and adopt different detoxifying mechanisms such as biosorpt ion, bioaccumulation, biotransformation and biomineralization. Bioremediation is a general concept that includes all those processes.
and actions that take place in order to biotransform an environment, already altered by contaminants, to its original status. Adhikari et al., (2004) also defined as bioremediation is the process of cleaning up hazardous wastes with microorganisms or plants and is the safest method of clearing soil of pollutants. Bioremediation uses primarily microorganisms or microbial processes to degrade and transform environmental contaminants into harmless or less toxic forms (Garbisu and Alkorta, 2003).

Microorganisms’ uptake heavy metals actively (bioaccumulation) and/or passively (adsorption) (Hussein et al., 2001). The microbial cell walls, which mainly consist of polysaccharides, lipids and proteins, offer many functional groups that can bind heavy metal ions, and these include carboxylate, hydroxyl, amino and phosphate groups (Scott and Karanjkar, 1992). Among various microbe-mediated methods, the biosorption process seems to be more feasible for large scale application compared to the bioaccumulation process, because microbes will require addition of nutrients for their active uptake of heavy metals, which increases the biological oxygen demand or chemical oxygen demand in the waste. Further, it is very difficult to maintain a healthy population of microorganisms due to heavy metal toxicity and other environmental factors (Ajmal et al., 1996).

Some microorganisms that live in soil and groundwater naturally use certain chemicals that are harmful to people and the environment. The microorganisms are able to change these chemicals into water and harmless gases, such as carbon dioxide. Many algae and bacteria produce secretions that attract metals that are toxic in high levels. The metals are in effect removed from the food chain by being bound to the secretions. Degradation of dyes is also brought about by some anaerobic bacteria and fungi (Colberg, 1995). To boost the world's food production rate to compensate for the increasing population, pesticides are being used. The extensive use of these artificial boosters has led to the accumulation of artificial complex compounds called xenobiotics. By introducing genetically altered microbes, it is possible to degrade these compounds. Plants can also be used to clean up soil, water or air; this is called
phytoremediation. Thus, Bioremediation has been proposed as a cost effective, environmental friendly alternative modern emerging technology which can be applied to a number of contaminants and site conditions.

The release of contaminants into the environment by human activities has increased enormously over the past several decades. In fact, although a few decades ago, man's greatest challenge resided in speeding up the industrialization process, today man attempts to find ways to deal with the growing industrialization and the associated problems (Thassitou and Arvanitoyannis, 2001). The relatively sudden introduction of pollutants into the environment has clearly overwhelmed their self-cleaning capacity and, as a consequence, resulted in the accumulation of pollutants. Soil pollution has recently been attracting considerable public attention since the magnitude of the problem in our environment calls for immediate action. Thus, it is essential to minimize poisonous effects of pollutants from soil and water, through the use of bioremediation technique (EPA, 2006).

**Types of Bioremediation**

According to EPA (2001 and 2002) on the basis of removal and transportation of wastes for treatment there are basically two methods. These are in-situ bioremediation ex-situ bioremediation.

**In-situ bioremediation**

In-situ bioremediation is no need to excavate or remove soils or water in order to accomplish remediation. The pollution is eliminated directly at the place where it occurs or at the site of contamination so may be less expensive, create less dust, and it is possible to treat a large volume of soil and cause less release of contaminants. In-situ biodegradation involves supplying oxygen and nutrients by circulating aqueous solutions through contaminated soils to stimulate naturally occurring bacteria to degrade organic contaminants. It can be used for soil and groundwater (Vidalı, 2001). Most often, In-situ bioremediation is applied to the degradation of contaminants in saturated soils and groundwater. It is a superior method to cleaning contaminated environments since it is cheaper and uses harmless microbial organisms to degrade the
chemicals and also a safer method in degrading harmful compounds. In-situ bioremediation can be two types.

These are intrinsic bioremediation and In-situ engineered bioremediation. In-situ bioremediation approach deals with stimulation of indigenous or naturally occurring microbial populations by feeding them nutrients and oxygen to increase their metabolic activity where as engineered In-situ bioremediation approach involves the introduction of certain microorganisms to the site of contamination. When site conditions are not suitable, engineered systems have to be introduced to that particular site. Engineered in-situ bioremediation accelerates the degradation process by enhancing the physicochemical conditions to encourage the growth of microorganisms. Oxygen, electron acceptors and nutrients (nitrogen and phosphorus) promote microbial growth (Evans and Furlong 2003).

**Ex-situ bioremediation**

This process requires excavation of contaminated soil or pumping of groundwater to facilitate microbial degradation. This technique has more disadvantages than advantages. Ex-situ bioremediation techniques involve the excavation or removal of contaminated soil from ground. Depending on the state of the contaminant to be removed, ex-situ bioremediation is classified as solid phase system and slurry phase systems. The Solid phase treatment includes organic wastes such as leaves, animal manures and agricultural wastes and problematic wastes like domestic and industrial wastes, sewage sludge and municipal solid wastes. Solid phase soil treatment processes include land farming, soil biopiles, and composting. Land farming is a simple technique in which contaminated soil is excavated and spread over a prepared bed and periodically tilled until pollutants are degraded.

The goal is to stimulate indigenous biodegradative microorganisms and facilitate their aerobic degradation of contaminants. Since land farming has the potential to reduce monitoring and maintenance costs, as well as clean up liabilities, it has received much attention as a disposal alternative (EPA, 2002). Composting is a technique that involves combining contaminated soil with nonhazardous organic
amendments such as manure or agricultural wastes. The presence of these organic materials supports the development of a rich microbial population and elevated temperature characteristic of composting (Cunningham, 2000). Biopiles are a hybrid of land farming and composting. Essentially, engineered cells are constructed as aerated composted piles. Typically used for treatment of surface contamination with petroleum hydrocarbons they are a refined version of land farming that end to control physical losses of the contaminants by leaching and volatilization. Biopiles provide a favorable environment for indigenous aerobic and anaerobic microorganisms (EPA, 2006). Slurry phase bioremediation is a relatively more rapid process compared to the other treatment processes. Contaminated soil is combined with water and other additives in a large tank called a bioreactor and mixed to keep the microorganisms, which are already present in the soil, in contact with the contaminants in the soil.

Nutrients and oxygen are added and conditions in the bioreactor are controlled to create the optimum environment for the microorganisms to degrade the contaminants. When the treatment is completed, the water is removed from the solids, which are disposed of or treated further if they still contain pollutants (Cunningham, 2000). Bioreactor is a containment vessel and apparatus used to create a three phase: solid, liquid, and gas, mixing condition to increase the bioremediation rate of soil bound and water soluble pollutants as water slurry of the contaminated soil and biomass capable of degrading target contaminants. In general, the rate and extent of biodegradation are greater in a bioreactor system than in-situ or in solid phase systems because the contained environment is more manageable and hence more controllable and predictable. Despite the advantages of reactor systems, there are some disadvantages. The contaminated soil requires pretreatment or alternatively the contaminant can be stripped from the soil via soil washing or physical extraction before being placed in a bioreactor (Von Fahnestock et al., 1998 and EPA, 2001).

**Advantage and disadvantage of in-situ bioremediation**

This method have many potential advantages as it does not require excavation of the contaminated soil and hence proves to be cost effective, there is minimal site
disruption, so the amount of dust created is less and simultaneous treatment of soil and groundwater is possible. It poses some disadvantages also as the method is time consuming compared to the other remedial methods, seasonal variation of the microbial activity due to direct exposure to changes in environmental factors that cannot be controlled and problematic application of treatment additives (EPA, 2001). Microorganisms act well only when the waste materials present allow them to produce nutrients and energy for the development of more cells. When these conditions are not favorable then their capacity to degrade is reduced. In such cases genetically engineered microorganisms have to be used, although stimulating indigenous microorganisms is preferred (EPA, 2002).

**Microorganisms used in bioremediation**

Microorganism’s uptake heavy metals actively (bioaccumulation) and/or passively (adsorption) (Hussein et al., 2001). The microbial cell walls, which mainly consist of polysaccharides, lipids and proteins, offer many functional groups that can bind heavy metal ions, and these include carboxylate, hydroxyl, amino and phosphate groups (Scott and Karanjkar, 1992). Among various microbe-mediated methods, the biosorption process seems to be more feasible for large scale application compared to the bioaccumulation process, because microbes will require addition of nutrients for their active uptake of heavy metals, which increases the biological oxygen demand or chemical oxygen demand in the waste. Further, it is very difficult to maintain a healthy population of microorganisms due to heavy metal toxicity and other environmental factors.

Fungi of the genera *Penicillium*, *Aspergillus* and *Rhizopus* have been studied extensively as potential microbial agents for the removal of heavy metals from aqueous solutions (Volesky and Holan, 1995). Xiao et al. reported a novel technology for obtaining highly efficient biosorbents from endophytes, a hyperaccumulator, which is more convenient than the traditional method of obtaining biosorbents (Xiao et al., 2010). Sun et al. evaluated the genetic diversity of endophytic bacteria from the copper-tolerant species of *Elshotzia apliendens* and *Commelina communis*, reporting
increased dry weights of roots and aboveground tissues compared to uninoculated plants (Sun et al., 2010). Further, they also reported significant amounts of (ranging from 63% to 125%) Cu content in inoculated plants compared to un-inoculated ones.

The bioremediation processes may be conducted by the autochthonous microorganisms, which naturally inhabit the soil/water environment undergoing purification, or by other microorganisms, that derive from different environments. There are a number of microorganisms that can be used to remove metal from environment, such bacteria, fungi, yeast and algae (White et al., 1997 and Vieira and Volesky, 2000). Microorganisms can be isolated from almost any environmental conditions. Microbes can adapt and grow at subzero temperatures, as well as extreme heat, desert conditions, in water, with an excess of oxygen and in anaerobic conditions, with the presence of hazardous compounds or on any waste stream. Because of the adaptability of microbes and other biological systems, these can be used to degrade or remediate environmental hazards. The main requirements are an energy source and a carbon source (Vidali., 2001). Because of the adaptability of microbes and other biological systems, these can be used to degrade or remediate environmental hazards.

Natural organisms, either indigenous or extraneous (introduced), are the prime agents used for bioremediation (Prescott et al., 2002). The organisms that are utilized vary, depending on the chemical nature of the polluting agents, and are to be selected carefully as they only survive within a limited range of chemical contaminants (Prescott et al., 2002). Since numerous types of pollutants are to be encountered in a contaminated site, diverse types of microorganisms are likely to be required for effective mediation (Watanabe et al., 2001). The first patent for a biological remediation agent was registered in 1974, being a strain of Pseudomonas putida that was able to degrade petroleum (Prescott et al., 2002; Glazer and Nikaido, 2007). These microorganisms can be subdivided into the following groups:

1. **Aerobic**

Pseudomonas, Alcaligenes, Sphingomonas, Rhodococcus, and Mycobacterium. These microbes have often been reported to degrade pesticides and hydrocarbons,
both alkanes and polyaromatic compounds. Many of these bacteria use the contaminant as the sole source of carbon and energy.

2. **Anaerobic**

There is an increasing interest in anaerobic bacteria used for bioremediation of polychlorinated biphenyls (PCBs) in river sediments, dechlorination of the solvent trichloroethylene (TCE) and chloroform.

3. **Ligninolytic Fungi**

Fungi such as the white rot fungus *Phanaerochaete chrysosporium* have the ability to degrade an extremely diverse range of persistent or toxic environmental pollutants. Common substrates used include straw, saw dust, or corncobs.

4. **Methylotrophs**

Aerobic bacteria that grow utilizing methane for carbon and energy. The initial enzyme in the pathway for aerobic degradation, methane monooxygenase, has a broad substrate range and is active against a wide range of compounds, including the chlorinated aliphatic trichloroethylene and 1, 2-dichloroethane (EPA, 2002). Bioremediation is not effective only for the degradation of pollutants but it can also be used to clean unwanted substances from air, soil, water and raw materials form industrial waste. With this in view, though many engineered processes for applying bioremediation have been developed but the inexpensive treatment of such sites has remained an elusive goal (Zeyaullah *et al.*, 2009).

**Microorganism based clean up system**

Remediation of environment niches such as soil, sediments and water amended with heavy metals can be achieved through biologically encoded changes in the oxidation state. Bioremediation is the microbe-mediated process for clearance or immobilization of the contaminants, including all possible toxins like hydrocarbons, agrochemicals and other organic toxicants. But for inorganic toxic compounds such as heavy metals, microbes are unable to simplify them into harmless compounds, and they should be used according to their specialization for the type of contaminants. Thus the
bioremediation strategy for heavy metals depends on the active metabolizing capabilities of microorganisms. Several microorganisms are known to require varying amounts of heavy metals as essential micronutrients for growth and development. For example, Fe\(^{3+}\) is essentially required by all bacteria while Fe\(^{2+}\) is important for anaerobic bacteria (Ahemad, 2014). However, the adsorption capacity depends on microbial total biomass and geochemistry of the system. Some oxyanions of metals do not interact with microbes, and their bioremediation is based on their catalyzed redox conversion to insoluble forms. These reduction or oxidation reactions take place due to enzymatic activity and biomass concentration of microbes.

Microorganisms have a great deal of undiscovered and unexplored potential for remediation of soil pollutants and increasing the production of agricultural crops with low input. Selection of rhizospheric microbes should be done based on an understanding of mechanisms involved in the adsorption and mobilization of heavy metals and trace elements in the soil to restore soil health. Microorganisms as metal accumulators possess an inherent novel remediation property for toxic metals in the soil. The study of genetics of such metal accumulator microorganisms can help us to transfer the traits in the microbes that are missing through the development of microarrays, which result in differentially expressed microbe genes. Detoxification and rehabilitation of contaminated soil with the use of microbes has emerged as the most safe, easy and effective technology.

Native soil microorganisms have been explored and harnessed for their ability to remove or detoxify toxic products released due to human activities in the environment viz. mining of ores, oil and gas extraction, pesticides, pigments, plastic, organic solvents, fuel and industrial processes (Garbisu and Alkorta, 2001). But the lack of information on the cellular responses of microbes towards utilization and interaction with trace element and heavy metal pollutants restricts their successful execution. Studies have been carried out to promote the use of modified microbes designed especially to increase sensitivity towards toxic metals. The biochemical route for the redistribution of the organic pollutant in the soil starts from various physical,
chemical and biological processes resulting in adsorption by soil particles and root tissues, volatilization, transport through water and air, microbial degradation and leaching, *etc.*

**Mechanisms of bioremediation**

Microorganisms are omnipresent that dominate in heavy metal-contaminated soil and can easily convert heavy metals into non-toxic forms. In bioremediation processes, microorganisms mineralize the organic contaminants to end-products such as carbon dioxide and water, or to metabolic intermediates which are used as primary substrates for cell growth. Microorganisms are capable of two-way defense *viz.* production of degradative enzymes for the target pollutants as well as resistance to relevant heavy metals (Ruchita Dixit *et al.*, 2015). Different mechanisms of bioremediation are known, including biosorption, metal-microbe interactions, bioaccumulation, biomineralisation, biotransformation and bioleaching. In which, bioaccumulation and biosorption methods employed in most of the studies and its process were quite different (Figure 2.2) (Table 2.2). Microorganisms remove the heavy metals from soil by using chemicals for their growth and development. They are capable of dissolving metals and reducing or oxidizing transition metals.

Different methods by which microbes restore the environment are oxidizing, binding, immobilizing, volatizing and transformation of heavy metals (Ruchita Dixit *et al.*, 2015). Bioremediation can be made successful in a particular location by the designer microbe approach, and by understanding the mechanism controlling growth and activity of microorganisms in the contaminated sites, their metabolic capabilities and their response to environmental changes. Many contaminants are organic solvents which disrupt membranes, but cells may develop defense mechanisms including formation of outer cell-membrane-protective material, often hydrophobic or solvent efflux pumps (Sikkema *et al.*, 1993). For instance, plasmid-encoded and energy-dependent metal efflux systems involving ATPases and chemiosmotic ion/proton pumps are reported for As, Cr and Cd resistance in many bacteria (Roane and Pepper, 2000).
**Bioremediation by adsorption**

Heavy metals can be biosorbed by microbes at binding sites present in cellular structure without the involvement of energy. Among the various reactive compounds associated with bacterial cell walls, the extracellular polymeric substances are of particular importance and are well known to have significant effects on acid-base properties and metal adsorption (Guiné et al., 2006). Studies on the metal binding behavior of extracellular polymeric substances (EPS) revealed a great ability to complex heavy metals through various mechanisms, which include proton exchange and micro-precipitation of metals (Comte et al., 2008; Fang et al., 2010). Recent studies have characterized and quantified the proton and adsorbed metals on bacterial cells and EPS free cells in order to determine the relative importance of EPS molecules in metal removal (Fang et al., 2011). Bioremediation research and practice are still hampered in the current scenario due to an incomplete understanding of genetics and genome level characteristics of the organisms used in metal adsorption, the metabolic pathway and their kinetics.

**Bioremediation by physio-bio-chemical mechanism**

Biosorption is the process which involves higher affinity of a biosorbent towards sorbate (metal ions), continued until equilibrium is established between the two components (Das et al., 2008). *Saccharomyces cerevisiae* acts as a biosorbent for the removal of Zn (II) and Cd (II) through the ion exchange mechanism (Talos et al., 2009). *Cunninghamella elegans* emerged as a promising sorbent against heavy metals released by textile wastewater (Tigini et al., 2010). Heavy metal degradation involves energy for the cell metabolic cycle. The combined active and passive modes of toxic metal bioremediation can be called bioaccumulation. Fungi have emerged as potential biocatalysts to access heavy metals and transform them into less toxic compounds. Some fungi such as *Klebsiella oxytoca*, *Allescheriella* sp., *Stachybotrys* sp., *Phlebia* sp. *Pleurotus pulmonarius*, *Botryosphaeria rhodina* have metal binding potential (D’Annibale et al., 2007). Pb (II) contaminated soils can be biodegraded by fungal species like *Aspergillus parasiticus* and *Cephalosporium aphidicola* with biosorption process (Akar et al., 2007). Hg resistant fungi (*Hymenoscyphus ericae*, *Neocosmospora*...
Vasinfecta and Verticillum terrestre) were able to biotransform a Hg (II) state to a nontoxic state (Kelly et al., 2006). Many of the contaminants are hydrophobic, and these substances appear to be taken up by microbes through the secretion of some biosurfactant and direct cell-contaminant association. Biosurfactants form stronger ionic bonds with metals and form complexes before being desorbed from soil matrix to water phase due to low interfacial tension (Thavasi, 2011).

Bioremediation may also involve aerobic or anaerobic microbial activities. Aerobic degradation often involves introduction of oxygen atoms into the reactions mediated by monooxygenases, dioxygenases, hydroxylases, oxidative dehalogenases, or chemically reactive oxygen atoms generated by enzymes such as ligninases or peroxidases. Anaerobic degradations of contaminants involve initial activation reactions followed by oxidative catabolism mediated by anoxic electron acceptors. The technique used to reduce the mobilization of heavy metals from contaminated sites by changing the physical or chemical state of the toxic metals is called immobilization. Solidification treatment involves mixing of chemical agents at the contaminated sites or precipitation of hydroxides (Evanko and Dzombak, 1997). Microbes mobilize the heavy metals from the contaminated sites by leaching, chelation, methylation and redox transformation of toxic metals. Heavy metals can never be destroyed completely, but the process transforms their oxidation state or organic complex, so that they become water-soluble, less toxic and precipitated (Garbisu and Alkorta, 2001).

Microorganisms use heavy metals and trace elements as terminal electron acceptors or reduce them through the detoxification mechanism, used for the removal of metals from the contaminated environment. Microorganisms remove heavy metals through the mechanisms which they employ to derive energy from metals redox reactions, to deal with toxic metal through enzymatic and non-enzymatic processes. Two main mechanisms for development of resistance in bacteria are detoxification (transformation of the toxic metal state and making it unavailable) and active efflux pumping of the toxic metal from cells (Silver, 1996). The basic redox (oxidation and reduction) reaction takes place in the soil between toxic metals and microorganisms;
microorganisms act as an oxidizing agent for heavy metals and cause them to lose electrons, which are accepted by alternative electron acceptors (nitrate, sulphate and ferric oxides). In aerobic conditions, oxygen acts as an electron acceptor, while in anaerobic conditions microbes oxidize organic contaminants by reducing electron acceptors. The microorganism takes energy for growth by oxidizing the organic compound with Fe (III) or Mn (IV) as an electron acceptor (Lovely and Phillips, 1988). Anaerobic degradation of organic contamination is stimulated with the higher availability of Fe (III) for microbial reduction (Spormann and Widdel, 2000).

Metals being used as terminal electron acceptors is called dissimilatory metal reduction (Ruchita Dixit et al., 2015). Biodegradation of chlorines from contaminants takes place through reductive dechlorination, where contaminants as chlorinated solvents acts as an electron acceptors in respiration. Microorganisms reduce the state of metals and change their solubility, like the Geobacter species, and reduce the Uranium soluble state (U6+) to insoluble state (U4+) (Lovely et al., 1991). Different defense systems (exclusion, compartmentalization, complex formation and synthesis of binding protein and peptides) reduce the stress developed by toxic metals. Heavy metal accumulation by microorganisms can be studied by the expression of metal binding protein and peptides (phytochelatins and metallothionein) (Ruchita Dixit et al., 2015). These metal binding protein transcription factors are known to mediate in hormone and redox signaling process in the context of toxic metal (Cd, Zn, Hg, Cu, Au, Ag, Co, Ni and Bi) exposure (Ruchita Dixit et al., 2015). Synechococcus sp. (cyanobacterial strains) has been reported with the expression of the smtA gene and production of metal-binding protein (Huckle et al., 1993). Ralstonia eutropha has been genetically modified to express mouse metallothionein on the cell surface and decrease the toxic effect of the Cd (II) in the contaminated sites (Valls et al., 2000). Expression of different proteins and peptides by the Escherichia coli regulates the range of accumulation of cadmium (Mejare and Bulow, 2001). Co-expression of precursor glutathione (GSH) along with phytochelatins (PC) resulted in the 10 fold increase in PC that finally increased cadmium accumulation two-fold (Kang et al., 2007). Natural resistant pathways for
heavy metals (Hg and Ar) in microorganisms have been regulated by metalloregulatory protein (Singh et al., 2008).

**Molecular mechanisms involved in bioremediation process**

Various mechanisms involved in the removal of heavy metals by microorganisms are known. In a genetically engineered bacterium *Deinococcus geothermalis*, Hg reduction has been reported at high temperatures due to the expression of mer operon from *E. coli* coded for Hg\(^{2+}\) reduction (Brim et al., 2003). Mercury resistant bacteria *Cupriavidus metallidurans* strain MSR33 was modified genetically by introducing a pTP6 plasmid that provided genes (merB and merG) regulating Hg biodegradation along with the synthesis of organomercurial lyase protein (MerB) and mercuric reductase (MerA) (Rojas et al., 2011). Modification of *Pseudomonas* strain with the pMR68 plasmid with novel genes (mer) made that strain resistant to mercury (SOone et al., 2013). Two different mechanisms for Hg degradation by bacteria (*Klebsiella pneumonia* M426) are mercury volatilization by reduction of Hg (II) to Hg (0) and mercury precipitation as insoluble Hg due to volatile thiol (H\(_2\)S) (Essa et al., 2002).

Genetic engineering of *Deinococcus radiodurans* (radiation resistant bacterium) which naturally reduces Cr (IV) to Cr (III) has been done for complete toluene (fuel hydrocarbon) degradation by cloned genes of *tod* and *xyl* operons of *Pseudomonas putida* (Brim et al., 2006). Microbial metabolites like metal bound coenzymes and siderophores mainly involved the degradation pathway (Penny et al., 2010). Genetically engineered microorganisms (GEM) are organisms whose genetic material has been altered using recombinant DNA technology to generate a character-specific efficient strain for bioremediation of soil, water and activated sludge by exhibiting enhanced degrading capabilities against a wide range of chemical contaminants (Sayler and Ripp, 2000). It offers the advantage of constructing microbial strains which can withstand adverse stressful situations and can be used as a bioremediators under various and complex environmental conditions.
Genetic engineering has led to the development of “microbial biosensors” to measure the degree of contamination in contaminated sites quickly and accurately. Various biosensors have been designed to evaluate heavy metal concentrations like mercury (Hg), cadmium (Cd), nickel (Ni), copper (Cu) and arsenic (As) (Verma and Singh, 2005). Genetic engineering of endophytes and rhizospheric bacteria for plant-associated degradation of pollutants in soil is considered to be one of the most promising new technologies for remediation of metal contaminated sites (Divya and Deepak Kumar, 2011). Bacteria like *Escherichia coli* and *Moraxella* sp. expressing phytochelatin 20 on the cell surface have been shown to accumulate 25 times more Cd or Hg than the wild-type strains (Bae *et al.*, 2003). However, one major obstacle for utilizing these GEMs in hostile field conditions is sustaining the recombinant bacteria population in soil, with various environmental conditions and competition from native bacterial populations (Wu *et al.*, 2006). Further, the molecular approaches have been applied to only limited bacterial strains like *Escherichia coli*, *Pseudomonas putida*, *Bacillus subtilis* etc. This means other microorganisms need to be explored for their application in heavy metal bioremediation through molecular intervention.