CHAPTER 2

REVIEW OF LITERATURE

2. Review of Literature

2.1. Background

From primeval period, metals have been an integral part of mankind. With gradual increase in its uses and their repertoire of demands in different eras of civilization has finally led to the development of modern industry and commerce. Industrial activities together with modernization, have led to large scale contamination of the environment with toxic heavy metals and other xenobiotic compounds affecting every strata of ecosystem including flora and fauna. Biro *et al.* [10] suggested that the toxic detrimental nature of heavy metal is due to their presence in transition form with incompletely filled *d* orbitals that have the ability to form complex unspecific compounds in the cells. Many toxic heavy metals even act as molecular 'mimics' of nutritionally essential trace elements to compete with important metallic cofactors for entry into cells and incorporation into enzymes. Even though metals play an important role in various biological systems, higher concentration of some metals might have toxic and deleterious effects on the cellular system and are reported to cause several diseases such as respiratory complications, emphysema, renal failure, bone disorders, immune suppression and various types of cancers in human beings [61]. It is now well known that heavy metals and other environmental xenobiotic compounds exert the toxicity through alteration of cellular redox status by generating the reactive oxygen species (ROS). The antioxidant system involving both enzymatic and non-enzymatic pathways play an important role in eliminating toxic effects of heavy metal induced oxidative damages [32].

Heavy metal pollution has become a major problem all over the world in recent times and is reported to have both direct and indirect impacts on the human health. Removal of metal contaminant from the environment is a real challenge as unlike organic contaminants, they cannot be destroyed but can only be transformed or contained [62]. Although physicochemical approaches are

available for metal remediation but from ecological and economical point of view these methods do not meet the criteria of specificity to treat target metals against a background of competing ions and for large scale subsurface contamination *in situ* [63]. Extensive studies in the recent past have been focused on biological approaches which may be applicable to remove highly toxic metals coupled with considerable operational flexibility and can be used both *in situ* and *ex situ* [62]. There has been recent focus on the use of resistant microorganisms that have evolved and adapted to the adverse environmental conditions in the field of heavy metal remediation. The significance of microbes for their role in biogeochemical cycling of toxic metals has been clearly elucidated [63] and their application in composting, sewage treatment and certain types of fermentation have been in practice by mankind since the beginning of the recorded history e.g. kitchen middens and compost piles dates back to 6000 BC. The first scientific use of bioremediation began over 100 years ago with the opening of the biological sewage treatment plant in Sussex, UK in 1891 [62]. Microbial flora with diversified taxonomy, acclimatization ability and varied catabolic traits can be exploited judicially in various biotechnological fields such as biofuel cells, microbe mediated nanomaterial synthesis, biosensors, bioremediation etc. [57]. Recent studies have also shown that microorganisms used as probiotics might be beneficial in minimizing heavy metal toxicity. Understanding of the molecular mechanism of heavy metal resistance and detoxification in the microbial system will help in resolving various issues related to environmental health, agriculture, energy crisis and healthcare [57, 61]. Focus of the present study is to characterize heavy metal resistant bacteria and decipher the molecular mechanism of metal detoxification using molecular and proteomic approaches.

2.2. Heavy metals

Broadly, the term 'Heavy metal' has been used to denote metals with an atomic number of 23 or more and with a density above 5 gm cm^{-3} [64, 65]. Different

authors have used different criteria to define heavy metals which are mainly based on density, atomic weight, atomic number or periodic table positions. Hawkes [66] suggested referring to heavy metals as all the metals in group 3 to 16 that are in periods 4 and greater. Of the 90 naturally occurring elements, 21 are non-metals, 16 are light metals and the remaining 53 (with arsenic) are heavy metals [64, 67]. Metals and metal compounds are natural constituents of the ecosystem with a wide range of oxidation states and coordination numbers. Their distribution in the environment is a result of geological processes (such as volcanic eruption, erosion, spring water, bacterial activity etc.) and other anthropogenic activities (fossil fuels, industrial and agricultural processes) [68, 69]. Once released into the environment adverse effects of toxic heavy metals are magnified and compounded by the bioaccumulation of metals in the absence of a definite route excretion from both animal and human body [68]. From the physiological point of view, metals fall into three main categories: i) those essential and nontoxic (e.g. Ca and Mg), ii) essential but harmful at high concentrations (e.g. Fe, Mn, Zn, Cu, Co, Ni and Mo), and iii) toxic (e.g. Hg or Cd) [70]. The increased use of metals in day to day life such as personal care products, home accessories, food products, vegetable etc. have exposed human being to metal toxicity but its wide applications and their role in progress of mankind cannot be denied.

Heavy metal such as chromium (Cr), occurring naturally is commonly found in the environment in two valence states, trivalent Cr (III) and hexavalent Cr (VI) which is widely used in industrial processes such as leather tanning, electroplating, metal finishing and chromate preparation processes thereby contaminating many environmental systems [63]. Similarly cadmium naturally occurring in ores together with zinc, lead and copper are used as stabilizers in PVC products, color pigment, paints, several alloys, phosphate fertilizers, fungicides, re-chargeable nickel–cadmium batteries etc. Metallic cadmium has mostly been used as an anticorrosion agent (cadmiation).WHO has referred

cigarette smoking and coal burning as two well-known sources of Cd toxicity [68] where as food is the most important source of cadmium exposure in the general non-smoking population in most countries. Metallic mercury is used in thermometers, barometers and instruments for measuring blood pressure. A major use of mercury is in the electrochemical process of manufacturing chlorine where mercury is used as an electrode. Mercury compounds have also been used to cure syphilis and as diuretics e.g. calomel (Hg_2Cl_2). The dental care staffs are the largest occupational group exposed to mercury and various studies have correlated the number of dental amalgam fillings or amalgam surfaces with the mercury content in tissues from human autopsy, as well as in samples of blood, urine and plasma [71]. Lead is also extensively used in paint, batteries, glass, petroleum, smelting alloying etc. and are found to accumulate extensively in bone and inhibits formation of heme and hemoglobin in erythroid precursor cells [61]. Elimination of lead from the body is a slow process and principally takes place via urine.

Arsenic which is widely distributed as metalloid occurs in the rocks, soil, water and air, and is mainly used in the production of pesticides, wood preservatives, and semiconductors to strengthen copper and lead alloys for battery manufacturing. Arsenic enters human body through food and in some area drinking water is a significant source of arsenic exposure. The contaminated soils such as mine-tailings are also considered as potential source for arsenic exposure. Like cadmium and mercury, nickel is also used in Ni-Cd batteries, pigments for ceramics and glass, non-precious dental materials and as catalyst for hydrogenation processes in the food, petroleum and petrochemical industries etc.

2.3. Biological importance of few heavy metals

In general there is a misconception on heavy metals that they are toxic but toxicity is found only if the concentration in an organism exceeds a certain

threshold point. Some of the metals such as calcium (Ca), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), potassium (K), nickel (Ni), zinc (Zn) etc. are essentially required for normal cellular processes in both prokaryotes and eukaryotes [65]. These nutritive and essential metals function as catalysts/cofactors for biochemical and enzymatic reaction, as stabilizers of protein structures and bacterial cell walls and also play an important role in maintaining osmotic balance [72, 73, 74]. Metals like iron, copper and nickel are involved in the cellular redox processes and other essential metals such as magnesium and zinc stabilizes various enzymes and DNA through electrostatic interactions. The presence of ferrous ion uptake system is important for bacteria that live mostly or occasionally under anaerobic conditions as Fe^{3+} can be used as electron acceptor [75]. Nickel is known to be present in some important enzymes such as hydrogenase, urease, dehydrogenase etc. bound to cysteine or histidine at the active sites. Potassium and sodium are known to regulate intracellular osmotic pressure [76] and some essential metals play vital role in regulating gene expression and bio-molecular activities. In contrast, toxic or non-essential metals such as cadmium and lead have no documented physiological function.

In plants, micronutrients are transition elements which are essential for biosynthesis and growth, nucleic acids, chlorophylls, carbohydrates lipids and secondary metabolites [77]. Supply of micronutrients is also essential for the integrity of the membranes [78]. Several studies have reported that non-essential heavy metals have stimulating or inducing effects at low dose, for example Cd is known to produce stimulating effects in the barley seedlings and similarly Pb & Ti in detached barley leaves [79, 80]. Manganese functions as an electron donor and plays an important role in catalysis of water cleavage during oxygenic photosynthesis [81]. In photosystem II of the photosynthetic process, four manganese ions are bound to a tetra nuclear complex together with calcium and chlorine [82, 83, 84]. In the cyclic light energy process, water

is oxidized to molecular oxygen and manganese ions are located close to tyrosine radicals and is required for the abstraction of protons from water [85, 86]. In the mammalian cells, chromium cation is reported to bind to a low molecular mass polypeptide in the ratio 4 Cr/peptide, resulting in the activation of insulin receptor tyrosine kinase [87] and this probably explains the reduced glucose tolerance in chromium starvation situation. Chromate, on the other hand, is known to be toxic, carcinogenic and allergenic (mason's allergy) in humans [88].

Heavy metals are also known to be a part of important enzymes e.g. manganese, copper, zinc and nickel required for the superoxide dismutase (SOD) activity which is an important antioxidant enzyme [64]. Besides copper/zinc superoxide dismutase, the function of copper is in the cytochrome C oxidase and other oxygen-dependent terminal oxidases an important part of respiratory chains of many organisms. Zinc is found to be associated with several enzymes and DNA binding proteins such as zinc finger proteins that bind to DNA and regulates gene expression in the eukaryotic systems [89]. Even though heavy metals are essential for variety of biological processes, high concentration have been reported to be toxic and associated with several disease conditions.

2.4. Heavy metals and human health/ associated diseases

Several studies have reported toxic and carcinogenic effects of heavy metals in humans. Prolonged exposures could result in dysregulation of the cellular pathways leading to subsequent toxicity [90]. Coordination chemistry and redox property of the metals helps them to escape the control mechanisms of homeostasis, transport, compartmentalization or binding to the designated tissue and cellular constituents. Dysregulation of the control mechanism leads to the binding of metals to unspecific protein sites or displacement of other metals from their natural binding sites. Reports suggest that heavy metal

interactions with nuclear proteins and DNA might cause oxidative deterioration of biological macromolecules [91]. Accumulation of metals may lead to the disruption of functions of vital organs and glands such as heart, brain, kidneys, bone, liver, etc. Exposure to the toxic metal and chemicals cannot be avoided as they can enter the body particularly through consumption of foods, beverages, skin, and the inhaled air [91].

Cadmium: The half-life of cadmium in humans is estimated to be between 15 and 20 years. Several reports suggests that the inhalation of cadmium fumes or particles can lead to life threatening, acute pulmonary disorders [92, 93] and various diseases such as anemia, non-hypertrophic emphysema, eosinophilia, anosmia, chronic rhinitis, growth impairment, hypoglycemia, reproductive disorders, schizophrenia, stroke, cerebral hemorrhage, cirrhosis etc. Kidney damage has been found to be reversible while the tubular damage is irreversible. Tubular dysfunction results in the excretion of low molecular weight proteins [such as β2-microglobulin and α1 microglobulin (protein HC)] or enzymes [such as N-acetyl-β-D-glucosaminidase (NAG)] [94]. Long-term exposure of cadmium may cause skeletal damage which was first reported in 1950's from Japan, where the *'itai-itai'* (ouch-ouch) disease (a combination of osteomalacia and osteoporosis) was caused due to cadmium-contaminated water used for irrigation of local rice fields. Cadmium is also reported to be carcinogenic and have been associated with cancers of the lung, prostrate, pancreas, and kidney [61]. Because of its carcinogenic properties, cadmium has been ranked as category #1 human carcinogen by the International Agency for Research on Cancer (IARC) of USA [61].

Mercury: Metallic mercury is an allergen which may cause contact eczema. Acute mercury exposure may damage lung, kidney and other vital organs. The neurological and psychological symptoms such as tremor, changes in personality, restlessness, anxiety, sleep disturbance and depression are characteristic of chronic poisoning. However, these symptoms are reversible

after cessation of the exposure [61]. Inorganic mercury do not affect the central nervous system due to the blood-brain barrier but methyl mercury poisoning leads to nervous system damage [95]. The earliest symptoms are paresthesia and numbness in the hands and feet which later might cause visual and auditory difficulties and higher doses might be lethal e.g. a disease occurred popularly known as Minamata catastrophe caused by methyl mercury poisoning from mercury contaminated fish which were discharged to the surrounding sea [61].

Lead. The half-life of lead in blood is about 1 month and in the skeleton for 20–30 years [96] and the symptoms of acute lead poisoning are headache, irritability, abdominal pain and various symptoms related to the nervous system. Recent research has shown that long-term low-level lead exposure in children might lead to diminished intellectual capacity, behavioral disturbance and concentration difficulties. There are also evidences that certain genetic and environmental factors can increase the detrimental effects of lead on neural development, thereby making children more vulnerable to lead neurotoxicity [97]. In severe cases of lead encephalopathy, the affected person may suffer from acute psychosis, confusion and reduced consciousness and in less serious cases might cause disturbance in the hemoglobin synthesis and long-term may cause anemia. IARC classified lead as a 'possible human carcinogen' in 1987 and the most likely candidates are lung cancer, stomach cancer and gliomas [98].

Arsenic: Acute arsenic toxicity might lead to gastrointestinal symptoms, severe disturbances of the cardiovascular and central nervous systems and eventually death. The survivors may suffer from bone marrow depression, hemolysis, hepatomegaly, melanosis, polyneuropathy and encephalopathy. Ingestion of inorganic arsenic may induce peripheral vascular disease which may lead to gangrenous changes (black foot disease reported in Taiwan) [61]. The populations exposed to arsenic from drinking water showed excess risk of

mortality from lung, bladder and kidney cancer [61]. There is also an increased risk of skin cancer and other skin lesion, such as hyperkeratosis and pigmentation changes. Studies on various populations exposed to arsenic by inhalation, such as smelter workers, pesticide manufacturers and miners have demonstrated the occurrence of lung cancer [61]. Reports are also available on diabetes and reproductive effects and weak cerebrovascular disease, long-term neurological effects and cancer of sites other than lung, bladder, kidney and skin [61].

Chromium: Chromium have been used as an antirust agent in water cooling, plating, leather tanning, production of textiles, dyes and pigments, metallurgical and chemical industries and in heat resistance. There are two major stable oxidation states of the element Cr (VI & III) as well as Cr metal out of which most dangerous and toxic form is the hexavalent chromium [88]. It has been reported that chromium acts as oxyanion molecular mimicry, therefore are transported and accumulated in the cells instead of sulfate or phosphate. The hexavalent chromium has structure similar with sulfate and phosphate at physiological pH and can enter the body and can even reach to the brain. Depending on the genetic susceptibility of an individual this could pose significant risk for cancer induction in any organ. All cells and organs possess the ability to take up hexavalent chromate, and many cells have the capacity to reduce Cr (VI) intracellularly to trivalent Cr which reacts with protein and DNA to produce toxicity and potentially causes cancer [99]. Chromium is recognized by the IARC and the US Toxicology Program as a pulmonary carcinogen. The increased risk of lung cancer occurs primarily in workers exposed to hexavalent chromium dust during the refining of chromite ore and the production of chromate pigments [99]. The individual studies suggest the possibility of an excess incidence of cancer at sites outside the lung but the results from these studies are inconsistent.

Nickel: Exposure of nickel-polluted environments causes variety of pathologic effects on human health. Sunderman *et al.* [100] reported the elevated incidences of lung and nasal cancer in the workers exposed to nickel. However, the exact mechanism of nickel-induced carcinogenesis is still unclear. Besides cancer, exposure of nickel causes variety of adverse effects on human health. Most important and frequent is nickel allergy in the form of contact dermatitis, others are lung fibrosis, cardiovascular and kidney diseases.

2.5. Heavy Metal Detoxification Mechanism

Several evidences suggest the existence of a concentration threshold between adaptive response and severe cell deregulation [101]. The organisms exposed to chronic level of toxicants can develop acclimatization that might confer increased resistance through important metabolic reshuffling [102]. Such organisms have evolved and adapted several mechanisms to tolerate and detoxify heavy metals. The toxic metal ions are able to cross membranes by various means such as molecular mimicry, where metals either compete for binding to multivalent ion carriers (such as Ca^{2+} channels) or after binding to low molecular weight thiols (such as cysteine) enter the cell by active transport (e.g. using amino acid transporters) [102, 103]. In another type of mechanism, metals bound to chelating proteins (such as metallothioneins) may enter the cell by endocytosis. Heavy metals can cause membrane depolarization and acidification of the cytoplasm [104, 105] and membrane injury which may lead to disruption of cellular homeostasis. Cellular adaptations such as exudation of chelating compounds and active efflux of metal ions by primary ATPase pumps can provide some degree of metal tolerance [104, 106] or even induce the synthesis of protective proteins. The antioxidants and related enzyme systems also play an important role in protecting cellular components from metal induced oxidative stress and acts through scavenging of free radicals, interaction with other antioxidants, bioavailability, ability to repair oxidative damage and metal chelating activity. In addition to the antioxidant proteins,

cells synthesize number of metal chelators such as metallothioneins (MTs) and phytochelatins (PCs) which are cysteine-rich polypeptides and have the ability to coordinate metals using the sulfhydryl groups on the protein [101].

2.5.1. Detoxification mechanism in prokaryotes

Microorganisms particularly bacteria have evolved several types of mechanisms to tolerate the uptake of heavy metals. These mechanisms fall under various facets of metal microbe interactions such as efflux of metal ions outside the cell, bioaccumulation/ biosorption, precipitation and complexation of the metal ions inside the cell, biotransformation (reduction & oxidation) of the heavy metal ions to a less toxic state [64], chelation (e.g. metallothionein, phytochelatins and organic acids), partitioning (vacuole) [107, 108] and alteration of the membrane permeability to the metal immobilization within the cell wall [109]. Currently three main export mechanisms for heavy metals are known: i) Transporters P-type ATPases or cation/H$^+$ antiporters which use ATP energy to pump metal ions out of the cytoplasm [110]. ii) CBA transporters which has three component trans envelop pumps of gram negative bacteria and act as chemiosmotic antiporters. iii) Cation diffusion facilitator (CDF) family transporters, which act as chemiosmotic ion proton exchangers. Presence of CBA transporters (RND protein) is exceptional and indicates high level resistance to heavy metal ions where as CDF transporters and P-type ATPase are commonly found among different bacterial species. Thus, these transporters form a part of the cellular homeostasis system [111].

Resistance systems are generally found on plasmids as well as in chromosomes (e.g. mercury resistance and cadmium efflux mediated by a P-type ATPase in *Bacillus* and arsenic efflux by chromosomal *Escherichia coli* genes). The first complete bacterial genome sequence i.e. 1.8 mega base pairs (Mbp) from *Haemophilis influenzae* [112] has listed homologs for *arsC*, *merP*, and *merT* that codes for arsenic and mercury resistance. Similarly, cyanobacterium

Synechocystis PCC6808 [113] have a homolog to the Czc cadmium, zinc, and cobalt resistance system and other genes apparently involved in toxic metal ion transport including some for arsenic and copper. The efflux pumps can be either ATPases (e.g. the cadmium and copper ATPases of gram-positive and the arsenite ATPase of plasmids of gram-negative bacteria) or chemiosmotic (e.g. the divalent cation efflux systems of soil *Alcaligenes* and the arsenite efflux system of the chromosome of gram-negative bacteria and plasmids of gram-positive bacteria).

Besides above mechanisms, certain enzymatic and non-enzymatic antioxidants have been reported to play important role in overcoming metal stress. Studies have shown that metals causes physiological stress through the generation of ROS such as O_2^-, H_2O_2, OH [40] and to neutralize the ROS different protective enzymes such as glutathione (GSH), ascorbic acid (ASA), superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR)and glutathione peroxidase (GPx) are expressed. These enzymes and non-enzyme systems are known to maintain the cellular redox state. SOD and CAT are known as the front line antioxidant enzymes which limit the balance of superoxide radicals and H_2O_2 whereas thiol compound GSH play a crucial part in oxidative stress relaxation [35, 41, 114, 115] by sequestering ROS. GR and GPx are responsible for maintaining the concentration of GSH in the cell. The enzyme GPx removes toxic peroxides formed during the normal course of metabolism and oxidizes reduced GSH to oxidized form (GSSG) [32, 116].

Different research groups have worked on proteome profile of various microbial systems to study the mechanism of heavy metal detoxification. Proteome findings have shown the expression (up- and down-regulation) of proteins with diverse functions suggesting the existence of several resistance mechanisms in bacteria. The protein expressed were from different functional categories including protein folding and repair, metal homeostasis, heavy metal efflux, energy metabolism, protein synthesis, antioxidant system etc. Studies

have shown that in response to higher concentration of chromium in Cr (IV) resistant *P. aeruginosa,* different proteins such as chaperons, 30S and 50S ribosomal proteins, elongation factor EF-TU, glutathione system and outer membrane proteins ere expressed [117]. Similar data were also reported by Daware *et al*. [118] in *K. pneumonia* under As (III) stress, where proteins related to carbohydrate and amino acid metabolism were expressed. The proteomic studies in response to metal stress were also reported in *P. fluorescens* [119]*, P. putida* [120] and *Caenibacter arsenoxydans* [121].

2.5.2. Detoxification Mechanism in Algae

Algae (both micro- and macro-) possess molecular mechanisms that allow them to discriminate non-essential heavy metals from those essential for its growth. Like higher plants, algae have attracted considerable attention for the capacity to eliminate heavy metals. It has been found that algae have developed the ability to produce peptides capable of binding heavy metals [122]. The organo-metallic complexes formed are partitioned inside vacuoles to facilitate appropriate control of the cytoplasmic concentration of heavy metal ions, thus preventing or neutralizing the potential toxic effect [122]. Stokes *et al.* [123] first discovered Mt III complex synthesis in the microalga *Scenedesmus acutiformis* in the presence of Cd^{2+}, Pb^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+}, Ag^+ and Hg^{2+} occurring in non-contaminated natural waters. Similar results are available in *Thalassiosira weissflogii* and *Scenedesmus subspicatus* [124]. Howe and Merchant [125] reported sequestration of approximately 70% of cytosolic Cd^{2+} by MtIII in *Chlamydomonas reinhardtii*. Torricelli *et al*. [126] working with two strains of *Scenedesmus acutus* (wild type and tolerant to Cr^{6+}) found higher level of cysteine in the tolerant strain. When the cells were exposed to Cd^{2+}, tolerant strain showed higher levels of reduced glutathione and MtIII compared with the wild strain. Tsuji *et al*. [127] suggested MtIII could play a role not only in detoxification of heavy metal, but also in mitigation of oxidative stress.

Additionally, MtIII might play an important role in essential metal ion homeostasis and sulfur metabolism in microalgae as well as in higher plants. In contrast to higher plants, the antioxidant response to oxidative and environmental stress has not been extensively investigated in algae at the molecular level [128]. However, in recent years several studies have been done showing algae as potential tools for bioremediation of heavy metals which is reflected from accumulation of metals to various degrees by different algae [129] and their survival in potentially toxic conditions. In the red algae *Mastocarpus stellatus* and *Chondrus crispus*, a correlation was found between the general level of ROS metabolism and oxidative and general-stress tolerance [130]. The bioaccumulation of Cd^{2+} by the red alga *Porphyra umbilicalis* and other seaweeds has also been detected [131]. Interestingly, some dinoflagellate algae have developed an important defense mechanism termed as encystment i.e the formation of cysts to survive under adverse conditions. When exposed to pollutant metals (Hg^{2+}, Cd^{2+}, Pb^{2+}, and Cu^{2+}), the dinoflagellate *L. polyedrum* may exhibit cell death or cyst formation depending on the dose [132]. It seems that survival of this group of algae at high metal concentrations might be due to cyst formation. This idea was supported by the fact that three species of dinoflagellates, *Amphidinium carterae*, *Lingulodinium polyedrum*, and *Prorocentrum micans*, can recover after metal-triggered encystment [133].

2.5.3. Detoxification mechanism in Yeast/ Fungi

Fungi produces metal chelating compounds such as siderophores and organic acids, (mainly oxalic acid and citric acid as metabolites), and their production is associated with the solubilization of insoluble compounds containing metal. The organic acids immobilize potentially toxic metals through formation of insoluble compounds such as metal-oxalate complexes and are considered as an important mechanism for metal detoxification [134]. The fungi also exudates different organic molecules such as di and tricarboxylic acids that do not belong to the matrix of the cell wall and are reported to chelate metal ions. Studies have shown that induction of oxalic acid efflux

is correlated with Cu tolerance in brown rot fungi. The over excretion of oxalic acid probably might contribute to the metal tolerance exhibited by *Beauveria caledonica* [135]. *Aspergillus niger,* a wild-type strain showed significant alterations in the level of certain enzymes and biomolecules to counter arsenate toxicity [136]. Among different levels of acid production, the level of oxalic acid was comparatively higher than other acids such as malonic, citric, shikimic, lactic, acetic, propionic, fumaric, formic, iso-butyric and butyric acid [137]. The metal ions binds with the chelators (PCs, MTs, organic acids) and chaperons and such chelators might contribute to metal detoxification by buffering cytosolic metal concentration, while chaperons can specifically deliver metal ions to organelles and metal requiring proteins. The main storage compartment of toxic compounds in fungi is the vacuoles where the transporters (Metal/H+ antiporters) help to remove and sequester potentially toxic cations from the cytosol [138]. Mycorrhizal fungi known to be important phytostabilizers of toxic heavy metals also protects plants by accumulating metallic pollutants which are stored in the vesicles as well as in fungal hyphae in the roots. Thus, they are immobilized and do not inhibit the growth and uptake of phosphorus and other micronutrients. These fungi also release various organic acids which increase the solubilization of insoluble phosphate compounds present in the soil. Arbuscular mycorrhizal (AM) fungi release glomalins that are certain metal absorbing glycoproteins known to increase the immobilization of toxic metal. Metallothioneins are also released by certain AM fungi, which also have been reported to reduce the heavy metal toxicity by chelation [139].

2.5.4. Detoxification mechanism in Plants

 Plants are also affected by the heavy metals. It can reduce photosynthesis, cause water imbalance, and disturb nutrient accumulation and many other adversities [140, 141]. However, some plants have evolved with a wide range of mechanisms at the cellular level which might be involved in the detoxification and increasing the tolerance against metal stress. Plants exposed to heavy metals experience oxidative stress leading to cellular damage and disturbance of cellular ionic homeostasis. To minimize the harmful effects of free radicals and ROS generated during oxidative stress by heavy metals, plants

have evolved an effective mechanism of defense by both antioxidants and non-enzymatic antioxidants that scavenge the generated ROS [142, 143]. Among the antioxidant defense system, ascorbate (AsA), glutathione (GSH), and related enzymes such as ascorbate peroxidase, glutathione peroxidase and glutathione reductase plays an important role in direct or indirect scavenging of ROS from plant cells. AsA is an essential compound in plant tissues that reacts rapidly with superoxide and singlet oxygen and H_2O_2 enzymatically through ascorbate peroxidase [144, 145, 146]. For alleviating the detrimental effects of heavy metal exposure and their accumulation, plants use chelation and subcellular compartmentalization mechanism. Phytochelatins (PC) are well-studied heavy metal chelators in the plants which are synthesized non-translationally from GSH in a transpeptidation reaction catalyzed by the phytochelatin synthase (PCS) [147, 148], an enzyme that is activated in presence of metal ions [149]. Therefore, availability of glutathione which also reduces ROS is very essential for PC synthesis in plants during their exposure to heavy metals [91,150]. Studies have found that nitric oxide (NO) is involved in the regulation of multiple plant responses to a variety of abiotic and biotic stresses. NO help plants to resist heavy metal stress either indirectly scavenging heavy metal induced ROS by increasing the antioxidant content and anti-oxidative enzyme activity or by affecting root cell wall components to increase accumulation of heavy metals. Reports also suggests that NO might act as a signaling molecule in the cascade of events leading to changes in the gene expression under heavy metal stresses e.g. induction of heat shock proteins [150,151]. Studies have revealed that plant possess several classes of metal transporters that might be involved in metal uptake and homeostasis in general, and thus could play a key role in metal tolerance. These include the heavy metal CPx-ATPases, Nramps and CDF (cation diffusion facilitator) family and the ZIP family [152]. Studies have also suggested that some plants resist a number of heavy metals including Zn and Cd by accumulation in the vacuole [153].

2.5.5. Heavy metals detoxification mechanism in animals

Several experimental studies on heavy metal detoxification in animal model system suggested that the multidrug resistance-associated protein subclass genes such as ATP binding cassette transporter gene and the mitogen activated kinase (MEK1) gene [122] showed increased sensitivity to heavy metal exposure. There is also growing evidence that phytochelatins might be important for metal detoxification not only in plants but also in many different animal species. Phytochelatins like metallothioneins are cysteine-rich peptides but unlike metallothioneins they are not genetically encoded, but are nonribosomal peptides produced from glutathione by the enzyme phytochelatin synthase (PCS) (EC 2.3.2.15) [154, 155]. The ubiquity of the PC synthase-dependent pathway in the animals and other than plants remains to be delineated, but there are many nematode species and parasitic protists such as *Eimeria, Leishmania, and Plasmodium* contain cDNAs whose predicted proteins are also PC synthase homologs and might be implicated in metal detoxification in animals [156, 157].

Metallothioneins (MT) are small cysteine-rich proteins that strongly bind to metal ions such as cadmium. These proteins were studied extensively in a wide variety of organisms, including prokaryotes, plants, invertebrates and vertebrates. The metal binding domain of MT consists of 20 cysteine residues, whereby the sulfhydryl groups can bind 7 moles of divalent metal ions. All the naturally occurring protein has Zn (II) in both binding sites, this ion may be displaced by other metal ions that have a higher affinity for thiolate such as Pb (II), Cu (I), Cd (II), Hg (II), Ag (I), Pt (II and IV), and/or Pd (II). MTs have been shown to be strongly metal-inducible in many different animal species and the evidences suggest that knocking out of MTs reduces tolerance to heavy metals. However, it is also clear that MTs are not the sole players in detoxification but it has been implicated in many biological roles beyond

detoxification including metal storage, transport and in metal ion homeostasis [158].

2.5.6. Lipoic acid and metal detoxification

Lipoyl synthase (EC 2.8.1.8) catalyzes synthesis of lipoic acid (LA) which is an essential cofactor required for the function of important metabolic pathways (multienzyme complexes). LA (1, 2 dithiolane pentanoic acid) is a dithiol which is very effective against oxidative stress due to the presence of two sulphydryl moieties. The antioxidant properties is present both in its reduced (dihydrolipoic acid) and oxidized form but reduced form has more antioxidant properties and might play an important role in recycling of other oxidized radical scavengers such as glutathione, ascorbate, tocopherol etc. The molecular mass of lipoic acid (mass 206) is relatively low as compared to tocopherol but larger than ascorbic acid and is soluble in both water and fat which facilitates its connectivity in the activity of antioxidants on the membrane with that of cytoplasm forming strong antioxidant network within the cell [159, 160, 161, 162, 163]. Increased levels of LA in response to metal stress have been predicted to play a pivotal role in the detoxification mechanism. A number of experimental as well as clinical studies emphasize the usefulness of LA as a therapeutic agent for diverse conditions, including diabetes, atherosclerosis, insulin resistance, neuropathy, neurodegenerative diseases and ischemia reperfusion injury. LA has also been reported to suppress the inflammatory response by inhibiting molecular signaling pathways activated by pro-inflammatory cytokines, such as TNF- α. TNF-α is a key activator of the NF- β B pathway, which mediates inflammatory responses and regulates the expression of several inflammatory mediators, including chemokines, cytokines and cytokine receptors [164, 165]. It is also reported to detoxify the divalent metal and subsequently ameliorates the cell membrane integrity [166]. Antidotal property of LA against Cd induced hepatotoxicity has also been reported [167].

2.6. Heavy metals and Environmental concern

Heavy metals are the main group of inorganic contaminants and a considerable large area of land is contaminated due to the uses of sludge or municipal compost, pesticides, fertilizers, and emissions from municipal wastes incinerates, exudates, residues from metalliferous mines and smelting industries [168]. Irrespective of the origin of the metals in the soil, excessive levels of many metals can result in soil quality degradation, crop yield reduction, and poor quality of agricultural products, posing significant hazards to human, animal, and ecosystem health [169]. Therefore, it has become essential to remove the accumulated metals from the contaminated area [91].

Heavy metals such as cadmium products are rarely re-cycled, but frequently dumped together with household waste contaminating the environment. The anthropogenic activities are the main source of cadmium, including industrial emissions and the application of fertilizer and sewage sludge to farm land, may lead to contamination of soils, and to increased cadmium uptake by crops and vegetables. High levels of air emissions may pollute areas near lead (Pb) mines or smelters and the airborne Pb can be deposited on the soil and water, and reaches human bodies *via* the food chain. Heavy metals also effect the growth of the plant. The leaf chlorosis, disturbed water balance and reduced stomatal opening are symptoms of metal toxicity in the plant and are part of heavy metal toxicity syndrome [170]. There is also decrease in chlorophyll and carotenoid content and the consequences affect the rate of photosynthesis and plant growth [171]. The decrease in the photosynthesis and its growth might be due to the disturbance in the electron transport causing electrons to be transferred to oxygen instead of the natural electron acceptors in chloroplasts and mitochondria, production of hydroxyl radicals by redox active metals in different oxidation states, inactivation and down regulation of enzymes of the antioxidant defense system and depletion of antioxidant substrates are some of the factors known [172].

2.7. Heavy metals and Remediation Methods/technologies

Various methods have been adopted for the removal of the heavy metals from the contaminated environment. The most common methods which are currently in use are chemical precipitation, chemical oxidation and reduction, ion exchange, filtration, electrochemical treatment, reverse osmosis (membrane technologies), evaporative recovery and solvent extraction. However, these methods are expensive and sometimes ineffective, and are not ecofriendly due to high energy requirements, unpredictable removal of metal ions and also cause generation of toxic sludge etc. [173, 174, 175, 176]. Some of the other commercially used methods are soil vapour extraction, soil flushing, excavation, solidification/stabilization, land farming etc. But these processes are not economically feasible and usually dependent on the concentration of the waste. The operational costs for treatment, energy consumption and disposable of the residual metal sludge are very high, therefore the search for novel and environment friendly techniques are needed.

With several disadvantages and limitation of the conventional methods, microbe mediated remediation has emerged as a promising approach and is considered as a potential alternative and economically attractive strategy for remediating metal contaminants. There are several advantages such as low operating cost, minimum ratio of disposable sludge volume, high efficiency in detoxifying very dilute effluents and also have potential for *in situ* remediation of contaminated groundwater and/or sediments [170, 177]. However, metal removal by microorganisms is a complex process that depends on various factors such as the chemistry of the metal ions, cell wall composition, physiology, and physicochemical factors such as pH, temperature, contact time, ionic strength, and metal concentration [178]. The understanding of the mechanism of detoxification is considered important to exploit the microbes for the remediation applications. Microbes particularly the bacteria have adapted to a variety of heavy metals and developed the tolerance which is often

plasmid-borne with capability to be transferred throughout the bacterial community by the lateral gene transfer. Heavy metal resistant microorganisms may be useful as indicators of potential toxicity to other forms of life and are important in studies of genetic transfer in heavy metal resistance mechanism [179, 180]. Heavy metals may therefore act as important selective agents driving the evolution of microbial communities. Thus with the abundance of diverse microorganisms in nature and their ability to acclimatize with harsh conditions like abilities to tolerate and sequester various toxic metals, it is of immense importance to identify and characterize such microbial strains with respect to their interactions with heavy metals. The genetic and molecular mechanisms regulating these interactions are essential factors determining the role of microbes in natural and perturbed environment with respect to metal biogeochemistry.

The present field of microbe based remediation has led to the development of innovative techniques that are in process or being utilized for detecting, concentrating and removing heavy metals from polluted sites. Currently there are different strategies that are implemented for *in situ* and *ex situ* bioremediation such as biostimulation, bioaugmentation, biotransformation (biooxidation & bioreduction), bioaccumulation and biosorption, natural attenuation etc. For biostimulation additional nutrients are added to the contaminated sites to increase the naturally occurring microbiota and in bioaugmentation, microorganisms are additionally incorporated that can biodegrade or biotransform a particular contaminant. The intrinsic bioremediation that occurs *in situ* and depends on the existing natural biological processes are known as natural attenuation [62]. Scientific reports have illustrated that different diversified bacterial system such as *Pseudomonas* sp. [6]*, Bacillus* sp. [173]*, Shewanella* sp. [181], *Cupriavidus metallidurans* [182] etc. are able to detoxify and sequester different heavy metals. Apart from bacteria, algae and fungi are also found to be suitable for the selective removal

and concentration of heavy metals. The main advantage of the algae is its high biomass production which benefits high adsorption and accumulation of heavy metals. The algal species such as *Spirogyra hyaline* for Cd, Hg, Pb, As and Co, *Sargassum fluitans* for Cu, Fe, Zn and Ni, *Fucus vesiculosus* for Zn and Ni, *Phormedium bohner* for Cr, *Ascophyllum nodosum* for Au, Co, Ni etc. and many other species have the capacity to uptake and accumulate metals and can be efficiently used in metal remediation and recovery processes. The ability of microalgae to accumulate metals within their tissues has led to their widespread use as biomonitors of metal availability in marine systems [183] and others such as microalgae are being used as biosensors as changes take place in their overall metabolism under metal stress. *Chlorella, Scenedesmus* and *Spirulina* are most widely used algae for metal removal [183]. Thus ecologically important algae can provide solutions to the challenges of environmental pollution [184, 185]. Fungi and yeasts accumulate micronutrients such as Cu, Zn, Mn and non-nutrient metals like Ni, Cd, Sn and Hg at higher concentration than nutritional requirement [186, 187]. Fungi belonging to the genera *Rhizopus* and *Penicillium* have already been studied as potential biomass for the removal of heavy metals from aqueous solutions [188, 189]. The different species of both live and dead cells of *Trichoderma, Candida, Zygorhynchus, Mucor, Saccharomyces and Fusarium* also have the ability to biosorb different metal ions from aqueous solutions [190, 191, 192]. Mycorrhizal fungi (*Glomus intraradices*, *Glomus mosseae* and some other species of *Glomus*) can also play a role in bioremediation of heavy metal pollution in soil via its fungal hyphae as well as in arbuscles [192, 193]. A variety of phytoremediation mechanisms such as phytostabilization, phytostimulation, rhizofiltration, phytodegradation, phytoextration, phytovolatilization etc. can be employed to remediate different types of contamination in soil, water and air. Compared with the organic pollutants only a few remediation techniques are available for metal pollutants and the use of plants to strip heavy metals from soil is an emerging tool [194]. Plants

accumulating extremely high amounts of one or more heavy metals are referred to as 'hyper accumulators'. More than 400 heavy metal hyperaccumulating plant species have been identified, 75% of which are Ni hyperaccumulators growing on ultramafic soils. The tropical hyperaccumulators are mainly trees and shrubs belong to the families *Violaceae, Flacourtaceae, Buxaceae* and *Euphorbiaceae* and also herbs from the family *Brassicaceae* [195]. The symbiotic relation of microbes and plants increases the microbial population and metabolic activity in the rhizospheres. This improves the physical and chemical properties of contaminated soil [196, 197, 198] as well as stimulates growth of the plants [199]. The rhizosphere is dominated by gram negative rods such as *Pseudomonas* sp. which are metal resistant and has the ability to detoxify heavy metals. It has been hypothesized that, when a suitable rhizosphere strain is introduced together with a suitable plant (e.g., by coating bacteria on plant seed), these well-equipped bacteria might settle on the root together with the indigenous population, thereby improving the efficiency of bioremediation process [200]. The mechanistic knowledge at biochemical and molecular levels on the metal detoxification might be exploited for the remediation and better management of heavy metal based waste.

2.8. Future prospects of Bioremediation

Recent technological development have led to the better understanding of microbe's role in the mineral cycling and application of such processes/mechanisms in the bioremediation of heavy metals. Both genomic and proteomic approaches have revolutionize many aspects of biology and have undoubtedly made an impact in the arena of environmental biotechnology. The availability of complete genome sequences for several environmentally relevant microorganisms will surely prove useful for understanding the precise mechanisms of environmentally relevant metal-microbe interactions. The different molecular markers expressed during stress conditions can potentially serve as indicators of metal stress in natural

microbial communities and the factors responsible for survival of the organisms can be used as biosensors. The present study is focused on the biochemical and proteomic approaches for understanding metal detoxification mechanisms in the bacterial system. The findings of the present investigation might have relevance in overcoming metal toxicity and might find applications in the field of bioremediation, biosensor or bio-mining.