Chapter I

GENERAL INTRODUCTION
1.1 HEAVY METALS

1.1.1 Definition

Heavy metals are the natural constituents of the earth crust constituting about 75% of the known elements in the biosphere (Sparks, 2005). Heavy metals are metals or metalloids termed as elements having density $\geq 5g/cm^3$ or 5 times more than water (Duruibe et al., 2007) including most of the transition elements (excluding Sc, Ti, Y), lanthanides, actinides (Nies, 1999). Of the 90 naturally occurring elements in periodic table, 53 elements are considered, heavy metals (Weast, 1984). Thirteen trace metals and metalloids silver (Ag), arsenic (As), bellium (Be), cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), nickel (Ni), lead (Pb), antimony (Sb), selenium (Se), titanium (Ti), zinc (Zn) are considered priority pollutants (Sparks, 2005). Unlike, the organic pollutants which are degradable into carbon dioxide and water, heavy metals are degradable to degradation as they can't be metabolized in body and persist in the environment (Gaur et al., 2012). They tend to bioaccumulate in the tissues, once it enters the body over a period of time, that leads to a significant bioaccumulation of heavy metal in the higher trophic level predators of the food chain and have long biological half-lives (van Lipzig et al., 2005, Lalonde et al., 2001).

1.1.2 Sources of heavy metals

Heavy metals are emitted into the environment by natural or anthropogenic activities. Rocks and metalliferous minerals are the natural contributors of heavy metals in the environment (Duruibe et al., 2007). However, in natural phenomenon the
weathering of the rocks are regarded as insignificant and non-toxic. But, the disturbance and acceleration of geochemical cycle contributed by human activities may in-turn escalate the heavy metals accumulation above its permissible limits, which could be toxic to natural biota (Wuana and Okieimen, 2011). Apart from natural sources, anthropogenic sources are the major contributors of heavy metal emissions especially the industrial discharges that have devastated the natural cycles of metals in many ecosystems (Jozefczak et al., 2012). In addition, industrial exploitation of heavy metals as in the component of appliances, tools and electronic devices, is also one of the causes of its increased concentration in the environment.

1.1.3 Heavy metal toxicity

Most heavy metals released into environment are toxic with no cellular functions occurring (Shi et al., 2002). Heavy metals can result in physical discomfort and sometimes life threatening illness including irreparable damage to vital body system (Ahluwalia and Goyal, 2007). Heavy metals are known to act as mutagens along with effect on the carbohydrate, amino acid, lipid metabolism (Strydom et al., 2006). Among all heavy metals Pb, Cd, Hg and As have been regarded as the most hazardous to human health (Nithya et al., 2011). The toxic effects of the heavy metals on human health have been studied and reviewed regularly by many international organizations as listed in the table 1.1.
Table 1.1: The table summarizes the heavy metal industrial uses, toxicity and their permissible limits (set by WHO) in the drinking water (adopted from Gaur et al., 2012; Wuana and Okieimen, 2011; Jarup, 2002).

<table>
<thead>
<tr>
<th>Heavy Metal</th>
<th>Permissible limit (WHO)μg/L</th>
<th>Uses of metals</th>
<th>Contaminating sources</th>
<th>Target organs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arsenic</td>
<td>10</td>
<td>Arsenical pesticides and wood preservatives, animal feeds, paints, dyes, and semiconductors.</td>
<td>Intake of ground water, food like fishes. Contamination of soil by pesticides, combustion of fossil fuel, Smelting of non-ferrous metals.</td>
<td>Central nervous cardiovascular system, GI tract.</td>
</tr>
<tr>
<td>Cadmium</td>
<td>03</td>
<td>Cadmium compounds used as stabilizers in PVC products, colour pigment, several alloys, in rechargeable nickel-cadmium batteries, as fertilizers, industrial emissions.</td>
<td>Breathing contaminated workplace air, and air near the burning of fossil fuels or municipal waste. Contaminated water and foods (i.e. shellfish, liver, kidney), cigarette smoking.</td>
<td>Lungs, stomach and kidneys, skeletal damage observed as itai-itai disease in Japan.</td>
</tr>
<tr>
<td>Chromium</td>
<td>50</td>
<td>In metal alloys, as pigments for paints, cement, paper, rubber, and other materials.</td>
<td>Released from electroplating processes and the disposal of Cr containing wastes.</td>
<td>Skin, ulceration, damages nervous system, kidney, and liver.</td>
</tr>
<tr>
<td>Copper</td>
<td>100</td>
<td>Used in production of alloys, in aircraft engines, magnets grinding/cutting tools, artificial joints, medical sterilization and research.</td>
<td>Copper normally occurs in drinking water from Cu pipes, as well as from additives designed to control algal growth.</td>
<td>Effects kidney, liver, stomach, intestine, blood system.</td>
</tr>
</tbody>
</table>
1.1.4 Mechanism of metal ions uptake and toxicity

Heavy metals are toxic to microbes, once inside the cell heavy-metal ions hinder the metabolism of structurally similar metals and can displace the essential metal ions from their native-binding sites (Bruins, 2000) such as chromate with sulfate, arsenate with phosphate. Thus, to differentiate between structurally similar metal ions, uptake system in microorganisms is tightly regulated. The microbial system has developed two types of uptake systems for metal ions. One is fast, unspecific, and driven by the chemiosmotic gradient across the cytoplasmic membrane of bacteria (Nies, 1999). This is not specific and used for uptake of variety of substrates, it is constitutively expressed (Nies, 1999). The second type of uptake system is substrate specific, is slower, often uses ATP hydrolysis as the energy source and is only expressed by the cell during the time of need, starvation, or a special metabolic situation (Nies and Silver, 1995). But, in the presence of high concentrations of nonessential metals, unspecific uptake systems are
constitutively expressed, causing the transport of nonessential metals inside the cell, which can be toxic to the cell (Nies, 1999).

Despite of metal toxicity many microbes are adaptive to metal contaminated locations, can grow and develop mechanisms of resistance for overcoming the toxicity (Gadd, 2010). Microbe’s resistance can vary from few percent in pristine environments to about 100% in heavily polluted conditions (Silver and Phung, 2009). The genes for metal resistance to a variety of metal ions are located on chromosomes, plasmids or transposons. Microbes cannot degrade the metals but transform the metal chemical properties via an array of mechanisms (Lloyd, 2002) and the necessity to develop resistance mechanism in the microbes is a way to protect the cellular components. Microorganisms have evolved different mechanisms due to the continuous exposure of toxic metals from the environment (Bruins et al., 2000; Silver, 1992) including metal exclusion by permeability barrier, active transport of the metal away from the microorganism, intracellular sequestration of metals by protein binding, enzymatic detoxification of a metal to a less toxic form, extracellular sequestration, reduction in metal sensitivity of cellular targets. Microbes can acquire one or a combination of other resistance mechanisms to overcome the metal toxicity (Bruins et al., 2000; Silver, 1992).

Numerous bacterial species are reported for exhibiting heavy metal resistance such as Pseudomonas and Bacillus sp.; Bacillus sp. JDM-2-1 and Staphylococcus capitis; Deinococcus Indus sp.; Bacillus circulans; Corynebacterium hoagie (Pepi et al., 2011; Zahoor and Rehman, 2009; Suresh et al., 2004; Yilmaz, 2003; Viti et al., 2002.
respectively). These bacterial species can be possibly used for heavy metal removal in contaminated sites as bioremediation processes.

1.2 ARSENIC

Arsenic isolated in 1250 A.D. by Albertus Magnus, synonymous with the word “poison” due to its past history (Mandal and Suzuki, 2002). It is a 33rd element and belongs to the fifth main group of the periodic table along with nitrogen, phosphorous, antimony and bismuth. Arsenic exhibits few properties of a metal as well as a non-metal, thus, referred as a metalloid. Moreover, arsenic is also referred as a metal and sometimes as a heavy metal in context to its toxicity (Jomova et al., 2011). It exists most commonly in the three oxidation states (+3, +5, -3) which are capable of forming organic and inorganic compounds in the environment and in the human body (Orloff et al., 2009).

Arsenic is ubiquitous in nature and stands to be the 20th abundant (0.0001%) element in the earth’s crust, 14th in the seawater, and 12th in the human body (Mandal and Suzuki, 2002). Arsenic is distributed in more than 200 mineral forms mainly as arsenates (60%), sulphides and sulfosalts (20%) and the rest 20% as arsenides, arsenites, oxides, silicates and elemental arsenic (As) in minor quantities (Mandal and Suzuki, 2002) and is associated with the ores of metals such as Au, Ag, Cu, Sb, Ni and Co (Lie’vremont et al., 2009).

Arsenic has been deleterious to mankind when exposed to this toxic metal through air, water and food. But, the most adverse effects of arsenic toxicity have been observed from drinking water. Arsenic ingestion can result in melanosis and keratosis and in severe
cases arsenicosis and arsenic poisoning. Arsenic prolonged exposure has been reported in development of type 2 diabetes, cardiovascular diseases and several cancers (lung, skin and bladder) (Abernathy, 2002).

Arsenic in the environment can be released through natural and human activities. Volcanic eruptions, weathering of rocks, forest fires are the natural contributors of arsenic (Jones, 2007). Among these the most significant source being weathering of rocks contaminating the ground water for drinking purpose (Liao et al., 2011) and mobilizing the arsenic in the environment while igneous activity has worsen the situation. Burning of fossil fuels, tobacco smoke and automobile exhaust are anthropogenic sources (Jones, 2007). In addition, arsenic being an active ingredient in mining, coal burning, glass making, wood processing, pesticides, electronic, paint, dye industry etc have also become source for arsenic contamination in the atmosphere (Mukhopadhyay et al., 2002). It has been estimated that an annual release of arsenic from volcanoes, microbial activity, and the burning of fossil fuels is approximated to be 3,000, 20,000, and 80,000 metric tons of atmospheric arsenic, respectively (Jones, 2007).

1.2.1 Arsenic and its uses

Arsenic has found its practical uses throughout its history since its isolation where arsenic lead pesticides had been used extensively over the past five decades contaminating millions of acres of land. Arsenic was in use for long in beauty products and arsenic-based pigments. Moreover, in past, arsenic was extensively used in medicinal formulations to treat various diseases such as malaria, syphilis, asthma, eczema, psoriasis.
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Apparently, in 1910, Paul Ehrlich was awarded the Nobel Prize for inventing the arsenic containing drug (trade name Salvarsan) for treating syphilis (Hughes, 2011). Although, arsenic use has been curtailed but its application in some fields are still in practice. Application of monosodium methylarsenate (MSMA), cacodylic acid, disodium methylarsenate (DSMA) and chromated copper arsenate (CCA) are still approved as pesticides and wood preservative respectively (Hughes et al., 2011). Also, in medicinal formulations, the use of As$_2$O$_3$ as chemotherapeutic drug for treatment of relapsed or refractory acute promyelocytic leukemia is FDA approved (Jones, 2007). Small quantities of arsenic metal are added to other metals to form metal mixtures or alloys with improved properties which are of great use in lead-acid batteries for automobiles, semiconductors and light-emitting diodes.

1.2.2 Arsenic: The global perspective

Arsenic, a human carcinogen, has been recognized as a major threat to human health by various organizations (EU, WHO, EPA). Arsenic ranks second next to microbial contamination in drinking water quality measurements (Lie`vremont et al., 2009).

Humans are exposed to arsenic through air, food and water but the most dramatic health effects are shown by contaminated drinking water. Past few decades, reports of high arsenic concentration have been recognized in water supplies of various countries contributing to be a major health concern (Mukerhjee et al., 2006). Thus, WHO has revised the maximum permissible limits of arsenic in drinking water reducing it from 50...
ppb to 10 ppb. However, in most of the developing Asian countries except Japan and Vietnam the permissible limit of arsenic is still 50 ppb which is set according to pre-1993 WHO guideline value. This might be due to the analytical constraints and problem in fulfillment of lower standard. Arsenic contamination in ground water has been reported in 70 countries exposing around 150 million populations across the globe from drinking arsenic-contaminated water (Akinbile and Haque, 2012). Out of which, 10 countries in South and south-east Asia were discovered to be affected with high concentrations of arsenic including Bangladesh, Cambodia, China, India, Laos, Myanmar, Nepal, Pakistan, Taiwan and Vietnam affecting 110 million people inhabiting these areas due to their dependence on As-contaminated water for drinking and irrigation purposes (Stroud et al., 2011; Brammer and Ravenscroft, 2009).
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Figure 1.1: World map of populations at risk. The figures give the number of people whose daily water consumption includes arsenic levels above 10 mg/l. The shades of grey indicate the number of persons contaminated, with the range of palest (the lowest numbers) to the darkest shade (the highest number) (Lie’vremont et al., 2009).

Of these, Bengal delta region found in Bangladesh and West Bengal, India, are the World’s two largest regions of groundwater arsenic contaminants that affected the huge number of people (Chowdhury et al., 2000). However, Bangladesh is the worst affected followed by India and China (Nordstrom, 2002). Arsenic poisoning in Bangladesh was regarded as “the worst mass poisoning in history” by the WHO (Mead, 2005). It has been estimated that in Bangladesh alone around 77 million inhabitants are exposed to arsenic contaminated drinking water (Jiang et al., 2013). Further, a report of Chakraborti et al. (2009) accounted 26 million people are at risk from drinking arsenic
contaminated water (above 10 µg/L) in West Bengal. Besides, West Bengal various other
states of India such as Uttar Pradesh, Bihar, Jharkand in Ganga plain are also affected by
arsenic, from which thousands are suffering from arsenic toxicity and millions are at risk
(Chakraborti et al., 2009). Nearly, 1,000000 tube-wells drilled into Ganges alluvial
region of Bangladesh and West Bengal were contaminated with approximately 1000 µg
l⁻¹ of arsenic (Meharg and Hartley-Whitaker, 2002). Since, in Bangladesh and West
Bengal crop irrigation relies on ground water which are contaminated with arsenic,
people are exposed to arsenic through ingestion of vegetation (Meharg and Hartley-
Whitaker, 2002). Before 2000, there were only five major incidents of ground water
contaminated arsenic including Bangladesh, West Bengal, India, and sites in China.
However, new sites of arsenic contaminated ground water sites have dramatically
increased ever since including China, Mongolia, Nepal, Cambodia, Myanmar,
Afghanistan, DPR Korea, and Pakistan (Mukherjee et al., 2006).

1.2.3 Forms of arsenic

Arsenic is present in pedosphere, the atmosphere, the hydrosphere, (soil, air,
water) contributed by natural and anthropogenic sources and exists in inorganic and
organic forms. Inorganic arsenic exists in four different oxidation states: arsenic [As(0)],
arsine [As(-III)], arsenite [As(III)], and arsenate [As(V)]. As(V) and As(III) are two
dominant states in the environment; these two modes are interconvertible via
oxidation/reduction of As(III)/As(V), where As⁵⁺ species are more stable in oxidizing
conditions while As³⁺ species are dominant under reducing conditions (Duker et al.,
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2005). Both these species are soluble at wide range of pH conditions (Bell, 1998). Under anaerobic conditions, microbial As(III) reduction gives rise to arsine gas which is highly toxic even in trace amounts (Duker et al., 2005) whereas, elemental arsenic occurs rarely and is not absorbed by body exerting no adverse effects. Arsenic species can be methylated to form compounds such as monomethylarsonic acid (MMAA), dimethylarsinic acid (DMAA) and trimethylarsine oxide (TMAO) by microorganisms (Gadd, 1993; Cullen and Reimer, 1989; Ridley et al., 1977; Woolson, 1977) and by animal’s excretory products, these compounds are less available in the environment (Oremland and Stolz 2005). The valence states of arsenic of environmental interest are the trivalent (III) and pentavalent (V) states. The trivalent arsenic compounds are more toxic than pentavalent compounds (Smedley et al., 1996; Cervantes et al., 1994). The speciation of arsenic decides its solubility, mobility, sorption behavior, bioavailability and toxicity (Tamaki and Frankenberger 1992). Pentavalent arsenic is sorbed onto clays, iron and manganese oxides/hydroxides and organic matters (Lievremont et al, 2009). It form complexes with iron to form ferric arsenate in the soil, that constraints its hydrologic mobility and availability. Studies have shown that the reduction of As(V) into As(III) results in the solubilisation and bioavailability of this element. While As(III) is adsorbed less strongly to fewer minerals, makes it relatively more mobile and available oxyanion (Oremland and Stolz, 2003) and considered to be more toxic and motile than As(V).
1.2.4 Arsenic toxicity

The predominant forms of arsenic in the environment are As(V) and As(III). The toxicity of As(V) is due to its similar structure and chemical properties with the phosphate ion, it competes with it when inside the cell, causing cellular disturbances especially, forms ester linkages with its hydroxyl groups resulting in the formation of unstable As-O bond as compared to stable P-O bond. The unstability of As-O bond is due to bonds length being 10% longer, that makes it unstable. As(V) results in arsenolysis where it uncouples the formation of ATP during oxidative phosphorylation or glycolytic pathways resulting in unstable arsenic anhydrides and hydrolyzing it into its components, ultimately depleting the ATP from the cell (Hughes et al., 2011). As(V) also replaces the phosphates in the DNA double helix causing mutagenesis, teratogenesis in the cell (Lie'vremont et al., 2009). On the other hand, As(III) binds to the sulphydryl groups of the enzymes and making them non-functional. As(III) is also known to inhibit pyruvate dehydrogenase (PDH) by binding to the sulphydryl group of dihyrolipoamide, a cofactor of PDH. PDH inhibition results in decreased ATP and citric acid activity (Bergquist et al., 2009). As(III) is known to inhibit more than 200 enzymes in human cells (Lievremont et al., 2009). It also forms strong bonds with the imidazolium nitrogens of histidine residues, which could possibly interfere with, for example, heme binding in cytochromes. In addition, As(III) results in the degradation of Fe-S clusters in proteins. Due to the protein inhibition and the higher bioavailability in aqueous environments, As(III) is considered more hazardous than As(V) (Kruger et al., 2013). Moreover, methylated trivalent arsenicals such as MMAIII are potent inhibitors of GSH reductase (Styblo et al., 1997).
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and thioredoxin reductase (Lin et al., 1999). The inhibition to these enzymes can result in cellular damage and leads to cytotoxicity (Hughes, 2002). The activity of the methylated trivalent arsenicals is greater than arsenite, MMA\textsuperscript{V}, and DMA\textsuperscript{V} suggesting that methylation is not solely the detoxification process (Hughes, 2002).

1.2.5 Arsenic geocycle

Arsenic ranks as the 20\textsuperscript{th} abundant element in the earth crust. Similar to geocycles of living system elements such as carbon, nitrogen, oxygen; toxic elements also follow geocycles including arsenic. As(V), the main arsenic compound in sea water is taken by marine organisms, like phytoplankton, algae, crustaceans, mollusks and fish (Mukhopadhyay et al., 2002) and convert into small organic compounds or organic storage forms that are then secreted into the environment. But, some arsenic is retained by phytoplankton and metabolized into complex organic compounds. The transformation of inorganic arsenic into lipid-soluble compounds might be an adaptive mechanism for marine phytoplankton (Mukhopadhyay et al., 2002). Moreover, complex algal organoarsenical compounds include dimethylarsenosugars (water-soluble arsenosugars) and arsenolipids (lipid-soluble compounds). These complex organoarsenic compounds are produced mainly by phytoplankton and macroalgae in the sea these organisms are later consumed and metabolized by marine animals. Fish and marine invertebrates retain 99% of accumulated arsenic in organic form while crustacean and mollusk retain higher concentrations of arsenic than fish. The main organoarsenic compound found in marine organisms is arsenobetaine which occurs in algae, clams, lobsters, sharks, shrimp and
Flounder. Transformation of arsenosugars and arseno lipids into arsenobetaine within the higher marine animals is unknown. Arsenobetaine is then metabolized by microorganisms in coastal seawater sediments to methylar senic acid and to inorganic arsenic, released in the environment, completing the biological cycle of this element in marine system (Mukhopadhyay et al., 2002).

Figure 1.2: Natural cycling of arsenic (Mukhopadhyay et al., 2002).
1.2.6 Arsenic uptake by microorganisms

Arsenic is not associated with life but for some microbes, arsenic act as the electron donor or acceptor or a part of electron transport chain. Since arsenic doesn't play any function in the cell cytoplasm, no specific uptake transporters have evolved. Instead, inorganic arsenic enters the cell through the transporters dedicated for other molecules due to its chemical analogy (Rosen and Liu, 2009).

Arsenate is an oxianion having chemical structure similarity to phosphate; it is taken up inside the cell by phosphate transporter. In *E. coli*, for example two phosphate transporters, Pit (phosphate transporter) and Pst (specific phosphate transporter) are the major conduits for As(V) uptake (Pa’ez-Espino *et al.*, 2009), where the Pit system is the major arsenate uptake system (Bhattacharjee and Rosen, 2007). However, when bacterial systems are constantly exposed to high concentrations of As(V) only Pst is expressed in order to reduce As(V) uptake (Kruger *et al.*, 2013). On the other hand, at neutral pH trivalent As(III) exists as an uncharged arsenic trioxide As(OH)₃ in solution, an analogue of glycerol. As(III) uptake by the cells has been shown by a branch of the aquaporin superfamily of transporters, GlpF an aqua-glycerolporin (Meng *et al.*, 2004). GlpF is the first recognized glycerol facilitator of *E. coli* and GlpF homologues have been reported in various bacteria (Tsai, 2009). Likewise, *S. cerevisiae* has evolved Yeast homolog of GlpF, Fps1p for uptake and sensitivity of As(III) (Bhattacharjee and Rosen, 2007). Another family of membrane transporters for As(III) uptake is glucose permeases (Liu *et al.*, 2004) which are induced by the cell in glucose starvation in contrast to Yeast Fps1p.
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As(0H)₃ (aqua-glyceroporins)  AsO₄²⁻ (Pit/Pst transporters)

Figure 1.3: Diagram of the different microbial processes involved in arsenic biochemistry in the environment. (1) Arsenic enters the cells through the phosphate transporters (Pit/Pst) or arsenite through aqua-glyceroporins (GlpF). (2) Once inside the cells, As(V) is reduced to As(III) by ArsC, which further extruded out of the cell by the specific pump ArsB. (3) As(V) can also be detoxified by complexation with Cys-rich peptides. In addition, (4) As(III) can serve as electron donor by oxidation to As(V). (5) As(V) can be used as the ultimate electron acceptor during respiration. (6) Inorganic arsenic can also be transformed into organic species in a methylation cascade (Paéz-Espino et al., 2009).
1.2.7 Arsenic detoxification strategies

Although arsenic is toxic, microbes have learnt to adapt and survive in arsenic-enriched environment (Belfiore et al., 2013; Baker-Austin et al., 2007; Anderson and Cook 2004). So as to thrive and grow in arsenic-contaminated environment microorganisms have evolved various strategies of detoxification where, the reduction of less soluble As(V) to more soluble As(III) is one of the phenomenon of tolerance termed as arsenate reduction process (Ji and Silver, 1995; Nies and Silver, 1995). In addition, methylation of As(III) and arsenite oxidation are two other processes. These reactions facilitate the bacteria from the toxic effects of arsenic as well as in some species, contribute to energy metabolism processes (Lievermont et al., 2009). The various strategies for arsenic detoxification are given below

1.2.7.1 Arsenite oxidation

Arsenite oxidation is a potential detoxification process employed by microorganisms to tolerate higher levels of As(III) by converting the more toxic form [As(III)] into less toxic form [As(V)]. In addition, As(III) is also involved to generate energy in heterotrophic as well as chemoautotrophic bacteria that uses As(III) as electron donor reducing oxygen or nitrate which serve energy to fix CO₂ and a source of carbon for bacterial growth. The latter process is carried by arsenite oxidase designated AioAB (Lett et al., 2012), a heterodimeric molybdenum enzyme from the DMSO reductase family. The Arsenite oxidase was first purified and characterized by Alcaligenes faecalis (Ellis et al., 2001). Later, this enzyme was characterized in various bacterial strains including
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Rhizobium strain NT-26 and Hydrogenophaga strain NT-14; H. arsenicoxydans (Santini and van den Hoven 2004; van den Hoven and Santini 2004; Weeger et al., 1999 respectively). The large subunit AioA (MW, 90 kDa) was found to contain two pyranopterin cofactors binding the Mo atom in the active site of arsenite oxidation and a [3Fe-4S] cluster for electron transport, while the small subunit (MW, 14 kDa) contains a Rieske-type [2Fe-2S] cluster and transfers the electrons to coupling proteins of the respiratory chain (Ellis et al. 2001; Anderson et al. 1992). Moreover, phylogenetic analysis indicated that the enzyme has been assembled from the basic redox building blocks suggesting that it might be a very ancient protein (Lebrun et al., 2003). In the primitive earth, As(III) may have served as main energy source for chemolithotrophic organisms by transforming more available As(III) into less available form As(V) that may have resulted in partial detoxification and allowing other microorganisms survival (Lievermont et al., 2009).

1.2.7.2 Arsenic methylation

Methylation of arsenic is a widely distributed phenomenon observed from microorganisms to higher animals. Methylation process involves the reduction of As(V) followed by oxidative addition of a methyl group and may result in the formation methyle arsenic to form non-volatile monomethylarsonic acid (MMA\textsuperscript{V}) and dimethylarsenic acid (DMA\textsuperscript{V}), and volatile trimethylarsine oxide (TMAO\textsuperscript{O}), trimethylarsine (TMA\textsuperscript{III}) and dimethylarsine (DMA\textsuperscript{III}) (Bentley et al., 2002). Methylation
process in microbial system is catalyzed by an enzyme ArsM (As(III) S-adenosylmethionine-transferase) and encoded by gene termed as arsM.

\[
\text{arsenate} \xrightarrow{\text{reductase}} \text{arsenite} \xrightarrow{\text{methyltransferase}} \text{methylarsonic acid}
\]

\[
\text{arsenate} \xrightarrow{\text{methyltransferase}} \text{dimethylarsinic acid} \xrightarrow{\text{reduction}} \text{dimethylarsine}
\]

\[
\text{arsenate} \xrightarrow{\text{methyltransferase}} \text{trimethylarsine oxide} \xrightarrow{\text{reduction}} \text{trimethylarsine}
\]

\[
\text{arsenate} \xrightarrow{\text{reductase}} \text{arsenite} \xrightarrow{\text{methyltransferase}} \text{methylarsonic acid}
\]

Figure 1.4: Predicted steps in methylation of inorganic arsenic resulting in the formation of trimethylarsine and dimethylarsine (Mukopadhyay et al., 2002)

1.2.7.3 Arsenate reduction

Reduction of As(V) into As(III) occurs by two mechanisms in prokaryotes. One is involved in using As(V) as the terminal electron acceptor (Dissimilatory reduction), while the other reduces As(V) to more toxic form As(III) via cytoplasmic arsenate reduction (ArsC) along with the extrusion of As(III) via efflux pump, ArsB (arsenic efflux).
1.2.7.3.1 Dissimilatory reduction

Arsenate may act as the terminal electron acceptor in anaerobic respiration and bacterial cells gain energy through it. Arsenate reduction is carried out by membrane-bound enzyme encoded in the arr operon which includes arrA and arrB genes (Macy et al., 2000). This mechanism is functional mainly in obligate or facultative anaerobic microorganisms, for example, the bacterium *Chrysiogenes arsenatis* arsenate respiratory reductase is a periplasmic, heterodimeric protein consists of two subunits with molecular mass of 87 kDa (ArrA) and 29 kDa (ArrB). Sequence analysis indicates that larger subunit (ArrA) is a Mo/Fe protein that shows sequence homology to the large dimethyl sulfoxide (DMSO) reductase class of proteins, and the smaller one (ArrB) contains a $[4Fe-4S]$ center protein and may be involved in the electron transfer to molybdenum cofactor of the ArrA subunit (Bhattacharjee and Rosen, 2007). The enzyme is substrate specific, doesn’t use other substrates such as nitrate, sulfate, selenate or fumarate. Arsenate respiratory reductase activity have also been described in other bacteria such as *Alkaliphilus oremlandii* (Fisher et al., 2008), *Shewanella* sp. strain ANA-3 (Malasam et al., 2008); *Clostridium* sp. strain OhILAs (Stolz et al., 2006), *Wolinella succinogena* (Stolz et al., 2006), *Alkaliphilus metalliredigenes* (Stolz et al., 2006), *Desulfuroporosinus* sp. strain Y5 (Perez-Jimenez et al., 2005), *Sulfurospirillum barnesii* (Malasam et al., 2004).
1.2.7.3.2 Arsenate efflux

Microbes possess genetic determinants to confer arsenic resistance which may be plasmid or chromosomal encoded systems and inactivation of these operons results in hypersensitivity to arsenic compounds (Mukhopadhyay et al., 2002). Bacteria confer the arsenic resistance generally by pumping the toxic arsenic throughArs efflux pump, thus reducing its concentration in the cell (Mukhopadhyay et al., 2002). Detoxification of cells is incurred by expression ofars operon, composed of 3 genes (arsRBC) encoding a transcriptional repressor, a transmembrane efflux pump and an arsenate reductase respectively. arsRBC is generally found in most chromosomal encoded gram negative bacteria while in plasmids and chromosomal gene systems of gram positive bacteria such as on Staphylococcus aureus pI258, or on the bacterial chromosome (Patel et al., 2007; Bruhn et al., 1996; Diorio et al., 1995; Sofia et al., 1994; Owolabi and Rosen 1990). However, in some plasmid-determined systems of Gram-negative bacteria an enlarged version of core genes was found in R773 and R46 plasmids of E. coli (Silver, 1998) and pKW301 plasmid of Acidiphilus multivurum AIU301 (Suzuki et al., 1998; Suzuki et al., 1997). An enlarged operon is composed of 5 genes (arsRDABC). ThearsA gene encodes for ATPase subunit providing energy to the ArsB efflux pump, whilearsD was identified as chaperone to enhance the efficiency of the ArsAB efflux pump for pumping the trivalent metalloids As(III) and Sb(III) (Lin et al., 2007) and increasing the affinity of ArsA for As(III). ArsD is a homodimer with three vicinal cysteine pairs in each monomer. As(V) enters through phosphate transporters inside the cell cytoplasm, where As(V) is enzymatically reduced to As(III) via arsenate reductase enzyme (ArsC) after
which As(III) is effluxed from the cell by transmembrane protein ArsB (Acr3 in some eukaryotes) or ArsAB complex. This efflux pump belongs to two different families, ArsB protein and ACR3 arsenite carrier gene family. The \textit{arsB} gene is generally encoded in Firmicutes and Gammaproteobacteria. It has 12 membrane spanning segments, which is similar to many carrier proteins (Wu \textit{et al.}, 1992). It confers resistance to As(III) and Sb(III) (Tisa and Rosen 1990; Chen \textit{et al.}, 1986) but having higher affinity for Sb(III). ArsB is an antiporter, coupling the efflux of trivalent metalloid As(III) to the electrochemical proton gradient (Meng \textit{et al.}, 2004).

Acr3 family is found in bacteria, archaea and fungi. Fungal members of this protein include \textit{S. cerevisiae} Acr3p efflux metalloid efflux protein, while \textit{C. glutamicum} has also shows three Acr3 gene homologues. Both yeast Acr3p and \textit{C. glutamicum} Acr3 shows more affinity towards As(III) over Sb(III), further, Acr3 doesn’t exchange As(III) with protons but coupling of Acr3 to proton motive force is unknown (Bhattacharjee and Rosen, 2007).

In addition, other \textit{ars} genes have been isolated from various microbes such as \textit{arsP} coding for a putative membrane permease, \textit{arsTX} encoding a thioredoxine system in \textit{Microbacterium} sp. A33 (Achour-Rokbani \textit{et al.}, 2010). \textit{arsN} coding for an acetyltransferase-like protein (Chauhan \textit{et al.}, 2009). Given that, the precise roles of these genes still need to be elucidated, suggesting some other resistance mechanisms might be functional in the microbes (Kruger \textit{et al.}, 2013).
Arsenate Reductase (ArsC)

Cytosolic arsenate reductase is involved in catalyzing the reduction of As(V) into As(III). It is present as three independent protein families that differ through their structures, reduction mechanisms and the location of their catalytic cysteine residues. These are named as

i) Glutaredoxin coupled arsenate reductase:

The family includes glutaredoxin (Grx) coupled arsenate reductase class borne by *E.coli* plasmid R773 ArsC, it catalyzes the reduction of As(V) to As(III) where glutaredoxin and glutathione act as reductants (Mukhopadhyay *et al.*, 2002).

ii) Thioredoxin coupled arsenate reductase:

This family involves homologues of the *arsC* of *S. aureus* plasmid pl258 (Ji and Silver, 1992) and *B. subtilis* chromosome, they are 14.8 kDa monomeric proteins that are unrelated to ArsC from *E.coli* plasmid R773 (Bhattacharjee and Rosen, 2007). It catalyzes the arsenate reduction by coupling to thioredoxin, thioredoxin reductase and nicotinamide adenine dinucleotide phosphate (NADPH) (Ji *et al.*, 1994).

iii) ACR2p family:

This family encodes eukaryotic arsenate reductase genes capable of reducing As(V) into As(III). It has been observed in *Saccaromyces cerevisiae* and *Leishmania major* where characterized as Acr2p in *S. cerevisiae* (Mukhopadhyay *et al.*, 2000) and LmAcr2 from *L. major* (Zhou *et al.*, 2004). Acr2p is of 130 amino acid, exists as homodimer and its sequence is discrete to bacterial arsenate reductase.
1.2.8 Arsenic and ROS

Arsenic is known to be a redox active metal (Flora et al., 2008) result in the formation of reactive intermediates, primarily radicals that react with oxygen resulting in production of well-known reactive oxygen species (ROS). In turn, induces oxidative stress, a situation resulting imbalance between levels of free radicals and cellular antioxidant defense in the cell.

Reactive oxygen species (ROS) have one or two unpaired electrons or open shell configuration including hydrogen peroxide, superoxide anion, singlet oxygen and hydroxyl radical that can directly or indirectly damage cellular DNA and protein. The toxicity of ROS is multifaceted. One significant effect is the initiation of cellular DNA damage including DNA oxidation and DNA strand breaks which is profoundly induced by hydroxyl radicals (Flora et al., 2008). ROS can also modify proteins through amino acids oxidation of thiol groups of cysteine, methionine, the imidazole ring of histidine, and the rings of tyrosine, phenylalanine, and tryptophan, etc among these cysteine and methionine are most susceptible to oxidation by ROS (Lushchak, 2001). Further, lipids are also vulnerable to ROS, especially polysaturated fatty acids resulting in a chain reaction that forms lipid peroxides, lysolipids (Lushchak, 2001). Auto catalytic oxidation of lipids mediates the formation of toxic malondialdehyde (MDA) (Marnett, 1999) resulting in depletion of lipids of the cell. MDA-guanine adducts formation may result in DNA-DNA or DNA-protein crosslinking (Valko et al., 2006).

Arsenic is known not only to generate ROS but also free radicals such as superoxide (O$_2^-$), singlet oxygen (¹O$_2$), the peroxyl radical (ROO'), nitric oxide (NO'),
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hydrogen peroxide (H$_2$O$_2$), dimethylarsinic peroxyl radicals (CH$_3$)$_2$AsO$_2$O$^\cdot$ and also the dimethylarsinic radical (CH$_3$)$_2$As (Flora et al., 2008). However, the exact mechanism of ROS induction is not elucidative, the possibility for the induction of ROS production in the cell initiates, with the oxidation of As(III) to As(V), resulting in H$_2$O$_2$ production:

$$\text{H}_3\text{AsO}_3 + \text{H}_2\text{O} + \text{O}_2 \rightarrow \text{H}_3\text{AsO}_4 + \text{H}_2\text{O}_2 \quad (\Delta r G^\circ = -40.82 \text{ kcal mol}^{-1})$$

The above reaction is a spontaneous, exergonic and standard Gibbs free energy change toward H$_2$O$_2$ formation of -40.82 Kcal/mol. Moreover, in the presence of transition elements such as Fe and Cu, hydrogen peroxide is decomposed (Fenton reaction) generating hydroxyl radicals:

$$\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot \text{OH} + \text{OH}^- \quad (\text{Fenton reaction})$$

These highly reactive hydroxyl radicals reacts in the region close to its site of formation, may be deleterious to DNA if in its close proximity leading to DNA strand breaks (Jomova et al., 2011). Trivalent arsenic is known to hinder the production of glutathione, which have a protective role against oxidative stress (Jomova et al., 2011; Miller et al., 2002).

In order to alleviate the toxic effects of oxidative stress the cell needs to either diminish the oxidative damage through stimulation of antioxidant compounds like glutathione and ascorbate or more importantly, by the induction of antioxidant enzymes [i.e., catalase, superoxide dismutase (SOD)]. For instance, SOD modulates the relative amount of O$_2^-$ and H$_2$O$_2$ by Haber-Weiss reactions and decreases the risk of OH$^-$ radical formation. While catalase (CAT) or glutathione peroxidase (GSH-Px) are involved in
detoxifying H₂O₂ into H₂O. Thus, the expression of antioxidant enzymes is essential to assuage the oxidative stress and improve the bacterial survival.

1.2.9 Metal removal strategies

To overcome the increased concentration of heavy metals in the environment various physico-chemical approaches are employed for their removal such as chemical precipitation, chemical oxidation or reduction, ion exchange, filtration, electrochemical treatment, reverse osmosis, membrane technologies and evaporation recovery. However, these techniques are cost-ineffective, not eco-friendly, produces toxic chemical sludge (Guo et al., 2010). Hence, attention has been focused past few decades onto approaches for use of microorganisms whereby exploiting the microbial potential in clean-up of heavy metal exposed sites (Zammit et al., 2012). Microorganisms can uptake metal, either actively (bioaccumulation) and or passively (biosorption) (Samarth et al., 2012). Bioremediation approach has emerged as a simple, safe, economic and eco-friendly option with no additional nutrient requirement, thus overcoming the drawbacks of physicochemical processes.

1.2.9.1 Biosorption

Biosorption is a physio-chemical process, defined as ‘the removal of substance from solution by biological material (Gadd, 2010). Biosorption is a passive process which involves entrapment of metal ions on the cell surface and latter biosorbed at its respective binding sites which is independent of the biological metabolic cycle (Malik, 2004).
Biosorption is a metabolism independent process of metal uptake through microorganisms either living or dead (Gadd, 2010). However, metal sequestration by viable cells is a better option than non-viable cells, due to the cell's ability of self-replenishment, continuous metabolic uptake of metals after physical adsorption and the potential for optimization through development of resistant species and cell surface modification (Malik, 2004). Biosorption employs various mechanisms of complexation, ion exchange, coordination, adsorption, chelation and microprecipitation which may be synergistically or individually involved (Hu et al., 1996). The microbial cell wall carries different negatively charged functional groups at neutral pH such as carboxyl (-COOH), amine (-NH2), hydroxyl (-OH), phosphate (-PO3) and sulfhydryl (-SH) groups, which might act as binding sites for metal ions interaction, and required for biosorption of positively charged metal ions (Kuyucak and Volesky, 1998). The microbial biosorbents can be specific or non-specific to metals (Mala et al., 2006). The microbial biosystems such as fungi, bacteria, yeast or marine algae and seaweeds have been used as biosorbents for heavy metal removal (Ahalya et al., 2005). Various species of Aspergillus, Pseudomonas, Sporophyticus, Bacillus, Phanerochaete, have been reported as efficient chromium and nickel reducers (Mala et al., 2006). However, fungi and yeast are among the potential microbes for metal biosorption due to excellent metal-binding properties (Gupta et al., 2000).
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1.2.9.2 Bioaccumulation

“Bioaccumulation” or sequestration (either intracellular or at the cell surface) is one of the mechanisms of metal resistance and it is metabolism dependent process. Bioaccumulation is referred as the accumulation of materials that are not essential elements of an organism such as accumulation of metals (Bains, 1993). In bioaccumulation metal ions can be sequestered through passive (like biosorption) or active uptake wherein, heavy metal ions are passed inside the cell via an active transport (Malik, 2004). According to Gadd (1990) metal accumulation constitutes of two phases an initial phase is rapid and involves physical adsorption or ion-exchange of metal at the cellular surface and followed by slower phase of active metabolism-dependent uptake of metal into the bacterial cells (Srinath et al., 2002; Gadd, 1990). During bioaccumulation, subsequently other processes like intracellular sequestration followed by localization within specific organelles, metallothionein binding, particulate metal accumulation, extracellular precipitation and complex formation can occur inside the cell (Srinath et al., 2002). In contrast to biosorption which is growth-independent, bioaccumulation is growth dependent and is mediated by viable biomass (Gupta et al., 2000). Various bacteria such as Streptomyces sp., Thiobacillus ferroxidans, Bacillus aureus, Zooglea sp., Pseudomonas aeruginosa and S. cerevisiae (Gupta et al., 2004); Ochrobactrum (Pandey et al., 2010); Bacillus sp. strain DJ-1 (Joshi et al., 2009) are reported to accumulate significant levels of different metals when treated with metals like cadmium, nickel, uranium, and thorium, arsenic, cadmium respectively.
1.2.10 Metal resistance and Exiguobacterium

Diverse microorganisms isolated from different places have shown to exhibit resistance to various metals including genus *Exiguobacterium*. The genus *Exiguobacterium* belongs to gram positive bacteria with morphological diversity (ovoid, rods, double rods, and chains), motile, facultative anaerobe/aerobe, low G+C content. The *Exiguobacterium* species has been isolated from diverse environments including Greenland glacial ice, hot springs at Yellowstone National Park, the rhizosphere of plants, the environment of food processing plants and mangrove region of Kerala, India (Pathak *et al.*, 2013; Vishnivetskaya *et al.*, 2009). Given that, *Exiguobacterium* strains have been examined for resistance to various heavy metals in few reports, especially in its resistance of chromate. Alam *et al.*, 2008 examined an *Exiguobacterium* strain resistant to multiple heavy metals such as Ni$^{2+}$ (6.73 mM), Zn$^{2+}$ (2.78 mM), Cd$^{2+}$ (2.48 mM), Cu$^{2+}$ (2.35 mM) and Cr$^{6+}$ (3 mM). Similarly, Pattanapipitpaibul *et al.* (2002) and Okeke *et al.* (2008) also reported chromium resistant *Exiguobacterium* strains (*Exiguobacterium* sp. GS1, *Exiguobacterium aurantiacum* respectively) isolated from aquatic habitats. Moreover, the reports of tolerance to chromate stress were examined in *Exiguobacterium* KCH5 isolated from chromium contaminated soil (Sarangi *et al.*, 2008). Further, Anderson and Cook (2004) was able to isolate the *Exiguobacterium* sp. WK6 strain from the arsenic contaminated site, it was capable to gain metabolic energy from As(V) under aerobic growth conditions and able to reduce As(V) to As(III) via a non-respiratory mechanism. In addition, Belfiore *et al.* (2013) studied the *Exiguobacterium* strain that exhibited high tolerance to arsenic such as 10 mM of As(III) and 150 mM of As(V).
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As(V). Studies have also shown that some of the *Exiguobacterium* strains can be used for its applications in biotechnology, bioremediation, industry and agriculture (Okeke *et al.* 2007; Lopez *et al.* 2005; Pattanapipitpaisal *et al.* 2002). Thus, it becomes interesting to explore the strain of this hereto little known bacteria isolated from different environment which had high resistance towards arsenic.

1.2.11 Heavy metal study via Proteomics

The term Proteome was coined in 1994 by Mark Wilkins, by definition it refers to the total set of proteins expressed in a given cell at a given time, the study of which is termed “proteomics.” Proteomics is a field that promises to narrow the gap between genome sequence and cellular behavior. The proteome delivers a dynamic overview of the genome unlike the genome which is a static phase. Proteomics rather than targeting a particular family of proteins it delivers the qualitative and quantitative aspect of the cellular proteome. It is a valuable tool for detecting slight changes in expression of individual proteins and modifications in amino acid sequence that may be the adaptive responses of organism towards an altered environment (Unwin *et al.*, 2006). The advent of proteomic approach has promoted the detection of comparative protein profiling of stressed and control conditioned cells which in-turn allows to perceive the altered expressional responses and contributes in assessing the molecular mechanism of stress response in organisms (Blackstock and Weir, 1999). Thus, cataloguing of these expressed proteins provides more precise and complete information compared to genomic/transcriptomic studies, since protein expressions are regulated at transcriptional
as well as translational levels, contributing to more details about mature proteins and their interactions (Fanous et al., 2008). In addition, proteomic approach is suitable for direct measurement of protein expression levels revealing the cellular activity information unavailable from any other approach.

Microbes including bacteria and fungi are among the first components of the biota in ecosystems affected by organic pollutants and heavy metals. Metals have shown to influence microorganisms by adversely affecting their growth, morphology, and biochemical activities (Jain et al., 2010). To overcome these challenges microorganisms adapt to the altered environment and their adaptive response can be studied via examination of changes in protein expression (Singh and Nagaraj, 2006). Thus, the proteins may be regarded as the main effectors of biological responses in an organism as they represent the end product of gene expression. Given that, the genomic analysis and their regulation are not adequate for studying microbial adaptation strategies, thus, post genomic analysis, including transcriptomics and proteomics maintain their importance (Schneider and Riedel, 2010). Thus, a proteomic approach can be a helpful tool for detecting the subtle changes in the protein expression or any modifications in the amino acid sequence in organisms which are responsive to altered environment and may contribute in the identification of new protein markers for metal toxicity (Nesatyy and Suter, 2008). In turn, provides the insight of adaptability of an organism under environmental onslaughts.
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Figure 1.5: Schematics for protein identification by mass spectrometry analysis. The sample is run for 1D/2D electrophoresis, digested with proteases and thereby the resulting peptides are mass fingerprinted or sequenced. The resultant sequence is submitted in databases for protein identification (Graves and Haystead, 2002).

1.2.11.1 Acquisition of protein structural information

1.2.11.1.1 Edman sequencing

This technique is the most basic method for protein identification with identification of N-terminal amino acid sequences through Edman chemistry. The N-terminal sequencing was introduced by Edman as early as in 1940. But with the introduction of mass spectrometers the use of this technique is waning in the field of proteomics.

1.2.11.1.2 Mass Spectrometry (MS)

Mass spectrometer has immensely grown, developed for past decades and has become favorable method for protein identification and amino acid sequencing. In past,
transferring biomolecules such as proteins, a large and polar substance into gaseous phase forwarded a challenge. It was Karas and Hillenkamp in 1985 that reported an energy-absorbing matrix could be used to volatilize small analyte molecules (Karas and Hillenkamp, 1988). But the phenomenal breakthrough in analysis of large molecules came in 1987 when K. Tanaka reported a mass spectrometric analysis of an intact protein with soft laser desorption (SLD) where gaseous macromolecules were generated through low-energy nitrogen laser. This laid the foundation of a technique called matrix-assisted laser-desorption ionization (MALDI) reported by Karas and Hillenkamp (1988). In general, a mass spectrometer comprises of three components such as an ion source, a mass analyzer and an ion detector. The ion source produces ionized peptide molecules that are then separated according to their mass to charge (m/z) ratios in the (electromagnetic) mass analyzer, and finally detected by an ion detector and recorded (Curreem et al., 2012). The mass spectrum is obtained in the form of a series of mass-to-charge (m/z) peaks versus ion intensity. The obtained m/z values of (singly or multiply-charged) ions of intact or fragmented molecules produced during the ionization process, are submitted on-line or in-house database to identify proteins/peptides (Curreem et al., 2012). Since then, MALDI has been explicitly used in protein / peptide identifications. The details of protein identification by MS can be divided into three components:

(i) sample preparation,
(ii) sample ionization, and
(iii) mass analysis.
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i) Sample preparation

Protein analysis is based on comparative protein profiling of two/more different samples which can be separated according to gel based and non gel based approaches to be presented and analyzed by MS.

i a) Gel based method

It is based on Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) for separation of protein mixtures wherein the separation is according to molecular weight of the proteins. However, the technique is not suitable for complex protein mixture like crude lysate due to its low resolving power. Thus, to increase the resolution of separated proteins, the proteins were resolved based on two distinct properties with the introduction of 2-Dimension gel electrophoresis (2-DE). The proteins are separated in 2-DE according to their isoelectric points in first dimension followed by their molecular weights (MW) in the second dimension. As a result, the resolving power of the gels is increased dramatically. The 2-DE was first reported by O' Farrell and Klose in 1975, ever since it continues to be a robust and established technique. It is a successful approach for studying changes in protein expression of organisms under environmental onslaughts. Further, the protein needs to be visualized in 2-DE gels either by protein staining that is done after the electrophoretic gel run or by protein labeling method that is done prior to the electrophoretic gel run. There are various staining methods employed for proteins visualization on gels. Below is the table that gives the overview of various stains.
### Table 1.2: An overview of various staining methods (Honore et al., 2004).

<table>
<thead>
<tr>
<th>Staining method</th>
<th>Detection limit</th>
<th>Advantage</th>
<th>Disadvantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coomassie Brilliant Blue</td>
<td>100 ng</td>
<td>Very easy to apply</td>
<td>Not sensitive for low abundance proteins</td>
</tr>
<tr>
<td>Colloidal Coomassie Brilliant Blue</td>
<td>50 ng</td>
<td>More Sensitive than CBB</td>
<td>Low range of linearity</td>
</tr>
<tr>
<td>Silver Staining</td>
<td>1-10 ng ng</td>
<td>Sensitive</td>
<td>Small dynamic range</td>
</tr>
<tr>
<td>Fluorescent SYPRO stains</td>
<td>2-8 ng</td>
<td>Easy to apply, more linear than silver stains</td>
<td>Expensive</td>
</tr>
<tr>
<td>Negative Stains</td>
<td>15 ng</td>
<td>Fast and easy</td>
<td>Not suitable for quantification</td>
</tr>
<tr>
<td>Radioactive labeling</td>
<td>&lt; 1 pg</td>
<td>Sensitive</td>
<td>More difficult to handle</td>
</tr>
</tbody>
</table>

Rather than staining the protein, the protein can be labeled prior to the running of the samples so as to visualize it on the gels. Previously the proteins were radiolabeled, but this method is labor intensive as it requires excision of the spots from the gel and counting in a scintillation counter. However, recently covalent labeling of proteins with fluorescent dyes such as propyl-Cy3 (Cy3) and methyl-Cy5 (Cy5) dyes were introduced. These dyes bind to the free amine groups of lysine residues and are just equivalent in sensitivity to silver stains (Honore et al., 2004).

### i b) Non-gel based methods

The need to get high throughput methodology for protein identification prompted the development of non-gel based methods. The technique doesn’t require gels and is suitable for hydrophobic and basic proteins. The advent of gel-free approach has been the application of liquid chromatographic separation, new protein chemistries and enrichment...
methods and the development of mass spectrometry and software for data analysis. In general, proteins are digested in solution, with specific proteases, commonly trypsin which results in different peptide fragments. These peptides are further pre-fractionated using one/more chromatographic approaches such as liquid chromatography using a reverse phase capillary column, in conjunction with MS analysis. The approach is fruitful for analysis of low abundance, hydrophobic proteins, large proteins (>180 kDa) and proteins with extreme pi values that are difficult to be analysed in gel based system (Honore et al., 2004). But, this technique provides an overview of qualitative rather than quantitative aspects of proteins.

ii) Sample ionization:

For analysis of biological samples by mass spectrometer, the molecules of the sample need to be charged and dry. Thus, the molecules must be converted into desolvated ions through either of the two commonly used methods such as

a) Electrospray ionization (ESI) and

b) Matrix-assisted laser desorption/ionization (MALDI).

In these methods, ions are generated from analyzed peptides with addition or loss of one or more protons. They are soft ionization methods employing generation of ions without considerable loss of sample integrity that ensures correct mass information, in turn protein/peptide identification (Graves and Haystead, 2002).
ii a) Electrospray ionization method

Electrospray ionization is a method used to produce gaseous ionized molecules from a liquid solution. A sample solution is passed through a microcapillary orifice of the mass spectrometer, and a potential difference applied between the capillary and a mass spectrometer inlet generates a fine spray of highly charged droplets. Upon solvent evaporation, the sizes of the droplets reduce, finally generating desolvated ions (Graves and Haystead, 2002). An advancement in ESI technology occurred with the development of nanospray ionization (Graves and Haystead, 2002) where the microcapillary tube has a spraying orifice of 1 to 2 μm and flow rates as low as 5 to 10 nl/min (170). In nanospray ionization the flow rates of sample uptake is reduced, thus increasing the time for sample analysis as compared to conventional electrospray (Graves and Haystead, 2002). In ESI, peptides require some form of purification after in-gel digestion, and this can be accomplished directly in the microcapillary tubes. To reduce this drawback electrospray sources are coupled with liquid chromatography (LC) systems like LC, reverse reverse phase LC (RP-LC) and reverse-phase microcapillary LC (RP-LC) (Graves and Haystead, 2002).

ii b) Matrix-assisted laser desorption/ionization (MALDI)

In MALDI, the sample is ionized by co-crystallization with matrix molecules and subjected to irradiation through a laser. The molecules of matrix absorbs the energy of laser beam, causing excitation of the matrix and a proton is donated to the sample molecules, generating singly charged ions that are consequently analyzed in mass
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spectrometer. Matrices consist of fairly low molecular weight organic acids to allow its vaporization upon laser radiation and also serve to minimize sample damage by absorbing most of the incident energy of laser. Once a charged ion is formed, a high voltage is used to eject the analyte from the ion source to the detector. Matrices commonly used in MALDI analysis are 2,5-dihydroxybenzoic acid or cyano-4-hydroxycinnamic acid.

iii) Mass analyzers

The mass analyzers resolve the molecular ions on the basis of their mass and charge in a vacuum. The table provides list of various analyzers commonly used in mass spectrometers.

Table 1.3: List of various mass analyzers (Graves and Haystead, 2002).

<table>
<thead>
<tr>
<th>Mass analyzers</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time of flight</td>
<td>Simplest analyzer, measures the m/z ratio by determining the time taken by the ion to traverse from the sample to the detector.</td>
</tr>
<tr>
<td>Quadrapole</td>
<td>Quadrapole (parallel metal rods) acts as a mass filter to allow the transmission of ions of a certain mass-to-charge (m/z) ratio.</td>
</tr>
<tr>
<td>Quadrupole ion trap</td>
<td>It works like &quot;normal&quot; quadrapole but ions are trapped and sequentially ejected, thereby increasing its sensitivity.</td>
</tr>
</tbody>
</table>

1.2.11.1.3 Types of mass spectrometers

Triple quadrapole: These spectrometers are generally used as amino acid sequencers where the analysis takes place in two stages. In first stage, the machine operates in MS
mode and ions of certain m/z ratio are filtered which are further carried for mass analysis in third quadrupole. In the second stage, mass spectrometer is operated in MS/MS mode where the selected peptide ions are fragmented by the process known as collisionally activated dissociation. This spectrometer is referred as tandem mass spectrometer (MS/MS) as it generates two different mass spectra.

**Quadrapole-TOF:** It is a hybrid mass spectrometer with a combination of different ionization sources with mass analyzers. This spectrometer consists of first quadrupole (Q1) and the quadrupole collision cell (q) of a triple-quadrupole machine that have been combined with a time-of-flight analyzer (TOF). The QqTOF mass spectrometer is involved in determining amino acid sequence of analyzed proteins along with characterization of protein modifications. The ionization source is ESI, which restricts it for large scale proteomics.

**MALDI-TOF:** MALDI is generally coupled to a TOF mass analyzer, in which flight time of the ion from the ion source to the detector is measured. This flight time is converted into a mass-to-charge ratio (m/z), determining the molecular weight of the ion. This spectrometer generates a list of experimental peptide masses, seldom referred as “mass fingerprints”. The obtained peptide mass fingerprint is searched against protein databases, thus identifying the parent protein. MALDI-TOF/MS is a widely used technique in proteomic research. It is considered as easy to use and relatively simple to automate for high-throughput methodologies.
MALDI-QqTOF: The MALDI-QqTOF mass spectrometer is developed by coupling a MALDI ion source with a QqTOF mass analyzer. This spectrometer permits the protein identification viz peptide mass fingerprinting and amino acid sequencing.

FT-ICR: A Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer is an ion-trapping instrument that can achieve higher mass resolution and mass accuracy than any other type of mass spectrometer. This spectrometer is involved in analysis of complex mixtures and are occupied in studying protein interactions and protein conformations.

1.2.12 Response of bacteria to metals: A proteomic approach

Mass spectrometry is still widely used technique for proteomic studies. Witzmann et al. (1999) was the first to report the use of mass spectrometry for protein identification in environmental proteomics. Later, Rodriguez et al. (2003) identified the altered expression of proteins in 2-D gel via mass spectrometry, following the exposure of clams to different concentrations of Acrolor 1254, Cu(II), tributyltin, and As(III) (Nesatyy and Suter, 2008). Since then, proteomics is increasingly used to study differential proteins under various metal stress responses ranging from microorganisms, plants and invertebrates. Microorganisms play vital role in heavy metal biotransformation and bioaccumulation or other relevant processes (Nesatyy and Suter, 2008). Exposure to diverse heavy metals has resulted in differential expression of proteins that have been documented in numerous findings and this technique has been used to unravel the mechanism of resistance in metal resistant organisms. The proteomic response in
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*Rhodotorula mucilaginosa* RCL-11 observed high expression of heat shock proteins under copper challenge by proteomic study (Irazusta *et al.*, 2012). Fanous *et al.* (2010) identified abundance of proteins involved in cell wall biosynthesis (1,4-alpha-glucan branching enzyme and nucleoside-diphosphate-sugar epimerase) upon cobalt stress, while under silver metal, proteins involved in energy metabolism (2-methylcitrate dehydratase and 1, 2-methylcitrate synthase) were expressed, suggesting the use of above proteins as biomarkers under Ag⁺ and Co toxicity. The comparative and proteomic analysis of *Pseudomonas aeruginosa* under Cr(VI) exhibited the differential expression of proteins involved in energy metabolism, stress proteins, protein biosynthesis. This analysis suggested the mechanism for Cr(VI) resistance through production of exopolysaccharide and complexation of metal ion outside the cell (Kilic *et al.*, 2010). Further, transcriptome profiling and mass spectrometry-based proteomic characterization of *Shewanella oneidensis* MR-1 reported the differential expression of proteins under chromate stress (Chourey *et al.*, 2006). Oxidative-stress related proteins were primarily the response of *Corynebacterium* when grown under sublethal concentrations of Cd, Hg (Fanous *et al.*, 2008). This study gave the deeper insight about the adaptive responses of bacteria against these metals. In addition, Kaakoush *et al.* (2008) also reported altered expression of proteins in presence of Cadmium chloride on *Campylobacter jejuni* proteins involved in energy metabolism, motility, and oxidative stress. Bagwell *et al.* (2010) showed the copper accumulation in *Kineococcus radiotolerans* along with the overexpression of proteins entailed in oxidative stress and defense, DNA stabilization and repair and protein turnover. The proteomic analysis of *Klebsiella pneumoniae* showed
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differential protein expression on the exposure to cobalt and lead identified L-isoaspartate protein carboxymethyltransferase type II and DNA gyrase A by MALDI/MS (Bar et al., 2007).

The proteomic analysis under arsenic challenge has also been studied with diverse bacterial species. The effect of arsenic stress on Caenibacter arsenoxydans revealed the differential expression of proteins involved in arsenite efflux and arsenate reduction (Carapito et al., 2005). Analysis of cellular responses to As(III) in Pseudomonas aeruginosa revealed the high abundance of heat shock protein (IbpA) and a putative allo-threonine aldolase (Glyl). Further, the study implied the importance of some of the proteins for resistance against As(III) in P. aeruginosa such as ArsB anion translocator, SOD, glutathione reductase, and the catabolite repressor, control protein Crc (Parvatiyar et al., 2005). The proteomic and transcriptomic analysis of Hemiimonas arsenicoxydans in the presence of arsenic indicated the expression of genes and proteins involved in arsenic detoxification, motility, exopolysaccharide synthesis, phosphate import or energetic metabolism providing the adaptive response information of the bacteria (Weiss et al., 2009). Moreover, arsenic exposure to Leptospirillum ferriphilum resulted in three arsenic resistance system (ARS) member proteins, along with the phosphate regulation and glutathione (GSH) synthesis which seemed to be involved in As(V) and As(III) tolerance (Li et al., 2010). 2D-PAGE revealed enhanced expression of heat shock proteins under As(III) stress in acidophilic archaeon Ferroplasma acidarmanus (Baker-Austin et al., 2007). Similarly, the study on Comamonas sp. strain CNB-1 observed tolerance to As(V) encoded by arsenic-gene cluster and identified the
altered expression of proteins responsive to As(V) stress. The identities belonged to heat shock proteins involved in protein folding, phosphate transporters, enzymes of carbon and energy metabolism that were identified by MALDI-TOF/MS (Zhang et al., 2007). Recently, As(III) toxicity to *Chromobacterium violaeceum* revealed altered expression of oxidative stress enzymes (SOD, GST, Grx), DNA repair and the metabolism of lipids, amino acids and coenzymes through two-dimensional differential gel electrophoresis (2D-DIGE) (Ciprandi et al., 2012). Daware et al. (2012) reported the altered expression of stress responsive proteins and antioxidant enzymes along with porins, amino acid metabolism enzymes in the presence of As(III) to *Klebsiella pneumoniae*. More recently, a proteomic analysis performed on *Exiguobacterium* sp. S17 revealed the enhanced expression of proteins entailed in energy metabolism, stress, protein synthesis and transport under arsenic stress (Belfiore et al., 2013). Identification and characterization of these proteins via proteomic analysis reveals the adaptive behavior of the organism towards different metals and helps to gain insight and better knowledge about the toxic mechanism of metals.
1.3 SCOPE OF THESIS

Metals have played an integral part in expansion of human civilization but the continuous use or abuse of these metals have raised the environmental issues that needs to be sought. Thus, various remedial approaches are available for decontamination of metal-polluted sites. Especially, biotechnological approaches that harness the microbial system have been found useful for this problem. Since microbe cannot disintegrate the metals, they can only alter the chemical properties through an array of various mechanisms. Perception of these various adopted detoxification processes by microorganisms may aid in improvement of these processes. Recent developments of proteomics have allowed new insight into bacterial interactions with metals. Thus, the aim of this work was to isolate and characterize the metal resistant microorganisms. To identify differential expressed proteins under arsenic stress in order to gain an insight on the physiological functioning of metal resistant organism

The specific goals of the undertaken study were:

1) To screen for the metal resistant microorganisms and to evaluate the arsenic resistant mechanism viz atomic absorption spectrometry, transmission electron microscopy and morphological perturbations through scanning electron microscopy.

2) To reveal the role of antioxidant enzymes under metal stress in the bacteria.

3) To determine the mechanism of arsenic resistance by 2-DE and identify the altered expressed proteins by MALDI.
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