CHAPTER 1:
Introduction and Review of Literature
The survival of individual organisms under stress is enhanced by physiological changes at the macromolecular level that enable the organisms to overcome different types of stress. Although, prokaryotes may be affected by fluxes of environmental factors; they are known to harbor an adaptive feature that enables them to circumvent many of these challenges. The major harmful stress condition, microbes face, is the elevated concentrations of heavy metals in the surrounding environment. Some heavy metals at low concentrations have physiological role in microbial metabolism, some of which are co-factors of enzymes involved in the key cellular processes, whereas others are needed for regulation of osmotic pressure, to stabilize molecules through electrostatic interactions, on the other hand some are nonessential and highly toxic (Bruins et al., 2000). At high concentrations, even very important heavy metal ions form nonspecific complex compounds, leading to toxic effects.

Natural sources contribute to heavy metal pollution to a less extent; whereas artificial sources resulted from different human activities, contribute decisively to the pollution of the environment. Control of environmental pollution has become the major and one of the most difficult tasks not only for the developed but also for the developing countries like India. In spite of Government regulations, lot of such waste is dumped around the cities and metropolis and deposited in natural water resources. In Pune, Mula river is contaminated with such waste materials (Imandoust and Gadam, 2007). Khatat et al. (2003) reported that elevated levels of toxic component in river water, indicated by decline in fish population in the river. There are numerous studies highlighting the deleterious effects of heavy metals. There are also reports, albeit few, explaining the mechanism of survival of the various organisms when exposed to heavy metal stress conditions. Yet there are many questions, which need to be properly answered. Therefore, it would be interesting and important to find out if there is any microbial growth in such contaminated water, which are those microorganisms and what is the mechanism of their survival.

1.1 Heavy metals

Heavy metal chemical elements having density/specific gravity >=5 g/cm³ or 5 times more than water (Duruibe et al., 2007) and are natural constituent of the earth crust (Sparks, 2005). Heavy metals are transition elements with incompletely filled d-orbitals.
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The d-orbitals provide heavy metal cations with the ability to form complex compounds, which may or may not be redox-active. In the periodic table, 53 elements are considered as heavy metals (Weast et al., 1984). Among them, 13 trace metals include antimony (Sb), arsenic (As), beryllium (Be), cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb), mercury (Hg), nickel (Ni), selenium (Se), titanium (Ti), zinc (Zn) and their metalloids are considered priority pollutants (Sparks, 2005). Out of these, Pb, Cd, Hg and As are regarded as the most hazardous to human health (Nithya et al., 2011). Heavy metals are stable and persistent environmental contaminants since they cannot be destroyed or degraded unlike organic pollutants into carbon dioxide and water and subsequently bioaccumulate in the food chain (Verma et al., 2001).

1.2 Sources of heavy metals

Heavy metals are widely distributed in the environment being released from both natural and anthropogenic sources. Natural sources contribute to heavy metal pollution to a lesser extent; whereas artificial sources resulted from different human activities, contribute decisively to the pollution of the environment. Exposure of human beings to heavy metals has risen dramatically in the last 50 years as a result of an exponential increase in use of heavy metals in industrial processes and products of electroplating, plastics manufacturing, fertilizers, paints, batteries, dyes, pigments, mining, leather, and metallurgical processes etc.

1.3 Heavy metal toxicity

Within the cells, most toxic interactions with heavy metals are possible because of the wide range of metal binding ligands found in the structural and biochemical components of living cells. In several studies, heavy metals have been shown to induce the formation of reactive oxygen species (ROS), in cells resulting in oxidative stress. ROS have one or more unpaired electrons. Two important forms of ROS are the superoxide (O$_2^-$) and the hydroxyl (OH$^-$) radicals. Superoxide and hydroxyl radicals are produced at low levels under normal physiological conditions when oxygen accepts electrons from redox enzymes, e.g., terminal oxidases and xanthine oxidases (Valko et al., 2004). Under normal conditions, superoxide is rapidly converted to hydrogen...
peroxide by superoxide dismutase (Desideri and Falconi, 2003) and hydrogen peroxide is subsequently removed by catalase and glutathione peroxidase (Michiels et al., 1994). However, heavy metals can induce the formation of ROS which is an overload for ROS detoxifying enzymes and other redox maintaining systems. The biological targets of these ROS are DNA, RNA, proteins and lipids in the cell. One significant effect due to ROS 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 directly attack polyunsaturated fatty acids in the membrane and initiate lipid peroxidation. A primary effect of lipid peroxidation is decrease in membrane fluidity, which alters membrane properties and can disrupt membrane-bound proteins significantly. This effect acts as an amplifier, for the generation of more free radicals and degradation of polyunsaturated fatty acids to a variety of products such as aldehydes. These aldehyde products are very reactive and can damage protein molecules.

Several classes in protein damages due to ROS, include oxidation of the sulphydryl groups, reduction of disulfides, reaction of aldehydes, modification of prosthetic group or metal cluster, protein-protein cross-linkage and peptide fragmentation (Fucci et al., 1983; Stadtman, 1990). All these modifications are deleterious to the cell, since they lead to loss of membrane function and protein, and block DNA replication or cause mutation (Cabiscol et al., 2010). Intracellular heavy metals can exert a toxic effect by forming coordinate bonds with anions, blocking functional groups of enzymes, inhibiting transport systems, displacing essential metals from their native binding sites and disrupting the cellular membrane integrity (Goyer, 1997; Nies, 1999).

1.4 Mechanisms of microbial heavy metal resistance and detoxification

Despite heavy metal toxicity, the microorganisms have acquired a variety of mechanisms to remove or detoxify toxic metals (Gadd, 2010). The detoxification mechanisms may be directed against one heavy metal or a group of chemically related metals. Bacterial resistance to heavy metals is heterogeneous at genetic and biochemical levels particularly at protein level. The genes for heavy metal resistance are located on chromosomes, plasmids or transposons (Cervantes et al., 2001; Nies, 1999). Microbes cannot degrade the metals but transform the metal chemical properties via an array of
mechanisms (Lloyd, 2002). There are five basic mechanisms of heavy metal resistance in microorganisms (Fig. 1.1) (1) Extracellular polymeric substances (2) Biosorption mechanisms; (3) Intracellular accumulation; (4) Microbial transformation; (5) Minimizing uptake of heavy metal or efflux them outside the cell (Gomathy et al., 2010; Nies, 1999).

Fig 1.1: Mechanisms of heavy metal resistance in microorganisms

1.4.1 Extracellular polymeric substances (EPSs) and exopolymers

Microorganisms form exopolymers which have negatively charged functional groups like phosphate, hydroxyl, succinyl and uronic acids. These are particularly efficient in binding heavy metals such as Pb\(^{2+}\), Cd\(^{2+}\) and U\(^{4+}\). The immobilization of Pb\(^{2+}\) by exopolymers has been observed in several bacteria, including Staphylococcus aureus, Micrococcus luteus, and Azotobacter spp. One of the classes of EPS is biosurfactants which are produced and excreted by many microbes. Biosurfactants have been investigated for their ability to form complexes with heavy metals like Cd\(^{2+}\), Pb\(^{2+}\)
and Zn$^{2+}$ and increases the solubility of metals. These biosurfactant complexed metals are non toxic to the cells.

1.4.2 **Biosorption mechanisms**

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 biological metabolic cycle (Malik, 2004). This is based on physical adsorption, ion exchange, chelation and chemical sorption. Microbial cell walls mainly composed of polysaccharides, proteins and lipids which have abundant metal binding negatively charged groups such as carboxyl(-COOH), sulphydryl (-SH), phosphate (-PO$_4$) and amine (-NH$_2$) groups, which might act as binding sites for metal ions (Kuyucak and Volesky, 1988). Among the microorganisms fungi and yeast are potential microbes for metal biosorption due to excellent metal binding properties (Gupta et al., 2000).

1.4.3 **Intracellular accumulation**

Bioaccumulation is one of the mechanisms of metal resistance and it is metabolism dependent process. Bioaccumulation is referred as the accumulation of materials that are non essential elements of an organism such as accumulation of metals (Bains and Raab, 2004). Extracellular or cell wall attached ligands are thought to bind toxic metals. These ligands transport the metals through the cell wall in a slow transport step. The metals are released inside the cell, incorporated into biochemical pathways, or trapped in an inactive form by complex formation with another high-affinity ligand. Various bacteria such as *streptomyces* sp., *Thiobacillus ferroxidans*, *Bacillus aureus*, *Pseudomonas aerugenosa*, *Saccharomyces cerevisiae* (Gupta et al., 2004); *Ochrobactrum* (Pandey et al., 2010) are reported to accumulate significant levels of different metals like cadmium, nickel, uranium and arsenic respectively.
1.4.4 Microbial transformation

Generally, microbial transformation of metals can be divided into two broad categories: redox conversions of inorganic forms and conversion from inorganic to organic form and *vice versa*, typically methylation and demethylation.

Some microbes can obtain energy by oxidation of metals such as Fe, Mn and As (Tebo *et al.*, 1997; Santini *et al.*, 2000). On the other hand, reduction of metals can occur through dissimilatory reduction where microorganisms utilize metals as a terminal electron acceptor for anaerobic respiration. For example, oxyanions of arsenic (Stolz *et al.*, 2006) and chromium (Quiñntana *et al.*, 2001) can be used in microbial anaerobic respiration as terminal electron acceptors. In addition, microorganisms may possess reduction mechanisms that are not coupled to respiration, but instead are thought to impart metal resistance. For example, aerobic and anaerobic reduction of Cr(VI) to Cr(III) (Quiñntana *et al.*, 2001); and reduction of Hg(II) to Hg(0) are widespread detoxification mechanisms among microorganisms (Wagner-Döbler *et al.*, 2000).

Microbial methylation plays an important role in the biogeochemical cycle of heavy metals, because methylated compounds are often volatile. For example, mercury Hg(II) can be biomethylated by a number of different bacterial species (e.g. *Pseudomonas* sp., *Escherichia* sp., *Bacillus* sp. and *Clostridium* sp.) to gaseous methylmercury Hg(0) which is less toxic.

1.4.5 Minimizing uptake of heavy metal or efflux it out of the cell

Most metal ions have to enter the bacterial cell in order to have a physiological or toxic effect (Nies, 1999). Most cells solve this problem by using two types of uptake system for heavy-metal ions: one is fast, unspecific and, since it is used by a variety of substrates, constitutively expressed. These fast systems are usually driven only by the chemiosmotic gradient across the cytoplasmic membrane of bacteria e.g. arsenate is transported by the fast Pit (phosphate inorganic trans-port) system and chromate by the fast sulphate-uptake system (Nies and Silver, 1995). The second type of uptake system has high substrate specificity, is slower, and often uses ATP hydrolysis as the energy source i.e. HoxN family for Ni$^{2+}$ and Co$^{2+}$ (Nies and Silver, 1995). After taking heavy metal inside the cell, some bacteria efflux it into the surroundings as a detoxification
mechanism. The intake and subsequent efflux of heavy metal ions by microbes normally includes a redox reaction involving the metal.

Microbes can acquire one or a combination of other resistance or detoxification mechanisms to overcome the heavy metal toxicity (Bruins et al., 2000).

1.5 Metal

1.5.1 Cobalt

Cobalt (Co\(^{2+}\)) is the 27\(^{th}\) element of the periodic table and plays an essential role in microorganisms as cofactor for many diverse metalloenzymes e.g. it is the active center of coenzymes called cobalamins, the most common example of which is vitamin B12. In bacteria, cobalt enters the cell mainly by CorA-transporters or exceptionally by a HoxN-type transporter (Smith et al., 1998; Smith and Maguire, 1995). In Gram-negative bacteria, cobalt resistance is based on the transenvelope efflux system driven by a RND transporter protein family whereas in Gram-positive bacteria and eukaryotes it is based on an efflux system driven by a CDF (cation diffusion facilitator) transporter protein family (Nies, 1999).

1.5.2 Nickel

Nickel (Ni\(^{2+}\)) is the 28\(^{th}\) element of the periodic table and small amount of it plays an essential role in microorganism as cofactor for many enzymes e.g. urease, hydrogenase, carbon monoxide dehydrogenase, superoxide dismutase (Hausinger, 1987). However, nickel is often toxic to bacteria at high concentrations. This toxicity is generally a consequence of nickel binding to sulfhydryl groups of sensitive enzymes or displacing essential metal ions in biological processes and also causes oxidative stress (Macomber and Hausinger, 2011). In bacteria and S. cerevisiae, it enters the cell mainly by the fast and unspecific CorA system (metal transport system, MIT) Mg\(^{2+}\) transport system (Hmiel et al., 1989). On the other hand highly specific nickel transporters are either Hox chemiosmotic transporters or ATP-binding cassette (ABC) uptake transporters, which use periplasmic nickel-binding protein (Nies, 1999). Nickel resistance studied in Ralstonia metallidurans strains CH34 and A31, which is based on nickel efflux driven by RND transporter. Efflux mediated
nickel resistance is best-known mechanisms of nickel detoxification.

1.5.3 Copper

Copper (Cu\(^{2+}\)) is the 29\(^{th}\) element of the periodic table. It's required by most organisms as co-factor for many enzymes, such as oxidases and hydroxylases participated in numerous catabolic pathways and electron transport (Mason, 1976; Nicholas et al., 2002) but is highly toxic when present in excess (Gaetke and Chow, 2003). Inside the cells, copper may be bound by various compounds to form copper complexes (Nies, 1999) and also displace other metals (i.e. Ni\(^{2+}\), Co\(^{2+}\), Mn\(^{2+}\), Zn\(^{2+}\)) from essential complexes. Therefore, the intracellular concentration of copper is controlled by copper transport systems encoded by cop operons, which maintain copper homeostasis (Cooksey, 1993). P-type ATPases seem to detoxify copper via efflux in some species, however, the best-characterized system is that of Enterococcus hirae, where the cop operon consists of four genes: copY, copZ, copA and copB (Solioz and Stoyanov, 2003).

1.5.4 Mercury

Mercuric (Hg\(^{2+}\)) is the 80\(^{th}\) element of the periodic table and binds to sulphydryl groups of enzymes, thereby inactivating vital cellular functions (Wagner-Döbler et al., 2000). There are three types of mercury resistance mechanisms reported in bacteria: reduced uptake of mercuric ions (Enterobacter aerogenes), methylation and reduction of Hg\(^{2+}\) to Hg\(^{0}\). The enzyme that catalyses the reduction of mercury is the intracellular, cytoplasmic, FAD-containing mercuric reductase. The reductase mechanism also involves a plasmid specified mercury specific transport system. It seems that the reductase and transport function might interact physically. An extensively studied resistance system is based on clustered genes in the mer operon (Summers and Silver, 1972), which allows bacteria to detoxify Hg\(^{2+}\) into volatile metallic mercury Hg\(^{0}\) by enzymatic reduction (Summers, 1986; Nies, 1999; Wagner-Döbler et al., 2000).

1.5.5 Arsenic

Arsenic is the 33\(^{rd}\) element in the fifth group of the periodic table. It is a metalloid as it exhibits properties of a metal as well as a non-metal. It is a well-known toxic
chemical and listed as a known carcinogen by the Environmental Protection Agency (EPA) and the World Health Organization (WHO, 2001). Certain bacteria have evolved a variety of mechanisms for coping with as well as to avoid arsenic toxicity (Baker-Austin et al., 2007; Anderson and Cook, 2004) by minimizing arsenic uptake or by immobilization or arsenic transformation.

1.6 Arsenic

1.6.1 Arsenic environmental sources

It is the 20th abundant element in the earth’s crust but is widely distributed in the environment being released from both natural sources, e.g. by volcanic action, during weathering of rocks and mining as well as from anthropogenic ones, such as pesticides (e.g. calcium arsenate dimethylarsonate, disodium methylarsenate), production of paints and dyes, application of dusts from burning of fossil fuels, semiconductor industry, processing of pressure-treated wood [e.g. chromated copper arsenate (CCA)], coal combustion, high-temperature combustion and smelting of metals (Cullen and Reimer, 1989). Use of arsenic in medicine as chemotherapeutic agent to treat various diseases such as protozoan, syphilis, asthama, also contribute as additional source of contamination (Welch et al., 2000).

Arsenic is present in reducing marine sediment, iron deposits, sedimentary iron ores and manganese nodules and is commonly associated with iron hydroxides sulfides (Mandal and Suzuki, 2002). Anthropogenic sources generally exceed natural sources by 3 to 1 in the environment. It occurs in the soil at the concentrations ranging from 0.1 to more than 1000 ppm, in atmospheric dust at 50 to 400 ppm and in sea water and fresh water the concentration may be 2.6 ppb and 0.4 ppb respectively. It has been estimated that an annual release of arsenic from volcanoes, microbial activity, and burning of fossil fuels is approximated to be 3,000, 20,000, and 80,000 metric tons of atmospheric arsenic, respectively (Jones, 2007). All these resources of arsenic either natural or anthropogenic, increases exposure of living being to arsenic or can enter into the atmospheric biogeochemical cycle.
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Arsenic environmental chemistry and its toxicity

In the environment, arsenic exists in four oxidation states viz., arsine [As(-III)], elemental arsenic [As(0)], arsenite [As(III)], and arsenate [As(V)], with the most common oxidation states being the pentavalent arsenate, As(V) and the trivalent arsenite, As(III) (Cullen and Reimer, 1989). The As (V) and As (III) are two dominant forms of arsenic in the environment; both forms are toxic to living organisms. These two forms are interconvertible via oxidation/reduction of As(III)/As(V), where As\(^{+5}\) species is more stable in oxidizing conditions while As\(^{+3}\) species is dominant under reducing conditions (Duker et al., 2005). Both these species are soluble within wide range pH mobility (Bell, 1998). The speciation of arsenic decides its solubility, mobility, sorption behaviour, bioavailability and toxicity (Tamaki and Frankenberger, 1992).

The toxicity of arsenic depends on its form and oxidation state. 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 bond length being 10% longer, that makes it unstable. Toxicity of As(V) is also because it inhibits the oxidative phosphorylation process by replacing cellular phosphate and can inhibit enzymes such as kinases. As(V) also replaces the phosphate in the DNA double helix causing mutagenesis, teratogenesis in the cell (Liévremont et al., 2009). On the other hand, As(III) binds to sulfhydryl groups of amino acids (-SH groups) thereby a wide range of enzymes such as pyruvate dehydrogenase (PDH) by binding to the sulfydryl group of dihydrolipoamide, a cofactor of PDH (Hughes, 2002) and making them non-functional. It also forms strong bonds with the imidazolium nitrogens of histidine residues (Rosen, 2002), 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 MMA\(^{III}\) are potent inhibitors of GSH reductase (Styblo et al., 1995) and thioredoxin reductase (Lin et al., 1999) which are involved in maintaining the intracellular redox homeostasis (Smits et al., 2005). The
inhibition of these enzymes can result in cellular damage and lead to cytotoxicity (Hughes, 2002).

1.6.3 Microbial arsenic resistance mechanism

Microorganisms play a significant role in the biogeochemistry of arsenic in the environment either by direct or indirect mechanisms (Oremland and Stolz, 2005; Bachate et al., 2009). Due to the abundance of arsenic in the environment, bacteria have evolved a variety of mechanisms for coping with as well as to avoid arsenic toxicity (Baker-Austin et al., 2007; Anderson and Cook, 2004) viz., (i) Minimizing arsenic uptake (ii) By immobilization and (iii) Arsenic transformation.

1.6.3.1 Minimizing arsenic uptake

Since arsenic don’t play any functional role in the cell, specific uptake transporters have not evolved. Rather, inorganic arsenic enters the cell through the outer membrane by non-selective porins and through the cytoplasmic membrane adventitiously via transporters of chemically analogous molecules (Rosen and Liu, 2009). As(V), which is a structural analogue of phosphate, enters the cell by phosphate transporter (Rosen and Liu, 2009). There are two phosphate (Pi) transporters, Pit (phosphate inorganic transporter) and Pst (phosphate specific transporter) which are major conduits for As(V) uptake (Páez-Espino et al., 2009). As(V) uptake occurs mainly through the Pit system rather than the more specific Pst system (Bhattacharjee and Rosen, 2007). However, when microorganisms are exposed to higher concentration of As(V), only Pst is expressed in order to reduce As(V) uptake (Kruger et al., 2013). On the other hand, in reducing and acidic environments, As(III) exists in the uncharged form [As(OH)₃] which is structurally similar to glycerol. Earlier studies suggest that As(III) enters into the cell via an aquaglyceroporin (GlpF) in E. coli (Meng et al., 2004) or by hexose permease as in Saccharomyces cerevisiae (Liu et al., 2004), and by minimizing or inactivating aquaglyceroporin or hexose permease microorganisms enable to tolerate As(III).
Fig 1.2: Schematic representation of processes evolved by prokaryotes to cope with arsenic: (1) Arsenic enters the cells through the phosphate transporters (Pit/Pst) or the aqua-glyceroporins (GlpF). (2) arsenic is immobilized in the environment by extracellular precipitation. (3) once inside the cells, As(V) is reduced by an arsenate reductase, ArsC, to As(III) which is extruded out of the cell by the specific membrane pump Ars(A)B. (4) inorganic arsenic can be transformed into organic species via a cascade of methylations. (5) As(V) is used as an electron acceptor during respiration by the dissimilatory arsenate reductase (ArrAB). (6) As(III) can serve as an electron donor via the As(III) oxidase, AoxAB or ArxAB.
1.6.3.2 Arsenic immobilization

This arsenic resistance mechanism is due to immobilization of the toxic ion before it enters into the cytoplasm by its precipitation outside the cell, thus preventing its entry into cells and interaction with essential components (Slyemi and Bonnefoy, 2012). The As(V), which is the dominant species under oxidizing conditions (0.2–0.5 V), may be removed by sorption onto or precipitation with Fe, Mn or Al hydroxides, Ca and Mg compounds as well as by organic matter (Smedley and Kinniburgh, 2002; Bissen & Frimmel, 2003). In reducing and acidic environments, the uncharged form As(OH)₃ becomes dominant and co-precipitates with oxides, as well as iron sulfides (Carbonell-Barrachina et al., 2000). Depending on the As oxidation state, which varies according to the local physicochemical conditions (mainly the redox potential and the pH), sulfate reducers and iron oxidizers could maintain this metalloid outside the cell by reaction with the product of their energy metabolism. Indeed, the final product of sulfur and sulphate reduction is H₂S which precipitates with As(V) to form insoluble sulfide complexes (Rittle et al., 1995; Newman et al., 1997; Battaglia-Brunet et al., 2009). In oxic environments, the acidophilic iron oxidizer Acidithiobacillus ferrooxidans, when grown with Fe(II) as an electron donor, scavenges As(III) with the newly produced Fe(III) to form schwertmannite or tooeleite (Duquesne et al., 2003; Morin et al., 2003). It was proposed that the As(III) is trapped with Fe(III) tightly bound to the extracellular polymeric substances (EPS) and that the Fe(II) oxidation site at the cell surface serves as the As(III) nucleation site (Duquesne et al., 2003). The EPS which surround bacteria could stably accumulate metallic ions (Harrison et al., 2007). Different bacteria have been shown to have this capacity to sequester metals outside the cell e.g. Herminiimonas arsenicoxydans (Marchal et al., 2010). However, this protection is limited, due to the saturation of the metal binding sites at relatively low level of arsenic.

1.6.3.3 Arsenic transformation

(a) Arsenate reduction

In prokaryotes, reduction of As(V) to As(III) occurs by two mechanisms, viz., dissimilatory reduction during anaerobic respiration, which involves As(V) as a terminal
Microbes possess genetic determinants to confer arsenic resistance which may be plasmid or chromosomal encoded system and inactivation of these operons results in their hypersensitivity to arsenic compounds (Mukhopadhyay et al., 2002). The number of genes in this operon can vary from three (arsRBC) to five (arsRDABC), arranged in a single transcriptional unit. In both cases, there are two components: a cytoplasmic arsenate reductase enzyme (ArsC) for the reduction of As(V) to As(III), which is subsequently extruded using an integral membrane protein, As(III) expulsion pump (ArsB or Acr3p). This efflux pump belongs to two different families, ArsB protein and Acr3p arsenite carrier gene family (Mukhopadhyay et al., 2002; Achour, et al., 2007). The well-characterized ArsB family includes membrane proteins of *E. coli* plasmid R773 and *Staphylococcus aureus* plasmid pI258, and it is prevalent in *Firmicutes* and *Gamma-proteobacteria*. This protein belongs to Na\(^+\)/H\(^+\) transporter family and is a 45 kilodaltons, inner membrane protein with 12-14 transmembrane helices (Rosen, 1999). The ArsB functions as antiporter using the membrane potential to extrude arsenite. On the other hand, much less is known about the second family of arsenite carriers, Acr3p and it is mostly present in *Actinobacteria* and *Alphaproteobacteria* (Achour et al., 2007). Members of Acr3p transporters showed a function similar to ArsB, but the two proteins have no significant sequence similarity. Even though Acr3p is much less characterized, it has been reported to be present in phylogenetically distant species than ArsB. Acr3p could be divided into two subfamilies, Acr3(1)p and Acr3(2)p, based on their phylogenetic dissimilarities (Achour et al., 2007). ArsA is a membrane associated ATPase that assists ArsB in As(III) efflux by providing the necessary energy via ATP hydrolysis. ArsR and ArsD are regulatory components primarily acting as a transcription repressor and repressor regulating the upper limit for operon activity, respectively.

In addition, other ars genes have been isolated from various microbes such as arsP coding for a putative membrane permease, arsTX encoding a thioredoxine system in *Microbacterium* sp. A33 (Achour-Rokbani et al., 2010) and arsN coding for an acetyltransferase like protein (Chauhan et al., 2009).
In bacteria, no As(V) extrusion pump has been identified to date. As a detoxification mechanism, the less toxic As(V) is generally converted to the more toxic As(III) by the cytoplasmic arsenate reductase (ArsC), which is then pumped out of the cell by As(III) efflux pumps. This As (III) extrusion pump is sufficient for As(III) resistance while ArsC is required for resistance to As(V) (Slyemi and Bonnefoy, 2012).

(i) Arsenate reductase

Arsenate reductase (ArsC) is small (13-16 Kilodaltons), monomeric and cytoplasmic enzymes that reduce As(V) to As(III). However, three distinct, but related, families of arsenate reductase have evolved and that differ through their structures, reduction mechanism and the location of their catalytic cysteine residues. Among them, two are thioredoxin (Trx) and glutaredoxin (Grx) coupled arsenate reductases and third is Acr2p family.

The Acr2p family encoded eukaryotic arsenate reductase genes capable of reducing As(V) to As(III), observed in Eukarya and Saccharomyces cerevisiae. Acr2p has a three-dimensional structure related to tyrosine phosphatases with the HCX5R motif involved in catalysis but lacking the glycine-rich phosphate binding motif (Mukhopadhyay et al., 2003).

The thioredoxin-coupled arsenate reductase family is typified by the ArsC protein from pl258 plasmid of Staphylococcus aureus, Bacillus subtilis, Archaeoglobus fulgidus DSM 4304 (Mukhopadhyay et al., 2002: Messens and Silver, 2006). These ArsC reductases use the cysteine thiol-coupling enzyme thioredoxin as the reductant and require the presence of thioredoxin reductase and NADPH to complete the catalytic cycle (Ji et al., 1994). The structure of these enzymes is remarkably similar to that of low molecular weight protein, tyrosine phosphatases (Zegers et al., 2001) and, indeed, the ArsC exhibits low level phosphatase activity.

The glutaredoxin-coupled arsenate reductase (Grx) family is represented by the ArsC of the E. coli plasmid R773. It catalyzes the reduction of As(V) to As(III) where glutathione (GSH) and the small thiol transfer protein glutaredoxin act as reductant (Mukhopadhyay et al., 2002). Reduction of As(V) is mediated by three cysteine residues: one in ArsC, one in GSH and one in glutaredoxin. As(V) binds to three arginine residues.
and then forms a covalent bond with the single catalytic cysteine residue. Cys12. The following step is the formation of a tertiary intermediate in which a disulfide bond is formed between Cys12 and a glutathione cysteine. Finally, glutaredoxin reduces As(V) and the disulfide bond leading to the release of As(III) and regeneration of reduced ArsC (Mukhopadhyay et al., 2003; Messens and Silver, 2006).

(ii) Dissimilatory arsenate reductases

Dissimilatory arsenate reduction is carried out by a terminal reductase, structurally and functionally different from the arsC arsenate reductases. As(V) is electrochemically positive with oxidation-reduction potential of +139 mV. Therefore, As(V) reduction coupled with the oxidation of an electron donor with a lower redox potential can be expected to provide sufficient energy to sustain bacterial growth (Ahmann, 1994). This mechanism is functional mainly in obligate or facultative anaerobic microorganisms, for example, Proteobacteria, Eubacteria, thermophilic Eubacteria and Crenarchaeota, from different environments (Silver and Phung, 2005; Stolz et al., 2006; Páez-Espino et al., 2009). These microorganisms can use a variety of electron donors for As(V) reduction including inorganic (e.g. hydrogen and sulfide), organics such as acetate, lactate, formate and pyruvate, and even more complex compounds such as benzoate, phenol and toluene (Stolz et al., 2006). Arsenate respiratory reductase activity has been described in many bacteria such as (Fisher et al., 2008), Clostridium sp. Strain OhILAs (Stolz et al., 2006), Sulfurospirillum barnesii (Malasarn et al., 2004).

Dissimilatory arsenate reductase (ArrAB) is a heterodimer composed of a large subunit of about 100 kDa (ArrA) and a small subunit of about 30 kDa (ArrB). ArrA, which belongs to the dimethyl sulfoxide (DMSO) reductase class of proteins, contains the As(V) binding site and the catalytic site with a bismolybdopterin guanine dinucleotide cofactor and one [4Fe-4S] cluster while smaller one (ArrB) contain a [4Fe-4S] center protein and may be involved in the electron transfer to molybdenum cofactor of the ArrA subunit where As(V) reduction takes place (Bhattacharjee and Rosen, 2007).
(b) Arsenite oxidation

In nature, microorganisms carry out As(III) oxidation using the enzyme As(III) oxidase, which is classified as a member of the DMSO reductase family. Microbial oxidation of As(III) is carried out by bacteria either heterotrophically or chemoautotrophically. Heterotrophic arsenite oxidizers can catalyze reaction by a periplasmatic enzyme that converts As(III) to As(V) while respiring oxygen but cannot grow without organic matter (Ellis et al., 2001; Simon Silver & Phung, 2005). Unlike the heterotrophs, the arsenite-oxidizing chemoautotrophic microorganisms use the energy and reducing power from As(III) oxidation for CO₂ fixation and cell growth under both aerobic (Duquesne et al., 2008; Garcia-Dominguez et al., 2008) and nitrate chlorate reducing (Oremland et al., 2002) conditions. Arsenite oxidation is a thermodynamically exergonic reaction (ΔGo' = -256kJ/reaction) and can provide sufficient energy for As(III)-oxidizing chemolithotrophs (Santini et al., 2000). The arsenite oxidase was first purified and characterized in Alcaligenes faecalis (Ellis et al., 2001).

As(III) oxidation is mediated by two distinct enzymes, one is the extensively studied AoxAB (also named AroBA or AsoBA), and recently found ArxAB. Enzyme AoxAB consists of two heterologous subunits: a large subunit (AoxB) belonging to the DMSO reductase family with a molybdenum cofactor together with a [3Fe-4S] centre and a small subunit (AoxA) contains a ‘Rieske’ [2Fe-2S] centre (Ellis et al., 2001; Duval et al., 2010). These two subunits are invariably encoded in an operon with the gene encoding the small subunit, aoxA, upstream from that encoding the catalytic subunit, aoxB. This relative gene order is conserved in all arsenite oxidizers analyzed to date including H. arsenicoxydans, Al. faecalis, Agrobacterium tumefaciens and Hydrogenobaculum sp. strain 3684 (Muller et al., 2003; Simon Silver and Phung, 2005; Kashyap et al., 2006; Clingenpeel et al., 2009).

Enzyme ArxAB also consists of two heterologous subunits: a small subunit (ArxB) containing four [4Fe-4S] iron-sulfur clusters and a large subunit (ArxA) containing a molybdenum cofactor and a [4Fe-4S] cluster (Richey et al., 2009). The ArxAB enzyme has been detected so far only in the Ectothiorhodospiraceae family ((Richey et al., 2009; Oremland et al., 2009). This arsenite oxidase appears to share more
characteristics with the respiratory arsenate reductase than with the arsenite oxidase AoxAB.

(c) Methylation/demethylation

Methylation of arsenic is a widespread phenomenon observed in microorganisms to human being. Methylation is originally thought as a detoxification step; but more recent observations indicate that methylated arsenic species are more toxic than their inorganic counterparts (Petrick et al., 2000; Dopp et al., 2010). Methylation process involves a series of steps in which the reduction of As(V) is followed by oxidative addition of a methyl group (Dombrowski et al., 2005) and may result in the formation methylate arsenic to form non-volatile monomethylarsonic acid (MMA\textsuperscript{V}) and dimethyl arsenate (DMA\textsuperscript{V}), dimethyl arsenite (DMA\textsuperscript{III}) and volatile trimethyl arsenic oxide (TMAO\textsuperscript{V}), trimethylarsonic acid (TMA\textsuperscript{III}) and dimethylarsine (DMA\textsuperscript{III}) (Bentley and Chasteen, 2002). In the microbial system it is catalyzed by an enzyme ArsM (As(III) S-adenosylmethionine methyltransferase) and encoded by gene termed as arsM. Generally, the methylation reactions do require S-adenosylmethionine (SAM) except in anaerobic bacteria which required methylcobalamin as the source of methyl group.

Microorganisms are thought to play a key role in regenerating As(V) by demethylation of methylated arsenic species to use them as carbon and energy sources. *Alcaligenes, Pseudomonas* and *Mycobacterium* are able to demethylate mono and dimethyl arsenic compounds (Bentley and Chasteen, 2002). Recently, a two-step demethylation of methylarsonic acid (MMA) to As(III) mediated by two distinct microorganisms was observed in MMA contaminated soil samples. This process involved a reduction of MMA to methylarsonous acid by *Burkholderia* sp. MRL followed by a demethylation to As(III) by *Streptomyces* sp. MRL (Yoshinaga et al., 2011). However, the mechanism of demethylation is still completely unknown.
1.7 Heavy metal resistance and *Klebsiella* sp.

There are several reports on resistance to heavy metal in genus *Klebsiella* isolated from varied sources as shown in Table 1.1.

**Table 1.1** The genus *Klebsiella* exhibit resistance to various heavy metals

<table>
<thead>
<tr>
<th>Heavy metal</th>
<th>Organism</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd(II). Cr (VI), Pb (II)</td>
<td><em>Klebsiella sp.</em></td>
<td>(Haq <em>et al.</em>, 1999)</td>
</tr>
<tr>
<td>Cd (II)</td>
<td><em>Klebsiella planticola</em></td>
<td>(Sharma <em>et al.</em>, 2006)</td>
</tr>
<tr>
<td>Ni (II). Cu (II)</td>
<td><em>Klebsiella oxytoca</em></td>
<td>(Stoppel <em>et al.</em>, 1995)</td>
</tr>
<tr>
<td>Ni (II)</td>
<td><em>Klebsiella oxytoca</em> ATHA6</td>
<td>(Alboghobeish <em>et al.</em>, 2014)</td>
</tr>
<tr>
<td>Hg(II)</td>
<td><em>Klebsiella pneumoniae</em></td>
<td>(Essa <em>et al.</em>, 2002)</td>
</tr>
<tr>
<td>Co(II). Ni(II)</td>
<td><em>Klebsiella pneumoniae</em></td>
<td>(Ainsworth <em>et al.</em>, 1979)</td>
</tr>
<tr>
<td>As(V)</td>
<td><em>Klebsiella oxytoca</em></td>
<td>(Maeda <em>et al.</em>, 1992)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Shakoori <em>et al.</em>, 2010)</td>
</tr>
<tr>
<td>Co(II). Pb(II)</td>
<td><em>Klebsiella pneumoniae</em></td>
<td>(Bar <em>et al.</em>, 2007)</td>
</tr>
</tbody>
</table>

1.8 Proteomics

The term ‘proteome’ was introduced by Marc Wilkins in 1994 and is used to describe all the protein contents encoded by the genome. The term ‘proteomics’ is used to describe the complete set of proteins that is expressed, modified following expression, by the entire genome in the lifetime of a cell, was first introduced in the 1990s (Wilkins *et al.*, 1996; Wasinger *et al.*, 1995). Proteomics provides direct information about the dynamic protein expression in tissue or whole cells, giving us a global analysis. Together with the significant accomplishments of genomics and bioinformatics, systematic analysis of all expressed cellular components has become a reality in the post genomic...
era, and attempts to grasp a comprehensive picture of biology have become possible. The fig. 1.3 shows the details of separation of proteins, mass spectrometry and bioinformatics tools for carrying out the proteomic analysis.

Proteomics has widely been used in a variety of basic and applied research and its analysis provides valuable information about changes in the synthesis, degradation, post translation modifications and interactions among proteins. One important aspect of proteomics is to study the expression levels and characterization of proteins in cells when exposed to different environmental stress. It is a valuable tool for detecting slight changes in expression of individual proteins and modifications in amino acid sequence that may be adaptive response of organism towards an altered environment (Unwin et al., 2006).

Proteomics is mainly of three kinds:

(a) **Expression proteomics:** Expression proteomics is the quantitative study of protein expression between the samples which differ by some variables. Several methods have been used in quantitative analysis of protein expression, mostly based on the conventional two-dimensional gel electrophoresis, which, when coupled with advanced mass spectroscopy techniques, has followed the rapid identification and characterization of thousands of proteins in a single gel.

(b) **Functional proteomics:** Functional proteomics is a broad term which embraces all proteomic approaches relating to devising its functions.

(c) **Structural proteomics:** Structural proteomics deals with the structure and nature of protein complexes present in a particular cell organelle.
Chapter 1

Separation of proteins 2-DE LC

Mass Spectrometry
Ion source MALDI ESI
Mass analyzer Ion trap TOF
Detector Quadrapole FT-ICR

Bioinformatics analysis
Mascot
Profound
Pepsea
Mawse
MS-Fit
Sonar
Sequest

2-DE Two-dimensional gel electrophoresis
LC Liquid chromatography
MALDI Matrix assisted laser desorption/ ionization
ESI Electrospray ionization
TOF Time of flight
FTICR Fourier transform – ion cyclotron resonance

Fig 1.3: Proteomics. Mass spectrometry and Bioinformatics
1.8.1 Methodological aspects of proteomic analysis

Proteome analysis is based on four technological aspects, 1) fast and simple procedure for purifying small amounts of proteins from complex mixtures, 2) a fast and sensitive method to generate limited, but sufficient, structural information from the protein of interest, 3) access to extended protein or DNA sequence databases and 4) computer algorithms capable of translating and linking the DNA sequence language with various types of protein structural information, such as N-terminal protein or internal peptide sequences, acid compositions, pl. peptide mass fingerprints, MS fragmentation patterns or sequence tags of selected peptides. These four pillars supporting actual proteomics developed during different time periods.

1.8.2 Separation of proteins

The separation of proteins or their fragments prior to further analysis is one of the key elements in proteomic analysis. The separation of proteins on the whole protein level is most often performed by gel-based electrophoretic or by liquid chromatographic methods. Separations or fractionations on the peptide level can be performed, for instance, by chromatographic methods or by peptide isoelectric focusing (Cargile et al., 2004a; Cargile et al., 2004b; Chick et al., 2008).

1.8.2.1 Non-gel-based separation in proteomics

Liquid chromatography coupled to mass spectrometry (LC-MS and LC-MS/MS) is a widely used and powerful technique for the analysis of proteins and peptides. In general, proteins are digested in solution with specific proteases, commonly trypsin which results in different peptide fragments. These peptide fragments are further pre-fractionated using one/ more chromatographic approach such as liquid chromatography using a reverse phase capillary column, in conjunction with MS analysis. This method is suitable for identification of hydrophobic, basic and large (>180 kDa) proteins. But, this technique provides an overview of qualitative rather than quantitative aspect of proteins.
1.8.2.2 Gel based Separation of Proteins

It is based on two-dimensional polyacrylamide gel electrophoresis (2D PAGE) in combination with mass spectrometry is currently the most widely used technology for comparative bacterial proteomics analysis (Gygi et al., 2000). This technique was introduced by O’Farrell in 1975. The high reproducibility of 2D PAGE is particularly valuable for multiple sample comparisons. Fig 1.4 represents the flow diagram for the proteomic analysis by 2D electrophoresis.

Fig 1.4: Schematic representations of proteomic analysis
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Fig 1.4: Schematic representations of proteomic analysis
(a) Sample preparation and protein solubilization

Appropriate sample preparation is essential for good 2-D results. Pre-treatment of samples involved complete solubilization, disaggregation, denaturation, and reduction to completely break up the interactions between the proteins. The ideal sample solubilization procedure for 2D-PAGE results in the disruption of all non-covalently bound protein complexes and aggregates into a solution of individual polypeptides. Lysis of sample from different types of cells and tissues may be achieved by homogenization, liquid nitrogen-cooled mortar and pestle technique, sonication, enzymatic lysis, detergents (e.g. NP-40, Triton X-100, CHAPS, CHAPSO, SDS), osmotic shock, repeated freezing and thawing with the addition of protease inhibitors. After cell lysis, it is necessary in most cases to inactivate interfering substances (nucleic acids and proteases) and to remove insoluble components by high speed centrifugation as well as 10% TCA.

(b) First dimension- Isoelectric focusing (IEF)

IEF is an electrophoretic method that separates proteins according to their isoelectric points (pI). Proteins are amphoteric molecules; they carry either positive, negative, or zero net charge, depending on the pH of their surroundings. The pI is the specific pH at which the net charge of the protein is zero. The charge separation based exclusively on the use of carrier ampholytes for many years. But equilibrium could not be achieved because of pH gradients instability with prolonged focusing time and have a tendency to drift, usually toward the cathode, over time. In addition, reproducibility of pH gradient profiles is limited by the batch to batch variability of carrier ampholyte preparations. Finally, the problems of pH gradients instability and irreproducibility were overcome by the introduction of immobilized pH gradients (IPG) of IEF. IPG strips allowed the generation of pH gradients of any desired ranges (broad, narrow or ultra narrow) between pH 3 to 12. Also it has a higher protein loading capacity.

(c) Second dimension- SDS-PAGE

SDS-PAGE is an electrophoretic method for separating polypeptides according to their molecular weights (Mr). SDS-PAGE consists of four steps: (1) Preparing the
second-dimension gel, (2) equilibrating the IPG strip(s) in equilibration buffer, (3)
placing the equilibrated IPG strip on the SDS gel, and (4) electrophoresis.

(d) Visualization

Important part of the proteomics is the ability to analyze and identify proteins from the gels at high throughput and sensitivity. Proteins can be visualized using a number of staining methods; however some of these are compatible with protein digestion and mass spectrometric analysis. Most of them involve binding of the dye of the stain ions to the protein. With the great variation in detection efficiency, accuracy to quantify the protein amount, compatibility with the mass spectrometry, complexity of procedures, and even its cost, the choice of the stain depends mainly on the experimental workflow. Visualization of 2-DE is somewhat different than SDS-PAGE; proteins are resolved as a spot instead to discrete band.

Among the various protein detection methods following 2-DE, coomassie blue (CBB) staining has a limited protein detection ranged from 50-100 ng within the spot. A major advantage of CBB is the compatibility with mass spectrometry (Neuhoff et al., 1988). The dye form complexes with basic amino acids of proteins such as tyrosine, histidine, arginine and lysine. The formation of protein-dye complexes stabilizes the negatively charged anionic form of the dye producing a clear protein spot with faint background color. Another method silver staining has gained wide popularity because of its sensitivity (1-10 ng). The proteins bind silver ions, which can be reduced under appropriate conditions to build up a visible image made of finely divided silver metal. However, some disadvantages including that basic protein are less efficiently stained than acidic ones (Chevallet et al., 2006; Lelong et al., 2009). A major drawback of silver staining that it is not compatible with mass spectrometry. By omitting glutaraldehyde from the sensitizer and formaldehyde from the silver nitrate solution the method becomes compatible with mass spectrometry analysis. This is because proteins within the gel to be analyzed should remain in its unmodified status. Recently, kits for MS-compatible silver staining are also available.

Rather than staining the proteins, the proteins can be labeled prior to the running of the sample so as to visualize it on the gel. 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 residue and are just equivalent in sensitivity to silver stains (Honoré et al., 2004).

(e) Image analysis

There are many sophisticated software systems for evaluation of 2D gel images. PDQuest version 8.0.1 (Bio-Rad laboratories), 6.0, Proteomweaver (Bio-Rad laboratories), Melanie (Geneva Bioinformatics), Image Master (Amershan-Pharmacia Biotech), Phoretix 2D (Phoretix International), ImageMaster 2D Platinum, Decyder 2D (GE Healthcare) are the main software which are used in various laboratories.

(f) Mass spectrometry

Mass spectrometry is an analytical tool used for measuring the molecular mass of a sample. Biomolecules are large and polar and thus their transfer into the gaseous phase was challenge. Tanaka (1987) showed that a low-energy nitrogen laser could be used to generate gaseous macromolecules, a technique that was further improved in matrix-assisted laser-desorption ionization (MALDI) by Karas and Hillenkamp (Karas and Hillenkamp, 1988). Mass spectrometry plays a significant role in biological sciences and is applied in a wide range of applications. Witzmann et al. (1999) was the first to report the use of mass spectrometry for protein identification in environmental proteomics. The ESI (electrospray ionization) and MALDI (matrix-assisted laser-desorption ionization) were the ionization techniques that led to the success of mass spectrometry in life sciences. In proteomic research, four types of mass analysers are mainly used as given in fig 1.3 (Aebersold and Mann, 2003).

MALDI MS

In MALDI the sample is co-crystallized with a molar excess of UV-absorbing matrix. Ion formation is accomplished by directing a pulsed laser beam at sample matrix crystals in a high vacuum. The energy of the laser excites the matrix, causing a proton to be donated to the sample molecules, creating charged ions. The matrix is a solid material that absorbs the laser radiation, resulting in the vaporization of the matrix and sample
embedded in it. Matrixes consist of fairly low molecular weight organic acids to allow facile vaporization, but large enough not to evaporate during sample preparation [e.g. α-Cyano-4-hydroxycinnamic acid (CHCA), 3, 5-dimethoxy-4-hydroxycinnamic acid (Sinapinic acid), 2, 5-dihydroxybenzoic acid (DHB)]. The matrix also serves to minimize sample damage from laser radiation by absorbing most of the incident energy. Once a charged ion is formed, a high voltage is used to eject the analyte from the ion source to the detector. Because MS is a highly sensitive technique, special care must be taken to prevent sample contamination. MALDI is most often coupled to a TOF mass analyzer, in which the 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. The obtained m/z values of (single 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).

(g) Protein identification

The development of algorithms and other bioinformatics tools for protein identification has been a great advance in biological MS (Mann et al., 2001). Proteins can be identified by MS using different techniques. The first, peptide mass fingerprinting (PMF), has been the most common and straightforward way to identify proteins in proteomic experiments. The second, peptide fragmentation analysis, utilizes fragment ion data (partial amino acid sequence) from a peptide together with its molecular mass. PMF is most often performed at the MS level with MALDI-TOF instruments and peptide fragment ion data are derived with tandem mass spectrometry (MS/MS) with MALDI-TOF/TOF or ESI MS/MS.

(i) Peptide mass fingerprinting (PMF)

In PMF, the protein is first digested with an endoprotease and the molecular masses of these peptides are then measured. The obtained set of peptide masses is unique for each protein. The acquired MS spectra are compared using database search algorithms with theoretical peptide masses calculated from each sequence entry in the database (Pappin et al., 1993; Henzel et al., 1993; James et al., 1993). The requirement
for a successful identification is that the protein or its very close homology is represented in a sequence database. The accuracy of measured peptide masses has the largest effect on the reliability of results by reducing the number of false positives (Clauser et al., 1999), so the mass accuracy and resolution of the used instrument are of great importance. A variety of scoring algorithms are available, some of which use a simple score based on the number of common masses between the experimental and theoretical spectra. More sophisticated scoring algorithms take into account the non uniform distribution of protein and peptide masses in the database.

(ii) MS/MS analysis

For MS/MS analysis the proteins are digested in the same manner as for peptide mass fingerprinting (PMF) and the sample is then analyzed by MALDI-TOF, generating a peptide mass fingerprint for the protein. The most abundant peptide ions are then subjected to MALDI-TOF/TOF analysis, providing information that can be used to determine the sequence. The results from both types of analysis are combined and searched using software (e.g. Mascot) to identify the protein.

1.9 Heavy metal resistant bacteria and their response to heavy metals – proteomic approach

An important aspect of proteomics is to study the expression levels and characterization of proteins in cells when exposed to different environmental conditions. In post genomic investigations, proteomics is one of the best strategies used to reveal the dynamic expressions of whole proteins in cells and their interactions and may contribute to the identification of new protein markers for metal toxicity (Nesatyy and Suter, 2008).

Exposure of microorganism to diverse heavy metals has resulted in differential expression of proteins that has been documented in numerous findings and proteomics has been used to unravel the mechanism of resistance which is given in Table 1.2.
### Table 1.2 List of differentially expressed proteins under heavy metal stress

<table>
<thead>
<tr>
<th>Name of the organism</th>
<th>Heavy metal</th>
<th>Altered proteins/ class of Proteins</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>Zn</td>
<td>DppA ABC periplasmic dipetide binding protein</td>
<td>(Easton et al., 2006)</td>
</tr>
<tr>
<td>Enterobacter liquefaciens</td>
<td>Co(II)</td>
<td>Mn-SOD</td>
<td>(Marrero et al., 2004)</td>
</tr>
<tr>
<td>Rhodobacter sphaeroides strain R26.1</td>
<td>Co(II)</td>
<td>Porphobilinogen deaminase, Aconitate hydratase</td>
<td>(Italiano et al., 2008)</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>Co(II) Pb(II)</td>
<td>L- isoaspartate protein carboxymethyltransferase type II and DNA gyrase A</td>
<td>(Bar et al., 2007)</td>
</tr>
<tr>
<td>Pseudomonas fluorescens</td>
<td>Pb(II) Cu(II) Co(II)</td>
<td>translational elongation factor EF Tu, enolase, OMPH1.</td>
<td>(Sharma et al., 2006)</td>
</tr>
<tr>
<td>Phanerochaete chrysoporium</td>
<td>Pb(II)</td>
<td>Antioxidant enzymes, transcription, DNA repair</td>
<td>(Volkan et al., 2011)</td>
</tr>
<tr>
<td>Arthrobacter sp. FB24</td>
<td>Cr(VI)</td>
<td>Amino acid and carbohydrate metabolism, Transaldolase and transketolase</td>
<td>(Henne et al., 2009)</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>Cr(VI)</td>
<td>Energy metabolism, stress proteins and protein biosynthesis</td>
<td>(Kılıç et al., 2010)</td>
</tr>
<tr>
<td>Pseudomonas putida UW4</td>
<td>Ni(II)</td>
<td>Stress responsive, anti oxidative proteins, heavy metal efflux proteins</td>
<td>(Cheng et al., 2009)</td>
</tr>
<tr>
<td>Halomonas sp.</td>
<td>Se, Ti</td>
<td>fatty acid synthesis, cell transport, antioxidant enzymes, DNA replication, transcription and translation</td>
<td>(Kabiri et al., 2009)</td>
</tr>
<tr>
<td>Corynebacterium glutamicum</td>
<td>Hg(II), Cd(II)</td>
<td>Zn-dependent alcohol dehydrogenase, Antioxidant enzymes, ABC-type dipeptide transport system, periplasmic component</td>
<td>(Fanous et al., 2008)</td>
</tr>
</tbody>
</table>
There are few reports of proteomic approaches being undertaken to study arsenate and arsenite stress on microorganisms. Weiss et al. (2009) and Parvatiyar et al. (2005) have used this approach to examine arsenic stress in *Heminiimonas arsenicoxydans* and *Pseudomonas aeruginosa*, respectively and have reported the role of antioxidant enzymes, superoxide dismutase (SOD) and glutathione reductase, in the mechanism of As(III) resistance. In the acidophilic *Ferroplasma acidarmanus* strain Fer1, synthesis of heat-shock proteins HSP60 and HSP70, which are involved in protein refolding, was enhanced when cells were exposed to As(III) (Baker-Austin et al., 2007). In *Comamonas* sp. strain CNB-1, synthesis of proteins associated with arsenate resistance and detoxification (GST), phosphate transport (Pst), heat-shock response (DnaJ/ DnaK/ HSP20), and energy generation and carbon metabolism (malate synthase and enolase) were enhanced when cells exposed to arsenate As(V) (Zhang et al., 2007). The DnaK/DnaJ chaperone system is essential for the recovery of stress-induced protein aggregates (Kedzierska, 2004); thus it was deduced that exposure to arsenate would cause significant protein misfolding in cells. The increased DnaK/DnaJ abundance would stimulate the recovery of the misfolded protein due to the arsenate/arsenite binding. In *Caenibacter arsenoxydans* numerous genes and proteins belonging to various functional classes including information and regulation pathways, intermediary metabolism, cell envelope and cellular processes were differentially expressed under As(III) stress, which demonstrates that bacterial response to As(III) is pleiotropic (Carapito et al., 2006). In the *Chromobacterium violaceum*, antioxidant enzymes (SOD, GST, Grx), DNA repair and the metabolism of lipids, amino acids and coenzymes were altered when cells were exposed to As(III) (Ciprandi et al., 2012). Also in our paper (Daware et al., 2012) we reported that in *Klebsiella pneumoniae* expression of stress responsive proteins and antioxidant enzymes were up-regulated and membrane transport/binding proteins, porins, and amino acid metabolism enzymes down-regulated under As(III) stress. Recently, in the *Exiguobacterium* sp. PS altered expression of stress responsive proteins and the metabolism of purine, carbohydrate and nitrogen when cells were exposed to As(V) (Sacheti et al., 2013). Also, Belfiore et al. (2013) reported that under arsenic stress enhanced the expression of proteins entailed in energy metabolism, stress, protein synthesis and transport in *Exiguobacterium* sp. S17. Identification and characterization of
Chapter 1

altered proteins under different heavy metal stress via proteomics reveals the physiological responses elicited by microbes during adaptation to heavy metal stress.

1.10 Present study

Microorganisms contain thousands of proteins and some of them must be involved in the regulation steps responsible for their survival under stress. Many aspects of metal-microbe interactions remain unexplained and unexploited. By evaluating the global pattern of gene expression in the microbe, under the influence of exogenous metals and the presence of an antibiotic compound, new insight into the understanding of cellular alterations under different perturbations can be obtained. The exact mechanism of tolerance, the role of various genes and proteins are not fully understood.

To understand the function of genes in a microorganism, it is not only required to know which genes are present, but also, which proteins are synthesized and under which conditions they accumulate in cells. There are a large number of specific proteins reported in various genera of bacteria which has shown an increase in their level of expression, upon adverse conditions, such as heat, toxic elements and nutrient limitations.

Great deal of efforts have been spent and being spent on investigating different mechanisms by which cell reacts when exposed to exogenous metals. One of the approaches is the use of recently developed technology of DNA micro array as a high throughput method for global analysis of gene expression to explore the cellular response to heavy metal toxicity. But it does not correlate well with relative protein abundance in the cell, as post translational modifications of proteins are not taken into consideration here. In post genomic investigations, proteomics is one of the best strategies used to reveal the dynamic expressions of whole proteins in cells and their interactions. A detailed protein profile of cells subjected to different types of exogenous stimuli, followed by high throughput functional analysis of proteins, can provide a direct link of the cell and its intracellular response with different components involved in cellular defense mechanisms and how they respond to different environments (i.e. heavy metal stress). Most investigations dealing with the interactions between heavy metal and bacteria have focused exclusively on the mechanisms of resistance that include the
transformation of metal by methylation, reduction or oxidation. In contrast, the other cellular functions involved in the adaptation of these microorganisms to toxic concentrations of heavy metal remain to date largely unknown. The knowledge of regulatory cellular pathways affected by heavy metal will provide a better understanding of the mechanisms implicated in the colonization of toxic environments supported by heavy metal cycling. Also this knowledge is important because it enables to understand the physiological responses elicited by microbes during adaptation to heavy metal stress.

This study, therefore, aims at the isolation and characterization of heavy metal resistant bacteria from polluted environment and understanding the molecular mechanism of arsenic tolerance in bacteria by proteomic approach.

The major objectives of thesis

1) To isolate and characterize bacteria with potential heavy metal tolerance.
2) To understand the heavy metal tolerance mechanism.
3) To find out differentially expressed proteins under heavy metal stress and their identification by proteomic approach.
4) To evaluate the role of differentially expressed proteins under heavy metal stress.
5) To establish the genetic basis of arsenic resistance in isolated bacterial strain.