Ecology and molecular analyses of marine bacteria resistant to arsenic

A Thesis submitted to Goa University for the Award of the Degree of

DOCTOR OF PHILOSOPHY

in

Marine Sciences

BY

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Research Guide

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Goa University,
Taleigao Goa
2014

CERTIFICATE

This is to certify that **Miss Geeta Suresh Nagvenkar** has duly completed the thesis entitled **"Ecology and molecular analyses of marine bacteria resistant to arsenic"** under my supervision for the award of the degree of Doctor of Philosophy.

This thesis being submitted to the Goa University, Taleigao Plateau, Goa for the award of the degree of Doctor of philosophy in Marine Sciences is based on original studies carried out by her.

The thesis or any part thereof has not been previously submitted for any other degree or diploma in any University or Institutions.

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DECLARATION

As required under the University Ordinance 0.19.8(vi), I hereby declare that the present thesis entitled **"Ecology and molecular analyses of marine bacteria resistant to arsenic"** is my original work carried out in the CSIR-National Institute of Oceanography, Dona-Paula, Goa and the same has not been submitted in part or in full elsewhere for any other degree or diploma.

The literature related to the problems analyzed and investigated has been appropriately cited. Due acknowledgements has been made wherever facilities and suggestions has been availed of.

Geeta Suresh Nagvenkar

STATEMENT

I hereby state that all necessary corrections/modifications as advised by the examiner for my Ph.D thesis entitled **"Ecology and molecular analyses of marine bacteria resistant to arsenic"** are incorporated.

Geeta Suresh Nagvenkar

Acknowledgment

At the end of my thesis it is a pleasant task to express my thanks to all those who contributed in many ways to the success of my study and made it an unforgettable experience for me.

First and foremost, Praises and Thanks to the Almighty God, for his showers of blessings throughout my research work.

I am extremely indebted to my guide Dr. N. Ramaiah, Scientist, National Institute of Oceanography, Goa for his continuous guidance and patience. I attribute the level of my thesis to his encouragement and support without which it would have remained as a dream. I am thankful for his invariably constructive criticism and advice during the completion of my thesis. His dynamism, vision, sincerity and motivation have deeply inspired me in completing my thesis.

I thank Dr. Satish Shetye, the former Director and Dr. S.W.A. Naqvi, Director, CSIR-National Institute of Oceanography, for giving me an opportunity to be associated with this institute.

I greatly acknowledge the Council of Scientific and Industrial Research, New Delhi, for awarding me the Senior Research Fellowship (SRF) that enabled me to complete my thesis work.

For this thesis I would like to thank my FRC committee members: Dr. Savita Kerkar, Dept. of Biotechnology, Goa University for her kind consideration to be my co-guide and Dr. Rakhee Khandeparkar my VC's nominee, for her valuable comments, support and encouragement.

My special thanks to Dr Samir Damare for his patience, time to time help and, personal attention in protein analysis which have provided me good and smooth basis for completion of my research work. I am sincerely thankful to Dr. A.C. Anil and Dr. Damodar B. Shenoy for their contribution.

I thank Professor G.N. Nayak, Head of the Department of Marine sciences, Goa University, for his constant help and support.

A special thank you to Dr. Sanjay Singh and Dr. Sagar Nayak for contributing immensely towards the completion of my thesis. I acknowledge the support provided by my friends and colleagues Dr. Veronica, Dr. Sultan, Veera, Seema, Ravidas, Mandar, Kaushal, Akhila, Priya, Elroy, Pankaj, Ujwala, Cindrella, Shahin, Prachi, Jasmine, Stecy, Nadine and Dhiraj. Thanks to Mr. Ram Murti Meena for helping me with the sequencing of samples.

I thank my friends Mr. Srinet Kothwale and Renita for cheering my mood and being with me in good and bad times and encouraging me to strive towards my goal.

This thesis is dedicated to my late grandparents Mr. Vinayak and Mrs. Premavati Nagvenkar. I owe my deepest gratitude to my parents Mrs. Sushma S. Nagvenkar and Dr. Suresh V. Nagvenkar for their love, care, prayers and sacrifices done throughout for educating me and preparing me for the future. I would also like to share the credit of my work with my siblings Dr. Dattaprasad, Mrs. Maya and Dr. Smita, and all family members for their love and encouragement. They supported me with immense patience during my research. Lastly, I thank my pets to cheer me up.

Besides these, there are several people who have helped me in the successful completion of my thesis whose name I could not include here for want of space.

Geeta S. Nagvenkar

Dedicated To My Beloved Family

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1.1 Introduction

Microbes modify many metals and, metalloids. At their ionic and/or molecular state/s that is. Their versatility in occupying almost all conceivable habitats and transforming infinite varieties of molecules are vital for Earth's ecological functioning. Infact, the environmental stability and functions are governed by microbes. This happens through their metabolic pathways. Ecosystems are adversely affected due to imbalance caused either naturally or via human activities.

Pollution leads to health effects, causes diseases, affects societal harmony, brings about economic losses, resulting in global changes and shifts in species diversity in all ecosystems subjected to its deleterious facets. In the natural environment, microbes interact with metals and metalloids in all their states. It is thus not surprising that they should "modify" them, often advantageously. Metal ions are changed by both prokaryotes and eukaryotes for various cellular functions. Only Eubacteria and Archaea possess representatives which can oxidize metallic ions such as Fe(II), Mn(II), Co(II), Cu(I), AsO_2 or reduce Fe(III), Mn(IV), Co(III), AsO_4 (Ehrlich, 1996). Further, these ions are reduced by some microbes respectively as Hg^{2+} or Ag^{+} to Hg^{0} and Ag^{0} without making use of the energy arising out of such processes (Summers and Sugarman, 1974). Interestingly, many microorganisms have a potential for resistance and/or tolerance.

Main focus here is on bacterial resistance/tolerance of bacteria to arsenic. Organic and metal pollution are of greater relevance, their detoxification is of practical significance. Among these, arsenic: As, a toxic metalloid, is a well known poison with atomic number 33 and molecular weight 74.9 that is ubiquitous in numerous environmental contexts throughout the globe. Albertus Magnus was the first to discover arsenic in 1250 (Rosen, 1999). It has the oxidation states of +5, +3, 0 and -3 resulting in a broad variety of arsenic compounds with diverse physical and chemical properties. Most common soluble forms being pentavalent [+5] As(V) and trivalent [+3] As(III) (Joshi et al., 2009). In the periodic table, As is placed under Group VA (metalloids). Metalloids are elements forming alloys with other metals. It also forms covalent bonds with C, H, O and S.Its mean abundance in the earth crust is 1.8 ppm, in soil it is 5.5 to 13 ppm, in streams it is less than 2 ppb (Mandal and Suzuki 2002) and in ground water it is generally less than 100 ppb.

In 2005, the Agency for Toxic Substances and Disease Registry (ATSDR)categorizedAs and its compounds as deadly poisons. Among the top 20 hazardous substances,As is ranked first by ATSDR and the USEPA. Nordstrom (2002) came up with 0.2 to 40 mg kg^{-1}As in soils as its typical range with a worldwide median concentration of 6 mg kg^{-1}. Also the more toxic, trivalent arsenite [+3] in its inorganic form (Hopenhayn,2006) is amenable for greater cellular uptakes (Valenzuela et al., 2009) than the less toxic and less mobile pentavalent arsenate [+5].

Global cycles of most elements have undergone significant modifications due to anthropogenic processes. This is no exception with non-essential/toxic elements (Goering et al., 1999). Drinking water As permissible limit is recently revised from 50 ppb to 10 ppb by the World Health Organization.

Mandal and Suzuki (2002) reported As to be much higher in concentration in drinking water in several parts of the world including India. In India, some portions of Jharkhand, Uttar Pradesh, Bihar, West Bengal, Assam, Manipur and Chhattisgarh have so far been found to be contaminated with arsenic at concentrations much above the permissible limit. The microbial transformation of As and associated genetic regulatory pathways have been studied intensively by Oremland and Stolz (2005) during the past decade, in part due to water quality crises in Bangladesh, India and southeast Asia.

Microorganisms posses numerous regulatory genes responsible for different alterations of As, such as methylation, reduction and oxidation. All these processes contribute to the fate, transport and biogeochemical cycling of As. The microbial transformation of arsenate and extrusion of arsenite via the *ars* operon (*ars*Cand *ars*B respectively) has been well studied and these genes appear widely distributed throughout the Bacterialand Archaeal domains.

Oxidation of As(III) is commonly observed in soil, sediment and natural waters, and several arsenite oxidases have been characterized. The trivalent arsenite is converted to 100 times less toxicpentavalent arsenate by the process of oxidation. Both heterotrophs andchemoautotrophs mediate As oxidation.These arsenite oxidizing bacteria possess *aox* gene that codes for arsenite oxidase enzyme responsible for arsenite oxidation (Branco et al.,2009; Cai et al.,2009). Thus oxidation of As[+3] to

arsenate is considered as a primary strategy of detoxification. This oxidation process can also be achieved by chemical methods but such methods are costly and result in secondary pollution. Hence, alternatively biological or bioremediation methods of arsenite remediation are preferred. Thus, realizing the importance of bioremediation of arsenite by biological oxidation I undertook the exploration and characterization of arsenite resistant bacteria. As Inskeep et al. (2005) suggest, distribution of *aox*-like genes in natural systems enables microbial oxidation of As(III) which is a critical component of the global As cycle.

My research has a focus on isolation of arsenite resistant bacteria from water and sediment samples followed by the identification of the bacteria. Further, the arsenite biotransformation potential in strains highly resistant (1000 ppm) to As is explored. The overarching aim here was to document ecological distribution of arsenic resistant bacteria (ARB) which is governed mostly by the degree of pollution due to arsenic. The main purpose of this study was to provide a current state of knowledge of metal-microbe interactions and to suggest scientific and technological approaches to address some gaps.

1.2 Objectives

- **Ecology and quantitative analyses of arsenic resistant bacteria (ARB) in the coastal waters**

  The rationale behind this objective was to study the prevalence and abundance of populations of bacteria tolerating arsenic which is of relevance in microbial ecology to understand the extent of its pollution and to realize the potential of such flora in detoxifying arsenic. Nature of the microbe-metal interactions is vital for metal toxicity alleviation.Investigation on the role of bacteria in the modification and sequestration of metals in natural environments is variously relevant. Study on the abundance of arsenic tolerant bacteria (ATB, capable of growth in media with less than 100 ppm As) and ARB (capable of growth in media with ≥100 ppm As) will prove useful in detecting metal pollution in the coastal regions. Keeping in view of lack of studies on ATB and ARB from the coastal regions, this major objective was pursued.

- **Taxonomic and molecular characteristics of ARB**

  Many strains of native bacteria can sense arsenic, and detoxify it efficiently. Detailed study on the estuarine and marine bacterial strains was undertaken for this purpose. Screening for Astolerance, biochemical identification, physiological, biochemical and molecular biological characterization and phylogenetic analyses of the bacterial isolates highly resistant to arsenic isolated from different locations of River Mandovi (midstream, estuarine and marine zones) were carried out. Further, uptake and biotransformation of arsenite by a few strains were also investigated to understand certain physiological and biochemical aspects of arsenic resistant bacteria from the study region.

- **Profiling of proteins and characterization of genes responsible for As detoxification**

  Many bacteria have an inherent capability to detoxify arsenic and the genes responsible for detoxification are thought as inducible both by arsenate and arsenite. Since bioremediation steps benefit greatly by knowing the presence of inducible promoters of the *ars* operons, a detailed analyses on this is relevant.

- **Cloning and sequencing of genes involved in arsenic transformation/ detoxification pathways**

  By cloning of, and/or screening for, genes responsible for arsenic resistance and, learning more about specific genes and proteins can help in understanding arsenite conversion to arsenate. Biochemical and molecular studies will help advance our information base. This will result in elucidation of tolerance mechanisms, structure, regulation and expressions of genes.

2.1 Preamble

In view of the environmental and human health adversities due to heavy metal pollution, there have been a large number of investigations on the role of microbes to solve/reduce the problem. This is particularly to recognize the fraction of native microflora capable of not only tolerating heavy metal toxicity but also to use their physiological pathways to investigate problems due to heavy metal contamination. Many metals are modulated by microbes using physiological processes microbial communities, or a versatile population within them, transform soluble and insoluble states. As Filali et al. (2000) point out some bacteria for instance many strains of *Pseudomonas* spp even use some heavy metal ions for energy and growth.

Under natural conditions, arsenic is cycled at the earth surface where the breakdown of rocks has converted arsenic sulfides into arsenic trioxide (Mandal and Suzuki, 2002; Oremland and Stolz, 2005). Furthermore, as Sanders (1980) and Tareq et al. (2003) report, arsenic is known to have multiple oxidation states where they are present in either organic or inorganic compounds in an aquatic environment. Both Zobrist et al. (1998) and Root et al. (2009) indicated that the mobility of arsenic inorganic compounds in contaminated aquatic and sediment environment is controlled by redox processes, precipitation, sorption, and dissolution processes.

2.1.1 Arsenic and its Uses

Although arsenic has been used as a poison, it has many chemical uses and is quite an important element. The first usage of arsenic in medicine could be dated around 2500 years ago where it was mainly consumed for the improvement of breathing problems as well as to give freshness, beauty, and plumpness figures in women (Mandal and Suzuki, 2002). Arsenic in the form of arsenical salvarsan (an As containing drug) was the initial antimicrobial agent used in the treatment of infectious diseases such as syphilis and sleeping sickness in 1908 (Rosen, 1999). Arsenic in the form of arsenic trioxide (As_2O_3) is one of the most common forms of arsenic, which is often used in manufacturing and agriculture industry and for medical purposes (Ratnaike, 2003).

Arsenic trioxide is also proven to be useful in criminal homicides due to its characteristic, which is tasteless, colorless, highly toxic, and soluble in water (Mandal and Suzuki, 2002). The high usage of arsenic trioxide in suicide cases had made it to

be often referred as the "inheritance powder" in the 18th century (Oremland and Stolz, 2005). During the 1970s, arsenic was mainly used in agriculture industry as a component insecticide in order to get rid of the insects (Cervantes et al., 1994; Mandal and Suzuki, 2002; Spiegelstein et al., 2005). As was also used as cotton desiccant and wood preservative in the United States (Mandal and Suzuki, 2002). Besides that, it is also being used in ceramic and glass industry, pharmaceutical industry, as food additive and, pigmenting agent in paints (Cervantes et al., 1994; Ratnaike, 2003). Arsenic in the form of 4-aminoben-zenearsenic acid (p-arsenilic acid, p-ASA) has been used as animal food additive for feeding of boiler chickens (Jackson and Bertsch, 2001).

2.1.2 Arsenic in the Environment

Affecting countless people worldwide and a widely distributed toxic metalloid, the As, is high in waste dump-yards (Pontius et al., 1994). It causes cancer and other chronic and acute problems, among an array of toxic effects.Among flora and fauna it complexes with C and H. Arsenic usually exists in four oxidation states: As^{-3}(arsine), As° (arsenic), As^{+3}(arsenite), and As^{+5}(arsenate) (Oremland and Stolz, 2005; Afkar, 2012). In water, two primary forms of arsenic are arsenite (As^{+3}) and arsenate (As^{+5}). Arsenite is ~100 times more toxic than arsenate (Neff, 1997; Mukhopadhyay et al., 2002; Muller et al., 2003) and is shown to inhibit various dehydrogenases (Ehrlich, 1996). With its ability to bind to sulfur bearing peptides/proteins and dithiols (eg. glutaredoxin) the AsO_2^- causes severe damages. As an identical analogue of PO_4, the AsO_4^{3-} can alter/cease phosphorylation. Unlike arsenate and arsenite, arsine is often available as highly toxic gases such as $(CH_3)_3$and H_3As (Oremland and Stolz, 2005) and often present in low concentrations in the environment.

Smelting of non-ferrous ores and river drainages are substantial local input sources for arsenic into marine environment. Mining leachates, increased land erosion and domestic sources (Cullen and Reimer, 1989) are other sources. Volcanoes and bed-rock leaching are the natural sources (Huysmans and Frankenberger, 1990; Rosen, 1999; Nair et al., 2003). Use of pesticides, burning of coal, gasoline, oil and wood (Hingston et al., 2002; Weis and Weis, 2002) also add As to aquatic systems.

Drainages of As containing pesticides, weedicides and other agro-chemicals (Diorio et al., 1995) heavily pollute the aquatic bodies. The As concentrations in freshwaters greatly differ but mostly under 10 µg L^{-1} (Quentin and Winkler 1974). The anthropogenic releases far exceed releases happenning naturally (Ferguson and Gavis, 1972).

2.1.3 Arsenic Biogeochemical Cycle

After the introduction of arsenic into the environment through natural or anthropogenic sources, it enters the biosphere (Figure 2.1). Its biogeochemical cycle impacts "mobility and the distribution of arsenic species"(Tamaki and Frankenberger, 1992; Quinn and McMullan, 1995). Vital role microorganisms play in As cycle is summarized byOremland and Stolz (2003) and Akai et al. (2004). They suggest accumualtion of arsenic in the subsurface waters due to microbial transformations. Masscheleyn et al. (1991), McLean et al. (2000) and Duquesne et al. (2008) reported arsenate to be the major species in well oxidized waters, and arsenite in reduced systems due to latter's slower redox alterations.

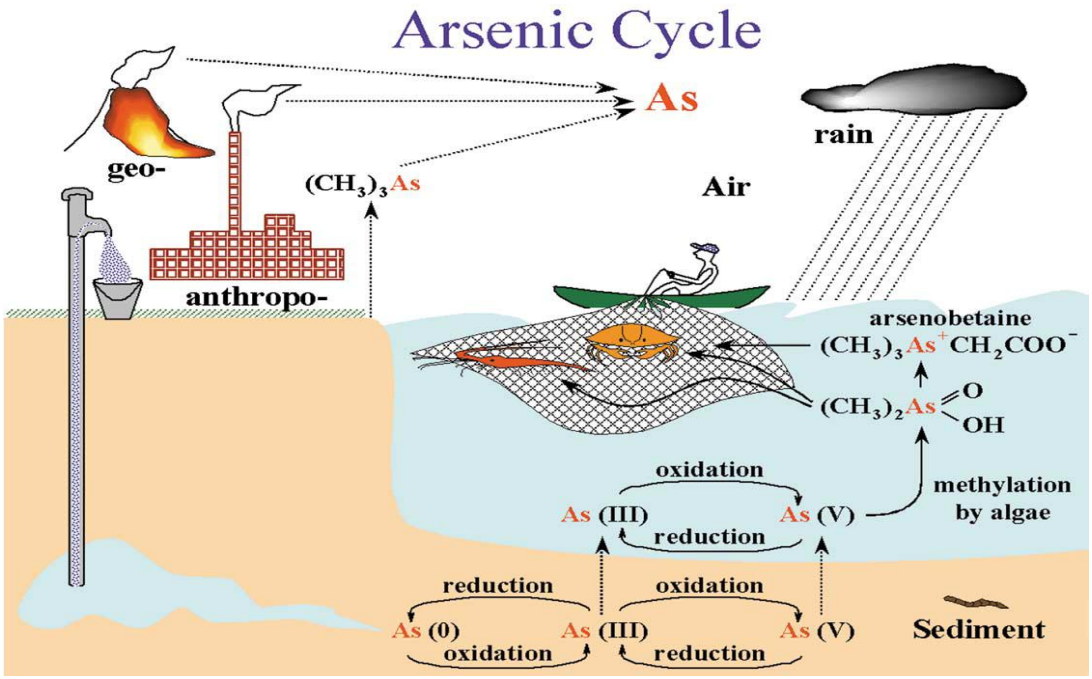

Figure 2.1 The global arsenic cycle (from Mukhopadhyay et al., 2002)

Among bacteria the ARB are reported to control cycling and speciation of As (Mandal et al., 1996; Smith et al., 1998; Fukushi et al., 2003; Oremland et al., 2004).

The versatility of *ars* mechanisms are reported to "affect the transformation between soluble and insoluble arsenic forms and toxic and non-toxic arsenic forms" (Turpeinen et al., 2002; Malasarn et al., 2004; Jackson et al., 2005; Zouboulis and Katsoyiannis, 2005). Some bacterial species resistant to a variety of antibiotics are known to possess toxic metal resistance (eg.*Staphylococcus* sp; Ug and Ceylan, 2003,*Escherichia coli*;Alam and Imran, 2014, *Salmonella typhimurium*;Garhwal et al., 2014 and, *Pseudomonas aeruginosa*;Matyar et al., 2010).

2.1.4 Toxicity of Arsenic

In Bangladesh, over 40 million consume As-laced groundwater (Smedley and Kinniburgh, 2002). Its presence and higher concentrations are a great concenrn elsewhere in the globe as well. Many studies on a variety of life-forms noted far higher tolerance than humans to As (Goering et al., 1999; Patel et al., 2007). Therefore, the USEPA in 2001 reduced the safe level of arsenic from 50 ppb to 10 ppb in drinking water.

The organic forms of arsenosugars, arsenobetaine, etc are less toxic, small and natural accumulations in food. Its presence in soil and water has become an increasing problem in many countries around the world, including Bangladesh, India, Chile and Taiwan (Lodh et al., 1996; Tseng, 2005; Hadi and Parveen, 2004), and natural geological source is one of the main causes of contamination (Ratnaike, 2003). Consumption of drinking water that has been contaminated by hazardous level of As could cause diseases such as arsenic dermatosis, lung cancer, liver cancer, uterus cancer, skin cancer and occurrence of skin and bladder and, hepatocellular carcinoma that will result in slow and painful death (Hadi and Parveen, 2004; Lu et al., 2004; Duarte et al., 2009).

As an element, As possesses some chemical similarities to phosphorous and has received attention because of its possible role in metabolic processes as a phosphate analogue (Tawfik and Viola, 2001; Knodle et al., 2012). Although known to be noxious to many higher life-forms,studies have shown that microorganisms use its ions as donars/acceptors of electrons (Ji and Silver, 1992a; Ahman et al., 1994; Cervantes et al., 1994; Macy et al., 1996; Newman et al., 1997). Some bacteria, as Oremland and Stolz (2003) note, modify its states during their physiological

processes. These processes bring about ionic modifications of As and aid in its transformation.

2.1.5 As Toxicity and Adaptive Responses of Native Microflora

Recently, there have been increasing concerns on arsenic toxicity issues. Arsenic contamination is reported from worldwide (Smedley and Kinniburgh, 2002; Mukherjee et al., 2006; Halem et al., 2009; Singh and Kumar, 2012). Consequent to human additions of over tens to thousands of times IMAC permitted limits, new amendments are available from some areas for water, soil, air, some life forms etc (Mukherjee et al.,2006; Armienta and Segovia, 2008; Sambu and Wilson, 2008). Since As is toxic in its inorganic forms its presence itself is an indication of impending harm. Arsenic poisoning of aquifers and possible health threats are reported from the Americas (Smedley and Kinniburgh, 2002), Asia (Chakraborti et al., 2002 and 2010) and also in central Europe (Lindberg et al., 2006) among human populations.

Arsenite is a toxic metalloid and its detoxification mechanism by most microbes frequently involves its oxidation to less toxic forms. The first arsenite oxidizing bacterium *Bacillus arsenoxydans* was isolated from South Africa by Green (1918). Since then numerous bacteria capable of arsenite oxidation are isolated and studied (Ilyaletdinov and Abdrashitova, 1981; Santini et al.,2000; Mokashi and Paknikar, 2002; Kashyap et al., 2006; Chang et al.,2010; Rehman et al., 2010). These encompass *Pseudomonas arsenitoxidans, Alcaligenes faecalis, Microbacterium lacticum, Agrobacterium tumefaciens* 5A, *Microbacterium oxydans* (Aksornchu et al., 2008), *Pseudomonas strutzeri, Pantoea* sp, *Pseudomonas* sp, *Agrobacterium* sp, *Aeromonas* sp, *Enterobacter* sp and *Comamonas* sp and *Pseudomonas lubricans*.

Once bioavailable, the arsenic stress to environmental microbiota is inevitable. In order to survive the As stress, microorganisms have been shown to develop resistance mechanisms. The arsenic resistant bacteria can survive in presence of arsenic but usually their growth declines with the increase in arsenic concentration and, at a point, the growth ceases. This dose is considered as minimum inhibitory concentration (MIC). Various workers have computed (e.g., Joshi et al., 2009; Dave et

al.,2010) As MIC for the isolates they studied and the MIC is reported to range from 2-120 mM.

The ARB occurrence and presence is a consequence of As contamination. Many ARB strains in some bacterial genera are known. Some of these are: *Acidithiobacillus* (Dopson et al., 2001), *Bacillus*, *Deinococcus* (Suresh et al., 2004a and b), *Desulfitobacterium* (Niggemyer et al., 2001) and *Pseudomonas* (Prithivirajsingh et al., 2001; De Vicente et al., 1990).

The As redox reactions are affected by bacteria in numerous ways. In the reducing situations as in ground water, As(V) is converted to As(III) for detoxifying the former. Bacteria can also convert arsenite to less toxic arsenate. Phillips and Taylor (1976) isolated several strains of *Alcaligenes faecalis* from raw sewage enriched with arsenite, which oxidized the arsenite to arsenate. Oxidation of arsenite to less toxic arsenate is brought about by *Pseudomonas* sp. (Turner, 1949; 1954; Turner and Legge, 1954). Arsenite oxidation is vital too in As cycling (Oremland et al., 2004). Several isolates were reported by Oremland and Stolz (2003) as capable of accepting electrons from arsenite and some bacterial strains detoxified arsenite by oxidizing it.

Arsenic oxidation is mediated by both heterotrophic and chemoautotrophic microorganisms. Some microbes gain energy from oxidizing arsenite (Inskeep et al., 2007), although this activity could be an exception limited to chemolithotrophic bacteria. Under standard conditions, arsenite oxidation is a thermodynamically exergonic reaction and can provide sufficient energy to support microbial chemoautotrophic cell growth (Ehrlich, 1996). Heterotrophic bacteria have not been shown to derive major energy from arsenite in growth experiments. In heterotrophic bacteria, this is generally considered to be a detoxification mechanism instead of supporting the growth.

Supposedly, the oxidation of arsenite is a detoxification process in some cases. Salmassi et al. (2002) isolated a bacterium namely, *Agrobacterium albertmagni* strain AOL15 is shown to oxidize arsenite as a detoxification step. The gene, *aox* is reported to perform arsenite oxidation to arsenate (Chang et al., 2009). Oremland and Stolz

(2003) suggested that arsenite [As(III)] impairs the function of many proteins and affects respiration.

2.1.6 Genetic Components Involved in As Transformation

A number of strains of Bacteria and Archeal capable of oxidase activities are reported (Muller et al., 2003). Various arsenite oxidizing bacteria that possess *aox* genes responsible for arsenite oxidation have been reported (Anderson et al., 1992; Vanden and Santini, 2004; Branco et al.,2009; Cai et al.,2009). The *aox*B gene acts as a functional marker for aerobic arsenite oxidizers and is responsible for the oxidation reaction which results in the formation of an enzyme, arsenite oxidase, facilitating arsenite oxidation to arsenate (Quemeneur et al., 2008). The bacteria possessing *aox*B are reported by Inskeep et al. (2007) and, Garrido and Joulian (2008).

2.1.7 As Oxidation and Efflux Mechanism

Selection of diverse ARB is likely in water and soil exposed to As for a long time. Under elevated arsenite concentrations such starins armed with efflux or oxidation and/or both processes are likely to perform efficiently. They can therefore be used as potential candidates for bioremediation. Further, by characterization of arsenite detoxifying bacteria and by determining the mechanism of arsenite detoxification, suitable *in situ* methodologies may be developed for the isolated strains and there is this potential of improvement in strains by genetic engineering. Most studies of arsenic resistant bacteria have been based on culturing them from environmental locations, which contain high concentrations of arsenic. For example *Deinococcus indicus* and *Bacillus indicus* were obtained from aquifers of West Bengal, India, *Staphylococcus* and *Citricoccus* from gold mine reactor (Sato and Kobayashi, 1998; Suresh et al., 2004a and b). However, research shows that common microorganisms such as *Escherichia coli*,*Pseudomonas aerugenosa* and *Staphylococcus* sp. also exhibit arsenic resistance.

2.1.8 Bacterial Resistance to Arsenic

Rosen (2002) and, Silver and Phung (2005) have provided overviews of As toxicity resistance possibilities. The following are the reported patways to deal with As toxicity: (i) “minimizing the uptake of arsenate through the system for phosphate

uptake" (Cervantes et al., 1994) (ii) "by peroxidation reactions with membrane lipids: (Abdrashitova et al., 1986), and (iii) "using microbial arsenic detoxification pathways involving the *ars* operon" (Silver and Phung, 1996). Among these the last is the best investigated (Stolz et al., 2006). In the main components are (ArsC), (ArsB) and (ArsA). When arsenate is taken in it is reducedand expunged using ArsB and ArsA-mediated ATP hydrolysis (Figure 2.2). Also ArsB can expunge As(III) if and when it enters the cell.

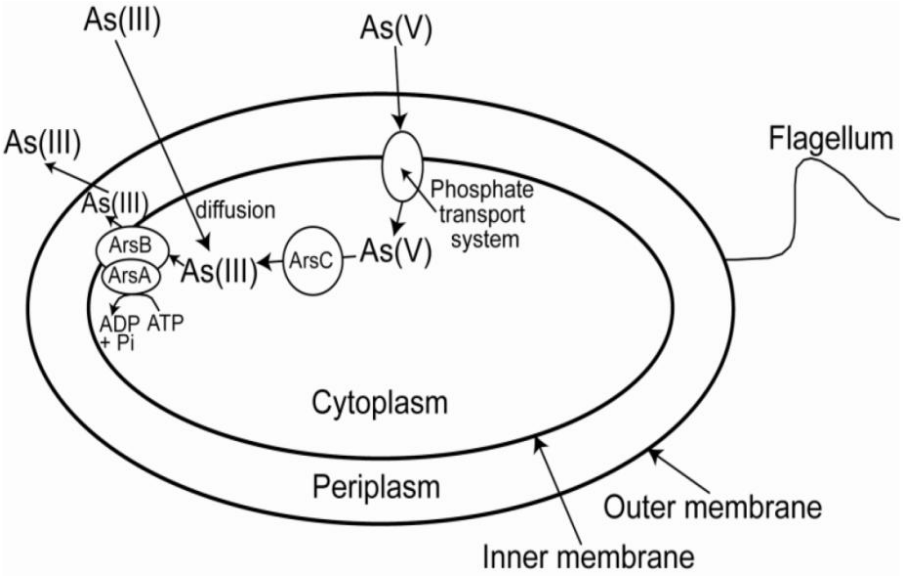

Figure 2.2 Bacterial As resistance mechanisms (after Hudson-Edwards and Santini, 2013)

The primary role detoxification process is to cope and grow in the presence of a toxic substance. Methylation is another means of As detoxification. Seveal methylation steps producing penta-, tri- and di- and mon- methyl plus gaseous arsene are involved (Stolz et al., 2006). Many strains capable of methylation processes are available (Cullen and Reimer, 1989; Dowdle et al., 1996; Newman et al., 1998; Stolz and Oremland, 1999; Salmassi et al., 2002; Silver and Phung, 2005). It is however important to note that microbial arsenite oxidation is quicker than chemical processing (Tamaki and Frankenberger, 1992). In this regard, microbes with right set of genes either chromosomal or plasmid are relevant. The mechanism of resistance made possible by efflux pumping and enzymes, as Silver (1996) proposed, are useful in detoxifying many ions of heavy metals.

Conversion of arsenite to less mobile less toxic arsenate that can be effluxed with ease is the primary survival mechanism. Many studies have recognized this fact (Ehrlich, 1996; Inskeep et al., 2002; Macy and Santini, 2002; Vanden and Santini, 2004). Genes that bring about detoxification are also studied from a variety of microbes (Silver and Phung, 1996; Weeger, 1999). Occurrence of bacteria from polluted locations capable of As tolerating are reported in several previous studies in particular by McNellis and Anderson (1998) and Kulp et al. (2004).

Arsenate resistance/tolerance in *Escherichia coli* is known to be achieved either by a chromosomal or plasmid encoded system. Silver and Nakahara (1983) suggest that the resistance mediated by chromosome activates a PO_4^{3-} uptake pump. This enables reduced uptake for arsenate by enhanced phosphate intake. Resistance enabled by plasmid-encoding lead to rapid expunging of aresenate (Mobley and Rosen, 1982). According to Bentley and Chasteen (2002), methylation of As could be other strategy for its detoxification. In methanogenic bacteria under anaerobic systems methane generating detoxification. In many fungi aerobic As detoxification process is modulated by unique methylation steps.

Encoded chromosomally or by plasmid, the *ars* operons are the mediators of As resistance.The two most common types of these operons contain either the five (*ars*RDABC) or three (*ars*RBC) gene operons (Silver and Phung, 1996)are the most common type in Bacteria. In Gram-negative bacteria such as *E. coli* R773, the former type is reported to be on the plasmids. The three gene ones can be on chromosome or plasmids. Examples are "*Staphylococcus aureus* pI258 and *Pseudomonas putida* KT2440" (Owolabi and Rosen, 1990; Diorio et al., 1995; Bruhn et al., 1996; Jimenez et al., 2002). The strain NRC-1 of *Halobacterium* sp. possesses a replicon pNRC100 which is a large extra-chromosomal unit. It contains *ars*ADRC-R2M-type.In this is a gene cluster for arsenic resistance. It is known to possess a putative arsenite methyltransferase (Wang et al., 2004). A unique circular plasmid pWCFS103 is credited to carry *ars*RDDB in *Lactobacillus plantarum* conferring As resistance (Wang et al., 2006). This has two regulatory gene copies but does not possess arsenate reductase gene *ars*C and *ars*D.

Now known for long, the plasmid-associated As efflux systems are reviewed extensively (Cervantes et al., 1994). While the composition efflux processes is known

to vary, up to five (*ars*RDABC) genes are reported from the plasmid pKW301of *Acidiphilium multivorum* and plasmids R773 and R46 of *Escherichia coli* (Suzuki et al., 1998), the transcription of genes in R773 are from a single operon.In the strain FR-008 of *Streptomyces* sp., a linear plasmid pHZ227 as reported by Lianrong et al. (2006) possesses *ars*RBOCTconferringAs resistanc. The expression of *ars* operon at basal and secondary stages in *ars*R and *ars*D genes is controlled by repressors. Where as structural systems of As resistance is encoded by *ars*ABC genes. Trans-membrane efflux pump is empowerd by ArsA which is an ATPase. This and ArsB form a complex to facilitate As effluxing. Located in cytoplasm the ArsC is a small arsenate reductase. Further, Dey and Rosen (1995) had reported that in the absence of ArsA too ArsB can expunge arsenite.

Only three genes, *ars*RBC are known to confer As resistance by plasmids in *Staphylococcus*(with pI258 and pSX267) and by chromosome in *E. coli* (Carlin et al., 1995) as well as *Pseudomonas aeruginosa* (Cai et al., 1998). The *ars*RBC and a fourth ORF of yet to be known function was reported from *Bacillus subtilis* (Sato and Kobayashi, 1998). Another system with *ars*RBC consisting of *ars*H a divergently transcribed gene was reported by Neyt et al. (1997) from *Yersinia enterocolitica* on its plasmid pYV which has Tn2502 reported to confer As resistance.

The *ars*H, whosefunction is not known yet, is reported to encode in *Y. enterocolitica* an NADPH-flavin mononucleotide oxidoreductase. Itspresence either in trans or cis form is essential for As resistance in *Sinorhizobium meliloti* and *Y. enterocolitica* (Neyt et al., 1997; Yang et al., 2005; Ye et al., 2007). Anonther gene *ars*Mis reported to encode "an arsenite *S*-adenosylmethionine methyltransferase" (Qin et al., 2006). It is known to detoxify As by methylating arsenite to trimethylarsine (a volatile form). The Gram negative rods, motile strain identified as *Zoogloea*ULPAs1 from soils found to biotransform arsenic, this strain is able to efficiently oxidize arsenite to arsenate. As Weeger et al. (1999) point out, this strain can be a good bioremediation candidate in As dosed places due to its increased resistance to As(III) as well as other toxic metals.

Many researchers (Diorio et al., 1995; Cai et al., 1998; Sato and Kobayashi, 1998; Suzuki et al., 1998; Butcher et al., 2000; Butcher and Rawlings, 2002; Maury et al., 2003) noted that the *ars* genes in bacteria are carried on either chromosomes or

plasmids. Their main organized types of operons are *ars*RDABC, *ars*RABCand *ars*RBC. The *ars* genes exist, in some cases, singly.

2.1.9 Arsenic Resistant Bacteria in Bioremediation Efforts

Potential and important biotechnological applications of metal-microbe interactions are in biobeneficiation of ores, bioleaching and/or in bioremediation of sites with metal pollution. Bioremediation is a better, cheaper and safer option thanlandfilling or incineration of the toxic materials.

Arsenic is a metalloid that causes harm to humans and environments.It is important to remove and reduce this pollutant from the environment through different approaches such as physical, chemical and biological. Technologies for removing arsenic from the environment should meet several basic technical criteria that include robustness, no other side effect on the environment, and the ability to sustain water supply systems for long terms and meet the quality requirement of physical, chemical and microbiological approaches (Duarte et al., 2009). Currently, there are many methods (Mahimairaja et al., 2005) for removing arsenic from the soil contaminated with arsenic, which could be divided into three categories, including physical, chemical and biological approaches.

Since most of the cases of arsenic poisoning are due to the consumption of water contaminated by arsenic, the process of cleaning up or reducing arsenic concentration in water becomes very important. Methods used in reducing arsenic levels in water are primarily divided into (i) physiochemical methods, which include filtration or coagulation sedimentation, osmosis or electrodialysis, adsorptions, and chemical precipitations and, (ii) biological methods such as phytoremediation by using aquatic plants or microbial detoxification of arsenic (Mahimairaja et al., 2005). Two important processes in the removal of arsenic from water by microorganisms are biosorption and biomethylation.

It is reported that biomethylation (by As(III) *S*-adenosylmethionine methyltransferase) is the reliable biological process for removing arsenic from aquatic media (Mahimairaja et al., 2005). Recently, Chen et al. (2013) noted that chromosomal insertion of arsenite *S*-adenosylmethionine methyltransferase (ArsM) into *Pseudomonas putida* KT2440 for potential bioremediation of arsenite from

environmental settings. The use of bioremediation to remove and mobilize arsenic from contaminated soils and aquifers could be effective and economic ways since a wide range of microorganisms have been found to be successfully degrading this pollutant from the environment. The iron oxidation biologically helps simultaneous removal of arsenic from contaminated groundwaters (Katsoyiannis and Zouboulis, 2006). Gihring and Banfield (2001) discovered some high temperature tolerant strains of *Thermus* from As-rich fluids. These can oxidize arsenite rapidly both in vitro and in vivo. In Spain, Canovas et al. (2003) isolated a filamentous fungus from the River Tinto which is highly acidic and high concentrations of toxic metals. This fungus can grow at ~15000 ppm arsenic. Whereas reference strains such as *Aspergillus nidulans* as *Saccharomyces cerevisiae* and *Escherichia coli* only withstand 20 fold lower this concentration.

Isolation from Australian gold mines and characterization of ARB are described by Santini et al. (2000). Capable of chemolithotrophic growth, some of these isolates use arsenite as electron donor and oxygen, the acceptor. Anaerobic utilization of arsenate as other electron acceptors is also known (Stolz and Oremland, 1999; Macy et al., 1996; Santini et al., 2000). Sizable As bioremediation achieved by employing ARB is reported by Chen et al. (1986).

Very slow remobilization of toxic metals (eg. Hg, Cd, As, Cu, Pb) through biogeochemical pathways leads to metal-organic complexation in marine sediments (Forstner and Wittmann, 1979; Muller et al., 2001; Gerlach, 1981; Barkay, 1987). This complexation leads to accumulation and biomagnifications via food chain, which is harmful to all life forms including humans.

Jeckel (1994) and Cavalca et al. (2013) propose different As removal steps through chemical processing on the basis of its oxidation and subsequent alkaline precipitation (Hering et al., 1997; Gupta and Chen, 1978; McNeill and Edwards, 1997; Bothe and Brown, 1999; Gregor, 2001). Disadvantages however are pollution and cost. Biological As oxidation was thus explored (Valls and Lorenzo, 2006). Starting with *Achromobacter* many bacteria capable of arsenite oxidization are collected (Green, 1918) and are followed by several, *Pseudomonas* (Turner, 1954; Turner and Legge, 1954; Ilyaletdonov and Abdrashitova, 1981) *Alcaligenes* (Osborne

and Enrlich, 1976; Phillips and Taylor, 1976), *Acidithiobacilus ferrooxidans* (Chandraprabha and Natarajan, 2011) and *Thermus* genus (Gihring et al., 2001).

Role of arsenite oxidase in detoxification is also explored (Anderson et al., 1992; Ellis et al., 2001). Adaptation to stress from metalloids and/or metals by soil flora is reported by Pennanen et al. (1996). Turpeinen et al. (2004) further reported the ARB diversity was greater in soils with elevated As, Cr and Cu concentrations.

Contaminated sites are tried with bacteria, fungi or yeast for bioremediating them (Strong and Burgess, 2008; Kumar et al., 2011). By harnessing microorganisms converting arsenite to arsenate, we can aim to clean up As contamination in the environments.The ability of marine bacteria to oxidize arsenite leading to its detoxification will be important (Oremland and Stolz, 2003). While a lot is known of genetics of bacterial resistance as well as detoxification of As in vitro, there is no certainty as yet on molecular mechanisms in microflora for in vivo trials. This study aims at understanding the fraction of marine bacteria capable of tolerance to high As concentrations, their ecology, molecular make-up for As resistance and at realizing their potential in bioremediation of As pollution.

Effluents containing loads of heavy metals are produced through uses of metals by industry. These are treated mostly chemically via ion exchange, precipitation or electrochemically (Wong et al., 2001). Chemical treatment is quite ineffective and expensive. Thus attention received for microbial bioremediation of heavy metals is recent and cognizant (Lei et al., 2000).

Physical method exhibits the simplest choice, but it was however limited to small scale operations. As Lim et al. (2014) point out, the chemical method had gained popularity by its high success rate; however, the remediation area can be exposed to other types of chemical contaminants. The usage of biological and phytoremediation methods might be the most practical methods for a small area but more research needs to be carried out especially in methylations, reduction, and oxidation using microorganisms for more effective method to remove the arsenic compound as they have a high potential application in the future.

The use of bioremediation to remove and mobilize arsenic from contaminated soils and aquifers could be an effective and economic way. This is because, a wide

range of microorganisms are found to be successfully degrading this pollutant from the environment. The sequencing of genome permits characterization of molecular basis of As reduction or oxidation. *Pantoea* sp. genome (strain IMH) draft assembly report by Tian and Jing (2014) suggests aerobic reduction of arsenate to arsenite. Presence of an intact "type III secretion system" in arsenic-gene island in the first reported draft genome-sequence of arsenite-oxidizing bacterium, *Achromobacter arsenitoxydans* SY8 by Li et al. (2012) implies its role/s for transport of many metals and metalloids. According to Jackson and Dugas (2003), the overall of the arsenate reductase/s phylogeny implies pivtal role for *ars*Cgene.

2.1.10 Major Studies in India on Arsenic Resistant Bacteria

Within the Deltas of Ganges, Brahmaputra and Meghna are highly As contaminated aquifers (Anawar et al., 2003; Stuben et al., 2003), with over a third of Bangladesh population being at As poisoning-risk (Smith et al., 2000). The chemical disintegration, transport and deposition of As rich rocks from Himalayas enriches downstream rgions. Biological alterations and solubilzation of As is cause that mobilizes As subsurface. Settings favourable to As-enriched aquifers are reported from deltas of Red River (Berg et al., 2001) and Mekong River (Polya et al., 2003) in Southeast Asia. Studies by Smedley et al. (2003), Karim (2000) and Das et al. (1996) suggest that Bengal basin aquifers as well as soil contain higher As concentrations than the WHO maximum contaminant level (MCL) of 0.01 ppm (0.13 mM).

Arsenite oxidizing strains have been isolated and studied by various researchers. Mokashi and Paknikar (2002) isolated *Microbacterium lacticum* from a municipal sewage sample by enrichment culture technique which exhibited tolerance to 50 mM arsenite. Achemolithoautotroph namely *Arthrobacter* sp. 15b was identified from a sewage treatment plant site by Prasad et al. (2009). Further, Bachate et al. (2012) isolated two heterotrophic arsenite oxidizing bacteria from garden soil that were closely related to genus *Bordetella* (MIC- 15 mM) and *Achromobacter* (MIC- 40 mM) based on 16S rRNA gene sequencing.

2.1.11 Marine Environment and Arsenic

Compared to terrestrial organisms, the marine forms differ greatly in both their As tolerance/resistance levels and in their ability to deal with different forms of As.

Former type contains just ~1 ppm As in their dry mass than the latter varying from several ppm to >100 ppm (Lunde, 1977). In norm-oxic brackish and marine waters, arsenate is the dominant form. As Neff (1997) reported, toxic arsenite a potentially cancer causing species is <20% of total As.

Studies so far indicate that marine life forms do complex inorganic As to organic ligands (Kumaresan and Riyazuddin, 2001). Such ability is not shown in their terrestrial counterparts. As is present in marine organisms in water and lipid soluble forms.The former types are very stable; breakdown by metabolic or chemical processes are difficult. In marine foods As is not in arsenite form, also not easily transformed in to inorganic form (Neff, 1997), it may not be detrimental when seafood is consumed.

Archaebacterium *Sulfolobus acidocaldarius* strain BC (Sehlin and Lindstrom, 1992), *Alcaligenes faecalis*, *Shewanella algae*, *β*-proteobacteria strain UPLAs1, *Alcaligenes faecalis*, *Comamonas terrae* sp. nov, some heterotrophic bacteria (*Herminiimonas arsenicoxydans*) and chemoliautotrophic bacteria are reported to have the ability to oxidize arsenite to a less toxic arsenate (Mahimairaja et al., 2005; Oremland and Stolz, 2005). Liao et al. (2011) reported that 11 arsenic reducing bacterial strains from seven different genera (i.e., *Pseudomonas*, *Psychrobacter*, *Citrobacter*, *Bacillus*, *Bosea*, *Vibrio* and *Enterobacter*) were isolated from environmental groundwater samples collected from well in Southern Yunlin County, West Central Taiwan that holds a significant impact in the biotransformation of arsenic that is present in the aquifer, and these communities of bacteria are well adapted to high arsenic concentrations that are present in the water.

Through screening selecting and examining literature/ data, it is possible to come up with an insight on arriving at solutions for problems associated with metal pollution. As such, an overview of current knowledge of arsenic transportation and the ecological processes and As transformation is essential. However, the literature reviews here foussed on arsenic resistant marine bacteria, their potential of As bioremediation and ARB capable of As detoxification. Detailed literature review with pertinent topic-wise aspects is in different chapters.

3.1 Introduction

Microbial ecology is central to basic understanding of the roles and functions the microbiota perform in their native habitats. Investigations on microbial populations specializing in ecological functions provide insights on the importance of such populations, communities, assemblages and/or species in efficient biotransformation of a given substrate. Thus, environmental studies are essential in studying prevalence and abundance of bacteria. For instance sewage outfalls, discharges and other dumpings affect type and loads of microflora in the coasts (Colwell et al., 1977; Marchand, 1986; Patti et al., 1987; Piccolomini et al., 1987; Nagvenkar and Ramaiah, 2009). Anthropogenic activities adversely affect many life processes diminishing efficiency in metabolic performances in the near-shore environs.

In the recent years, there have been concerns on the release of arsenic compounds into environment and their toxicity to a variety of native organisms as well as human beings. Studies on metal tolerating native flora are therefore, important to realize their metal detoxifying potential as well as to understand the extent of metal pollution.

In any environment, presence of arsenic resistant bacteria is likely to be linear with arsenic contamination level. Arsenic is toxic to life, but certain microbes endowed with As detoxification mechanisms use its species either as electron acceptors or donors (Ahmann et al., 1994; Cervantes et al., 1994; Newmann et al., 1998; Stolz et al., 2002). Such microorganisms can be used in the process of bioremediation of water bodies or soils that are contaminated with arsenic.

Although arsenic resistant bacteria (ARB) are obtained from non polluted systems (Chopra et al., 2007), their higher incidence in any habitat is an indication of their possible exposure to As.Some observations (Nickson et al.,1998) on concentrations of As in sediment, water and biota are far more than those endured by humans.Microbial alterations affecting As distribution and mobility in the environment govern its natural cycle (Tamaki and Frankenberger, 1992; Quinn and McMullan, 1995). Arsenic biotransforming and/or modulating bacteria are obtained by isolating them from total arsenic tolerant bacteria at a given sampling site. These

investigations help realize the potential of metal-tolerance flora which can detoxify other harmful substance/s as well.

Widelydistributed though not quantitatively abundant in soils and waters arsenic (As), a toxic metalloid, is generally released into the environment from natural (rock-weathering) as well as agricultural (pesticides), industrial (painting)or mining sources (Cullen and Reimer, 1989; Pepi et al., 2007). Leaching naturally (Lee et al., 2005) and added anthropogenically, arsenic keeps accruing in seawater and marine sediments. Worldwide many areas are arsenic polluted (*sensu*Mukherjee et al., 2006; Halem et al., 2009), presenting a threat to people and environment.

So far, most focus on As-resistant floral abundance and types is from freshwater regions and rare arereports from tropical estuaries. Since coastal regions are major disposal sites attention to microbes dealing with arsenic species will prove useful to obtain natural microflora with a potential to work well under varied and wide range of environmental situations. Therefore for understanding the As cycling and the impact of land drainages in to nearshore regions of higher biological productivity, this study was planned and investigated. Modifications following cellular uptake of As (and/or its compounds) can lead to bio-magnification, move up to reach humans via food chain (Chen et al., 2009; Liu et al., 2009) resulting, as Barwick and Maher (2003) ponder, in chronic and/or acute ailments.

3.2 Materials and Methods

3.2.1 Sampling Locations

The sampling locations for this study are shown in Figure 3.1. The region receives many types of effluents including barge-produced wastes. Prior to draining off Mormugao into Arabian Sea, both Mandovi and Zuari rivers flow through iron and manganese mining areas. They bring in substantial quantities of land debris (Shankar et al., 2004; Kessarkar et al., 2009; Maya et al., 2011; Parab et al., 2013) during the rainy months of June-September. This is besides, ~10 million litres of sewage per day (Sawkar et al., 2003; Nagvenkar and Ramaiah, 2010). Further, prior to Oct 2012, ~300 barges were daily moving iron-ore from upstream mines to load ~15-20 ore-carriers in the confluent harbor for shipment (Nair et al., 2003).

3.2.2 Sampling

Sampling was carried out during May (pre-monsoon), November (post-monsoon) and September (monsoon). Sampling was carried out from upstream of both rivers, in Marmugao Bay and from offshore. Samples (sediment and water) were obtained from the oligohaline 'midstream' (~5-<10 psu), and lower stretches (~10-25 psu) during monsoon as well as pre and post-monsoon. Sampling from an adjacent marine location (~30 psu) was done only during post-monsoon. Samples for microbiological analyses in particular were from sites 'M' (offshore); 'MS' (Mandovi) and 'Z' (Zuari mouth).

Niskin samplers (5 L capacity, General Oceanics, FL) were used for water sampling from 1m below the surface to thwart micro-layer laden contamination. For bacteriological analyses sub-samples were taken into polyethelene bottles (pre-sterilized). Portions of sediments from a Van veen grab were collected aseptically and analysed within 3-4 h of collections.

3.2.3 Enumeration of Total Viable Counts

Total viable counts (TVC) were enumerated from samples of midstream zones by spread plating a known aliquot onto nutrient agar (composition (NA): peptone 5.0 g, NaCl 5.0 g, yeast extract 1.5 g, beef extract 1.5g, agar 15 g, deionised water 1000 ml, pH 7.4 ± 0.2). TVC from estuarine and marine water samples were enumerated using NA prepared respectively with 25% and 50% aged seawater. From each zone, sediment sample TVC were counted by plating 0.1 to 0.3 ml aliquots in triplicates from 10^{-2} and 10^{-3} dilutions on NA plates prepared in 50% and 25% seawater (for marine and estuarine zone samples) and NA plates with no added seawater from midstream samples.

3.2.4 Isolation of Arsenic Resistant Bacteria

Nutrient agar was added with arsenic trioxide (As_2O_3) to grow, enumerate and differentiate bacteria tolerating As. For this, 0.1 and 0.2 ml aliquots in triplicate were spread onto NA containing 15, 25 and 50 ppmAs. Sediment were also processed suitably and plated. All plates were incubated upto 48 h at room temperature

(27±2.5°C). TVC were noted and expressed on dry weight basis after sediment samples were dried to consistency.

3.2.5 Evaluation of As Tolerance

To determine arsenic resistance and response to As stress, bacteria from water and sediment were grown in medium with high concentrations of As. After incubation, many isolates with different morphologies were selected from nutrient agar plates containing 50 ppm As. They were individually streaked for purification and plated again on 100, 200, 300, 500 and 1000 ppm arsenic to ensure that they do grow in high As concentrations. For collecting strains tolerating 1000ppm As concentration, many colonies were selected and streaked on NA with 1000ppm As. These were maintained on slants as well as in glycerol stocks at -80°C. Isolates growing on NA containing 100 ppm As or more were termed as Arsenic Resistant Bacteria (ARB).

3.3 Results

In the study area, some of the physical and chemical parameters are as follows. Depth range: 3.8 to 20 m (deeper offshore). Water temperature ranges in May 2005: 28.0ºC to 32.6ºC; in November 2005: 27.6 to 28.9ºC and in September 2006: 27.4ºC to 29.5ºC. Salinity range: 7.3 to 36.3 S in monsoon/postmonsoon; 34.7 to 36.7 S in premonsoon. Respective NO_3, PO_4 and SiO_4 concentrations range in May: 0.01-12.57; 0.05-0.51 and, 0.02-10.1 µM and 0.01-4.28, 0.02-1.8 and, 0.4-19.3 µM in November. Chlorophyll concentrations range: 1.22 to 11.45 (May) and, 1.17 to 6.14 (November) mg m^{-3}.

ARB were recorded from all samples from coastal, estuarine and marine locations. The TVC in water samples from 'midstream' in all three months were higher (2000 to 9600 ml^{-1}) than those from estuarine (1020-1600 ml^{-1}) location (Table 3.1). Similar trend was also found at the estuarine and marine locations. In the general, the % of TVC in water samples growing on NA with 15 ppm As was higher (~20 to 36%) than that with 25 ppm As (~12 to 18%) or with 50 ppm As (~6 to 13%). Compared to water samples, TVC was higher in sediment samples (Table 3.1). Tolerance of bacteria to arsenic trioxide was far higher. For instance, those growing on 50 ppm As were able to actually tolerate as much as 132 ppm arsenic trioxide.

Post-monsoon samples had higher TVC *vis a vis* monsoon or pre-monsoon periods (Figure 3.2). Similarly, the TVC on NA decreased with increasing As (Table 3.1).

As high counts were seen on NA containing 50 ppm As, other higher As concentrations (*viz*., 100, 200, 300, 500 and 1000 ppm) were included to NA for isolating strains growing on them (Table 3.2). With increased As concentrations, % of isolates tolerating higher As concentration decreased (Figure 3.3).

3.4 Discussion

In this chapter, current levels of marine flora growing in NA with 15, 25 and 50 ppm arsenic and their seasonal differences are reported. To evaluate the tolerance of a select set of flora to such high As concentrations many isolates grown initially on nutrient agar with 50 ppm were grown in higher As concentrations of 1000 ppm.

To assess the significance of microbes in any environment, knowledge of their general load is obligatory. With such data evolving advisories is possible as to control or regulate their quantities. The reported As concentrations are 0.11 to 0.78 in water and, 5.84 to 9.72 in Mandovi and in Zuari sediments, 5.07 to 10.20 ppb (Nair et al., 2003). It is evidenced here that percent of bacteria tolerating As is very high. As such, their metabolism is suggested (Ahmann et al., 1994) to play a greater and very direct function in arsenic cycling. Certain heterotrophs are reported to use arsenite as an auxiliary source of energy as Newman et al. (1998) suggested,whereas, many others may oxidize arsenite as a means of detoxification. Several isolates are reported to derive energy from As(III) oxidation and, perform oxidative As(III)-detoxification (Oremland and Stolz, 2003). Anyanwu and Ugwu (2010) isolated arsenic resistant bacteria, *Bacillus* sp. from a sewage treatment plant resistance to arsenic concentration of 300 ppm. Takeuchi et al. (2007) isolated bacterial strains highly resistant to arsenic such as *Vibrio alginolyticus* (resistant upto 730 ppm As), *Marinomonas communis* (510 ppm), *Bacillus subtilis* (220 ppm), *Rhodococcus equi* (140 ppm) and *Pseudomonas aeruginosa* (100 ppm) frommarine and non marine environment. *M. communis* used as potential candidates for bioremediation of arsenic contaminated water.

These results are useful to notify the preponderance of isolates in different locations those that are tolerating high As concentrations and growing. Ecological significance of such native flora needs to be evaluated. Their % in most sampling sites being substantial, it is possible to suggest that a large fraction of aerobic heterotrophs apparently seems to be physiologically geared up to deal with As and/or other polluting toxicants. De et al. (2008) reported that native bacterial populations tolerant to Hg could resist and proliferate when many other toxic substances are present. ARB too could similarly deal with many other toxicants.

Strategies of resistance to-and uptake of trace metals in bacteria from seawater are shown to be numerous. In that some species are reported to develop resistance to Hg and/or other toxic metals (Osborn and Ehrlich, 1976; Ramaiah and De, 2003; De and Ramaiah, 2006; De et al., 2007 and 2008). Further, marine microbes do undergo certain selection adaptations in the presence of toxicants to become resistant. Therefore As resistance is apparently mediated chromosomally or by transposons in most isolates investigated in this study.

The As tolerance/resistance or its myriad molecular or ionic forms in native microflora is of high significance. While the As concentration in the two rivers was moderate (Nair et al., 2003), it is pertinent to note that the existence of isolates highly resistant to As. This is suggestive of the fact that many folds higher resistance in the bacteria from water or sediment in this region can be expected. Studies by Achour et al. (2007) and Jackson et al. (2005) also reported similar inferences drawn from this work.

To date, Many ARB are studied from different ecological settings and documented (Rosen, 2002; Silver and Phung, 2005). Environmental bacteria reportedly possess numerous As resistance mechanisms (Mukhopadhyay et al., 2002; Rosen, 2002) with *ars* operons encoded either chromosomally or by plasmids conferring the essential charactersistic. Notable among these operons are the three (*ars*RBC) gene and the five (*ars*RDABC) genes operons (Silver and Phung, 1996). To evaluate these further, analyses of the distribution and preponderance of ARB in sediment and water samples in various different regions were carried out. This was to relate the abundance of ARB populations with that of molecular mechanisms extant in a select set of isolates.In chapter 8 results on PCR screening for few of the *ars* genes are provided.

Ecologically speaking, prevalence of a large fraction of native bacterial flora in Mandovi-Zuari estuarine complex resistant to As at as high as1000 ppm, implies greater capability of toxic As biotransformation. Observations from this study are useful to recognize that already a large percent of CFU, in diverse group, is capable of high tolerance for As and that it may be experiencing elevated physiological or genetic modifications as to endure changing ecological settings. As pollution bioremediation with such strains does have a potential.

4.1 Introduction

Prolonged exposure to toxic metals such as Zinc, Copper, Mercury and Nickel (Baath et al., 1998; Muller et al., 2001; Ramaiah and De, 2003) as well as arsenic (Turpeinen et al., 2004) is confirmed to alter the microbial community. Bachate et al. (2009) and Cai et al. (2009) report that some strains within a species in many environs apparently have developed resistance to As. There is a lot of evidence on As resistance among many species of microorganisms (Mergeay, 1991; Diorio et al., 1995; Tsutomu and Kobayashi, 1998; Gihring et al., 2001). Takeuchi et al. (2007) and Oremland et al. (2009) have reported on the important role of ARB in regulating cycling and speciation of As through a variety of *ars* gene-mediated mechanisms in the marine systems. Chang et al. (2008) suggested that the ARB do affect the soluble, insoluble states of As among toxic and less/non-toxic forms of As.

For their transformational ability, ARB are extremely vital in detoxifying As compounds taken up by phosphate transporters, aqua-glyceroporins, and active extrusion system. Also many of the As compounds are reduced by arsenate reductases via dissimilatory reduction mechanism (NiDhubhghaill and Sadler, 1991; Sanders et al., 1997; Bun-ya et al., 1996; Wysocki and Chery, 2001; Silver and Phung, 2005; Liu et al., 2002). Some species of autotrophic and heterotrophic microorganisms use As oxyanions for regeneration of energy. Certain species of microorganisms for example strains of *Aeromonas*, *Exiguobacterium*, *Acinetobacter*, *Bacillus* and *Pseudomonas* are shown by Ahmann et al. (1994) and, Anderson and Cook (2004) to be able to use arsenate as their nutrient in respiratory process. Detoxification operons are a common feature of arsenic resistance in microorganisms (Stolz et al., 2002; Silver and Phung, 2005). Hence, use of bioremediation could be an effective and economic way to reduce this pollutant from the environment.

As is a known toxicant. However, some microflora adapt to exposure to As and through methylation, reduction or oxidation, biotransform certain ionic forms of As (Osborne and Ehrlich, 1976; Sanders and Windom, 1980; Anderson and Bruland, 1991). As also noted by many studies (Weeger et al., 1999; Macy et al., 2000; Oremland and Stolz, 2003), several bacterial strains posess abilities to convert inorganic forms As by reduction and/or oxidation reactions.

A few studies have examined ARB from marine locations and their potential to detoxify arsenic. Description of bacterial isolates resistant to arsenic, and growing well at 1000 ppm As (as As_2O_3) was carried out by examining some of their biochemical properties and antibiotic sensitivity. Molecular identity of these 13 and all the remaining isolates of ARB was established through 16S rDNA sequencing.

4.2 Materials and Methods

4.2.1 Isolation of Arsenic Resistant Bacteria (ARB)

ARB from sediment and water samples was isolated from different sampling sites during pre-monsoon, post-monsoon and monsoon months. All samples were spread plated onto nutrient agar. A select set of isolates from NA containing 50 ppm As was purified to evaluate their growth on 100, 200, 300, 500 and 1000 ppm. Thirteen morphologically different isolates from water and sediment samples growing in NA containing 1000 ppm As were characterized and used for quantifying their ability to remove/detoxify/oxidise As_2O_3 following the method of Koroleff (1976) as is described in Chapter 5.

4.2.2 Characterization of ARB

These ARB maintained on NA with 10 ppm As were tested for their biochemical characteristics according to Holt et al. (2000). The following are the major tests carried out.

Gram staining was done on 24 h old cultures and observed microscopically.

Shape was made out microscopically while checking out for Gram character.

Motility was also examined microscopically by hanging drop method.

Enzyme profiles: Production or elaboration of various enzymes (listed in Table 4.1) by these isolates was carried out as per Holt et al. (2000).

Growth on various carbon sources: The isolates were grown in liquid medium with eithersucrose, dextrose, lactose, fructose, maltose, raffinose, galactose, inositol, glycerol, mannose, cellobiose, mannitol, arabinose or rhamnose etc to check for their ability to grow on these carbon sources.

Other tests: Reduction of NO_3, oxidation-fermentation reactions, production of methyl red, Voges-Proskauer reaction, and H_2S production were tested following Holt et al. (2000).

4.2.3 Growth Characteristics of ARB

All thirteen ARB isolates were examined for their growth in the nutrient broth containing 200 ppm As. Growth of estuarine isolates was examined in NB prepared with 25% aged seawater. All isolates were also examined for their growth in NB without added As. Both sets were held on a shaker (at 150 rpm) at 26±2°C and optical density at 660 nm taken for over 5 days once every 3 h. For measuring OD, identical volume of 1 µl culture of 24 h old was added to each flask to avoid wide variation in cell numbers.

4.2.4 Antibiotic Sensitivity

An array of antibiotics such as Streptomycin, Penicillin-G, Kanamycin, Tetracycline, Oxytetracycline, Chloramphenicol, Neomycin and Gentamycin, were used to test the sensitivity of these select set of ARB, which are used in certain other experiments (see chapters 5, 7 and 8). Sensitivity assay was done as follows. The ARB were grown for 24 h and lawn cultures made by spreading on NA plates. All antibiotic discs were placed before incubating for 24 h at room temperature. Once the zones of inhibition were noted, the antibiotic sensitivity was evaluated as per manufacturer's (Hi-Media, Mumbai) protocols.

4.3 Results

The ARB strains were identified by an array of physiological and biochemical tests. Their responses were different in their morphological traits and biochemical properties (Table 4.1). Seven of the 13 isolates were Gram negative. The isolates were short (<2 µm) or large rods (2-5 µm) or cocci in shape. Isolates 1, 2, 3, 5, 7, 10 and 12 were short rods (SR) and, 6 and 8 were long rods (LR) while 9, 11 and 13 were cocci. All but 4, 9, 10, 11, 12 and 13 were motile. Taxonomic identity of these isolates is mentioned in the legand of the Table 4.1. All tested isolates were negative for Methyl red and Voges Proskauer tests. All isolates utilized citrate. Except for isolates numbered 6 and 12 urease was elaborated by other isolates. None of the isolates were

positive for Phenylalanine deaminase (PA) and H_2S production. Nitrate was reduced by isolates 1, 5, 7, 8 and 11. Presence of various enzymes in different isolates can be discerned from the responses listed in Table 4.1.

All isolates produced catalase but most of them were oxidase negative except 7 and 13. Except 7, 11 and 12 fructose was utilized by all other isolates. Arabinose was utilized by only 7. Except 6 and 10 most isolates were unable to utilize sucrose as source of carbon. Gelatinase was produced by 4, 5, 6, 8, 10 and 13, amylase by only 3, 6 and 10. Out of thirteen isolates, three isolates (1, 3 and 4) used up glucose through fermentation and ten via oxidation. 1 and 3 belonged to Enterobacteriaceae, 2 and 7 to *Pseudomonas* sp., 4 to *Corynebacterium* sp., 5 to *Xanthomonas* sp., 6 to *Acinetobacter* sp., 8 to *Flavimonas* sp., 9 to *Micrococcus* sp., 10 to *Bacillus* sp., 11 to *Staphylococcus* sp., 12 to *Rhodocccus* sp. and 13 to *Planococcus* sp.

The optical density of all ARB strains in the NB with and without 200 ppm As (Figures 4.1a, b and c), is useful to note that in media with As, the generation times were longer.Prolonged lag phase in NB with As was seen for many isolates.Growth increment was greater between 12 and 54 h and between 36 and 96 h in NB without As and with As, respectively. Isolate 4,a *Corynebacterium* sp., and 5, *Xanthomonas* sp., grew quite rapidly in the presence of As (Figure 4.1a and b). However, their growth was rapid between 12 and 36 h in the medium without As. Growth maxima were seen in media with As, between 36 and 51 h. So also isolates 1 (an Enterobacteriaceae strain), 2 (*Pseudomonas* sp.), 9 (*Micrococcus* sp.), 10 (*Bacillus* sp.), 11 (*Staphylococcus* sp.) and 12 (*Rhodocccus* sp.) grew rather well in the medium with As (Figure 4.1a and c respectively). Growth maxima in media with As were between 36 and 54 h while it was rapid between 12 and 36 h in the absence of As. However, not much difference was seen in the growth of isolates 8 (*Flavimonas* sp.), 3 (Enterobacteriaceae) and 13 (*Planococcus* sp.) in the presence and absence of As. Their growth was higher between 12 and 36 h in both the media. Exponential growth phase was much later in the presence of As among the other isolates. Isolates 6 and 7 belonging to *Acinetobacter* sp. and *Pseudomonas* sp. grew very slowly in the presence of As and attained short exponential growth phase between 81 and 85 h.

The isolates showed variation in sensitivity to different antibiotics (Table 4.2). Except for chloramphenical, penicillin-G, and oxytetracycline many ARB were

sensitive to other tested antibiotics (Figure 4.2). Interestingly, isolates 5, 11 and 12 were resistant to oxytetracycline and chloramphenical. Isolates of Enterobacteriaceae (1 and 3), *Pseudomonas* sp. (2), *Corynebacterium* sp. (4), *Acinetobacter* sp. (6), *Micrococcus* sp. (9) and *Bacillus* sp. (10) were sensitive to all antibiotics tested except penicillin-G. Isolates of *Flavimonas* sp. (8) and *Planococcus* sp. (13) were sensitive to all antibiotics tested in this study.

4.4 Discussion

As many as 500 ARB isolates (355 from water; 145 from sediment samples) were collected from coastal environments of Goa and grown on NA containing 10 ppm arsenic as As_2O_3. Some of them were taken up for testing different biochemical characteristics. Morphological, physiological and nutritional properties of 13 of them were examined following Holt et al. (2000) using different tests.

Resistance to As and other toxic metals in many bacteria is reported (Osborne and Enrlich, 1976; Osborn et al., 1997; Meinhardt et al., 1997; Nies, 1999; Ravel et al., 1998a, b, 2000a, b). Several researchers have reported detoxification of As by ARB, irrespective of the habitats they are obtained from (Turner, 1954; Osborne and Enrlich, 1976; Weeger et al., 1999; Gihring et al., 2001; Salmassi et al., 2002; Bruneel et al., 2003). In contaminated sites, such set of strains are vital for upkeeking the ecological processes. The following genera are reported to harbor strains resistance to arsenic: *Arthrobacter*, *Acinetobacter*, *Pseudomonas* (Turner, 1954; Turner and Legge, 1954; Ilyaletdonov and Abdrashitova, 1981),*Microbacterium, Agarobacterium* (Silver et al., 1981; Mergeay, 1991; Ji and Silver,1992a, b),*Comamonas*, *Stenotrophomonas*, *Arthrobacter*, *Rhodococcus* (Tsutomu and Kobayashi, 1998; Weeger et al., 1999),*Achromobacter*,*Alcaligenes* (Diorio et al., 1995), *Agrobacterium*, *Bacillus*, *Thiomonas* (Green, 1918),*Acidithiobacilus ferrooxidans* (Chandraprabha and Natarajan,2011),*Desulfomicrobium*,*Cyanobacteria*,*Sulfurospirillum*, *Microbacterium* (Butcher et al., 2000), *Ochrobactrum*,*Escherichia*, *Achromobacter* (Rokbani et al., 2010), *Staphyalococcus*, *Citrobacter*, *Klebsiella*, *Shigella* (Cai et al., 2009), *Thiobacillus* and *Thermus* (Gihring et al., 2001; Salmassi et al., 2002; Bruneel et al., 2003).

During this study, isolates capable of detoxifying 10 ppm As were found to belong to 11 different genera viz. Enterobacteriaceae,*Pseudomonas*, *Corynebacterium*, *Xanthomonas*, *Acinetobacter*, *Flavimonas*,*Micrococcus*,*Bacillus*, *Staphylococcus*, *Rhodocccus* and *Planococcus*.There were differences in their responses to biochemical and physiological characteristics among the ARB strains assigned to the same genus. Their ability to grow on a wide variety of carbon sources suggests their nutritional versatility. Their high tolerance to As, is a certain indication of their potentialto process many toxicants and grow even in adverse conditions.

Isolates growing in media with 200 ppm As posess abilities to tolerate such high As concentrations. Apparently isolates examined in this study have the potential to endure and grow in media with highAs.Likely, certain specific genes and/or plasmids confer this ability as already boted by Silver and Phung (1996).As resistance is achieved by enzymatic detoxification or expunging (Silver et al., 2002). Extended lag phase observed in this study in some ARB strains might imply that they do undergo physiological adaptation. As Summers and Silver (1978) and Silver et al. (2002) also noted all cultures examined in this study seem to possess inducible As reductases that apparently enable rapid growth of ARB once they adapt to toxic milieu.

Further, quite a substantial percent of bacteria capable of growing in thepresence of As was evidenced. Except for six non-motile isolates of *Corynebacterium* sp., *Micrococcus* sp., *Bacillus* sp., *Staphylococcus* sp., *Rhodocccus*sp. and *Planococcus* sp., others were Gram negative. Although Canovas et al. (2003) reported of a fungus tolerating ~1500 ppm As no such reports available for bacteria. Existence of a plethora of strains capable of tolerating and growing in high As concentrations is proven from this study. This study also portrays the need to investigate the importance of marine flora growing in such high As concentrations.

Marine microbes are reported to aquire resistance when there is selection pressure in the presence of toxicants.Thus resistance, in particular by Gram -ve isolates, to various antibiotics and to As might be chromosomally- or transposon- or plasmid- mediated in the isolates studied here. Though As resistant isolates were resistant also to many antibiotics, elucidation of the ARB relationship between these two resistance processes needs further studies. Shakoori et al. (2010) reported that *Klebsiella oxytoca*and *Bacillus anthracis* were sensitive against ampicillin while *Citrobacter freundii* being resistant to it. Further, *C. freundii*and *B. anthracis*were found sensitive to erythromycin, kanamycin, nalidixic acid, and tetracycline while *K. oxytoca*was found resistant against such antibiotics. Also, these three bacterial strains tested were found sensitive to amoxicillin, chloramphenicol, neomycin, oxytetracycline, streptomycin, and polymixin-B but resistance to bacitracin.

Greatest resistance toAs was exhibited in many isolates examind for this investigation than any isolate from marine regimes. These isolates are of great

relevance in microbial ecology and ecologists can vitalise contemporary concepts on As resistance to recognize the significance of As resistance in view of increasing trends of heavy metal and other pollution all over the globe. In marine ecosystem, As resistance and uptake of toxic/trace metals follow different pathway (Robinson et al., 1990; Misra, 1992). As mentioned above and reported by Hideomi et al. (1977), marine microorganisms, in particular bacteria seem to develop resistance following selection pressures in the media with toxicants.

Since earlier studies on marine bacterial isolates capable of As biotransformation have focused on a single strain of *Bacillus* sp. by Banerjee et al. (2013),species specific comparison ofthe strains *Bacillus baekryungensis*,*Staphylococcus arlettae*, *Rhodocccus erythropolis* and *Planococcus maritimus* is not done. However, these strains seem to be better biotransformers of As than that reported by Banerjee et al. (2013). When compared between them the strains examined in this study did differ in certain biochemical traits. Therefore, deciphering their genetic make up is warranted to reveal marine bacterial As resistance strategy. Through a very simple experiment, this study with simple experiment has demonstrated biotransformation potential of ARB by growing them in initially medium 10 ppm As and by selecting ARB growing in NB containing 1000 ppm As.

5.1 Introduction

Contamination due to heavy metal is a serious concern all around the world due to their harmful, toxic effects to animals, plants and mankind and, as of current global scenario, unsuccessful removal from the native environments. No known essential biological function of either As, Cd, Hg or Pb is reported from the commity of life forms (Gadd and White, 1993). And they are in fact highly deliterious (Hideomi et al., 1977). These metals tend to accumulate in sediments and go through aquatic food chain to ultimately reach humans, where they begin producing acute and/or chronic health conditions (Forstner and Wittmann, 1979) and numerous mutations at the DNA level (De Flora et al., 1994). Thus, damage by such toxic substances tends to be quite harmful.

The toxic metalloid As, a categorised (IARC, 1987; USEPA, 1998)) human carcinogen in all its oxidation states, gets into natural systems either by human releases (Cullen and Reimer, 1989). It binds to dithiols and/or proteins of sulfhydryl groups. As Ehrlich, (1996) suggested, it is highly toxic due to it inhibiting certain dehydrogenases. Its biogeochemical cycle strongly depends on microbial alterations which affect its natural biogeochemistry and its distribution and, mobility (Mukhopadhyay et al., 2002). Although As is generally toxic to life, it has been demonstrated that because certain microorganisms use its species as donors (Ahmann et al., 1994) or acceptors (Cervantes et al., 1994) of electrons, its toxicity is not of the same degree among different life forms. Many strains efficiently detoxify As (Ji and Silver, 1992b; Newmann et al., 1997; Stolz et al., 2002).

Bacterial isolates performing As conversions are reported from diverse habitats (Macur et al., 2004; Cummings et al., 1999; Oremland et al., 2005). Remineralization of essential elements biological functioning by marine microbial flora are important for mitigating toxicities associated with pollution and toxic substances (De et al., 2003). Pepi et al. (2007) proposed three dissimilatory systems that metabolize or tolerate As. They are (a) *ars* operon of many bacterial and plasmid genomes, (b) *arr* genes functioning anaerobically and (c) *aso* genes, the initial donor system conferring arsenite resistance under aerobic situations (Silver and Phung, 2005), These systems function depending on oxidation ($As_2O_3$to As_2O_5) followed by high pH precipitation (Bothe and Brown, 1999; Gregor, 2001).

The bioavailability, mobility and toxicity of As are governed by its oxidation states (Chang et al., 2008). Biotransformation of As has been studied in both aquatic (Maher and Butler, 1988) and terrestrial (Mandal and Suzuki, 2002) environments. Continuous dispersion or addition of As by human and/or natural processes are increasing its concentrations in marine environment. Therefore, their removal or detoxification from environment is of serious concern. Several chemical processes are known to remove arsenic. The downside of such processes though are, added pollution generation and high cost. Hence alternative choice of As remediation by knowing its oxidation state would prove useful. Yong and Mulligan, (2004) stated that natural attenuation is ideal,As bioremediation in sediments is promising since it takes advantage of potential of indigenous microorganisms assisting in its removal by coprecipitation or adsorption.

In view of all the above mentioned concerns, it was aimed to check on the As or alteration/removal potential by certain ARB strains isolated during this study. ARB strains from waters and sediment samples collected from different locations with Asalteration/removal potential is not evaluated as yet. The ability of 1000 ppm As tolerant ARB to remove/detoxify As was studied employing medium containing 10 ppm As.

5.2 Materials and Methods

5.2.1 Bacterial Isolates

Thirteen ARB isolates examined in this study were isolated from midstream, estuarine and marine locations off Goa (See Figure 1 in chapter 3). The ARB strains growing in NA with 1000 ppm As were tested. Their biochemical characteristics, their identification and test conditions are reported earlier (Nagvenkar and Ramaiah, 2010).

5.2.2 Arsenic Transformation by ARB

The following 13 ARB isolates namely 1 and 3 (Enterobacteriaceae); 2 and 7 (*Pseudomonas* sp.); 4 (*Corynebacterium* sp.); 5 (*Xanthomonas* sp.); 6 (*Acinetobacter* sp.); 8 (*Flavimonas* sp.); 9 (*Micrococcus* sp.); 10 (*Bacillus* sp., Accession No. KJ719328); 11 (*Staphylococcus* sp., Accession No. KJ719326); 12 (*Rhodocccus* sp.,Accession No. KJ719354) and 13 (*Planococcus* sp., Accession No. KJ719380)

were studied. These ARB strains were inoculated individually into the NB containing 10 ppm As (using arsenic trioxide). For spectro-photometricmeasuring As, 50 ml broth from each flask was drawn on all sampling days. Cell-bound and biotransformed As was measured by passing one aliquot of 25 ml broth through 0.22 μm filters. The filtrate was dried and As concentrations measured in duplicate. As concentrations were measured from other 25 ml unfiltered broth.

Method of Koroleff (1976) was followed to measure As concentrations. Being isomorphic with phosphate ions the arsenate ion forms also yellow and blue complexes of molybdate (Koroleff, 1976). As in the case of phosphate measurements, the formation of arseno-molybdenum blue offers the greatest sensitivity for measuring As. The following steps were adopted to quantify As in broth. As per Koroleff (1976), filtrates were acidified with 0.5 ml 9N H_2SO_4 and, 50 ml filtrates from strains collected from midstream, 500 mg of solid seawater mix (composition: 310 mg NaCl and 190 mg $MgSO_4.7H_2O$) added. Into 50 ml filtrate of seawater NB, $MgSO_4$ (190 mg) was added. Into each filtrate, 2 ml oxidation solution (composition: 5 g potassium peroxidisulphate [$K_2S_2O_8$] in 100 ml distilled water) was added and, held at 121°C for 30 min under 15 PSI pressure.

Following this, each filtrate was divided into two equal volumes (flask Aand B) of26 ml each. To one of them, 0.30 ml of iodate solution (w/v: 0.15 g KIO_3 in 100 ml distilled water) and 0.70 ml of ascorbic acid (w/v: 7 g $C_6H_8O_6$ in 100 ml distilled water) were added. After 5 min, 0.5 ml of freshly prepared mixed reagent (composition: 9 ml ammonium heptamolybdate solution [$(NH_4)_6Mo_7O_{24}.4H_2O$], 25 ml 9N sulphuric acid, 1 ml potassium antimony tartrate solution [$K(SbO)C_6H_4O_6$] and 65 ml distilled water) was added and held at room temperature for 90 min. To other flask, 0.5 ml of ascorbic acid was added and kept aside for 75 min. Then, 0.25 ml of sodium thiosulphate reagent (w/v: 1.7 g $Na_2S_2O_3.5H_2O$ in 100 ml distilled water and, 50 mg of Na_2CO_3) was added. After 15 min, 0.5 ml of mixed reagent was added and allowed to stand for 5-7 min. The ascorbic acid and mixed reagent being common, the iodate solution was added to flask A and, thiosulphate solution to flask B. All analyses were done in replicates of two sub-samples. The amount of arsenic calculated from the expression:

$$\mu g \text{ at As } L^{-1} = F (A_a - A_b),$$

Where, A_a is absorbance of solution in flask A;

A_b is absorbance of solution in flask B and,

F, calibration factor

The above procedure was performed in full with distilled water for determining the reagent blank. Further, to obtain the calibration factor, a dilute standard solution (DSS) containing 0.4 µg at As L^{-1} in distilled water was prepared. In brief, 100 ml DSS was made up to 200 ml with synthetic seawater (composition: 6.4 g NaCl, 2.8 g $MgSO_4.7H_2O$, 0.03 g $NaHCO_3$ in 200 ml distilled water) and, 2 ml of 9N sulphuric acid added. Similarly, a blank solution (BS: equal volumes of synthetic seawater and distilled water) was also treated with 2 ml of 9N sulphuric acid. Two milliliters of oxidation solution was added into both DSS and BS and held under 15 PSI pressure at 121°C for 30 min. Following this the steps followed were the same as done for flask A above but for holding these flasks for 120 min before reading the absorbance using 10 cm column. The factor F was calculated from the expression,

$$F_{10\ cm} = 0.2 / A_{st} - A_b$$

Where, A_{st} is mean absorbance of standards and,

A_b is the mean of the blank

5.3 Results

Quantification of As biotransformation was carried out for thirteen ARB strains: Enterobacteriaceae (1 and 3), *Pseudomonas* sp. (2 and 7), *Corynebacterium* sp. (4), *Xanthomonas* sp. (5), *Acinetobacter* sp. (6), *Flavimonas* sp. (8), *Micrococcus* sp. (9), *Bacillus* sp.(10), *Staphylococcus* sp.(11), *Rhodocccus* sp.(12) and *Planococcus* sp.(13). As detoxifying potential was checked to see if effective bacterial bioremediationwas achievable using these ARB strains. From the quantification of arsenic removal by thirteen ARB strains, it was observed that amount of As removed was substantial (>85% of initial concentration) within 120 h by most of them. By 24 h, the As biotransformed was 45% in the growth medium and it was ~ 80% in 48 h and 92% in 120 h by Enterobacteriaceae (3). In all the cell-free filtrates on all

sampling days, the As concentrations were invariably low. Cell-boundAs built up only marginally between day 2 and 4 and by 120 h, it was very low in all thirteen strains (Figure 5.1, 5.2 and 5.3).

In case of Enterobacteriaceae (1) it was observed that amount of As removed was significant (>90% of initial concentration) by 120 h. It was 50% by 48 h and close to 80% by 96 h. Similarly in case of Enterobacteriaceae (3) it was observed As biotransformed was as much as 50% by 48 h and close to 80% by 96 h. Amount of As removed was >90% of initial concentration within 120 h (Figure 5.1). Bacterial isolates namely *Pseudomonas* sp. (2) and *Corynebacterium* sp. (4) removed As (>85%) within 96 h. Indeed, it converted 95% As by 120 h. Biotransformed As was close 40% by 24 h and 70% by 72 h in case of *Micrococcus* sp. (9), it biotransformed as much as 92% (Figure 5.3) of the As by 120 h.

The percent As biotransformed was comparatively less in the case of *Acinetobacter* sp. (6). By 24 h it removed 30% of As and ~60% by 72 h.It biotransformed >70% of initial As concentration from the growth medium by 120h (Figure 5.2). The percent As biotransformed was the highest by *Planococcus* sp. (13) and *Micrococcus* sp. (9) followed by Enterobacteriaceae (3), *Xanthomonas* sp. (5), *Rhodococcus* sp. (12), *Pseudomonas* sp. (2 and 7), *Staphylococcus* sp. (11), *Bacillus* sp. (10), *Corynebacterium* sp. (4), *Flavimonas* sp. (8) and *Acinetobacter* sp. (6). However time taken for complete reduction was different among the tested strains. Isolate Enterobacteriaceae (1 and 3), *Pseudomonas* sp. (2 and 7), *Micrococcus* sp. (9), *Xanthomonas* sp. (5), *Staphylococcus* sp. (11), *Rhodococcus* sp. (12) and *Planococcus* sp. (13) reduced it completely (as much as>90%) in 120 h, while others needed more duration for completing the reduction coinciding with their attaining peak density. By the strain of *Acinetobacter*, the reduction was not total.

In the case of *Staphylococcus* sp.(11) it was observed that amount of As removed was significant (90% of initial concentration) by 120 h (Figure 5.3). The As removed was 50% by 48 h and 75% in 96 h. Similarly in case of *Flavimonas* sp. (8) and *Bacillus* sp. (10) removal was ~40% by 48 h and 70% by 96 h. Amount of As removed was close to 85% of initial concentration within 120 h (Figure 5.2 and 5.3). Bacterial isolates namely *Xanthomonas* sp. (5), *Pseudomonas* sp. (7), *Rhodococcus* sp. (12) and *Planococcus* sp. (13) removed As (>80%) within 96 h. Indeed, it

biotransformed as much as 90% of the As by 120 h (Figure 5.2 and 5.3). In the case of *Planococcus* sp. (13) it was observed that amount of As removed was significant (90% of initial concentration) by 120 h (Figure 5.3). The As removed was 45% by 48 h and 85% by 96 h.

5.4 Discussion

Arsenate is chemically similar to phosphate. Its resistance in microflora, in particular bacteria, is related in its specific energy-dependent membrane pumps. Being a toxic analogue, arsenate is used in phosphorylating activities (Summers and Silver, 1978) and arsenite on the other hand, can bind to dithiols and/or proteins. Detoxification of As_2O_5 and oxidative transformation are microbially mediated and serve in As cycling in aquifers and soils (Oremland and Stolz, 2003). Mobilization of arsenite from soil to aquifer is a serious threat. Osborne and Ehrlich (1976) proposed that arsenite oxidation is a detoxification step. Also methylation is other mechanism of detoxification. The uptake of As and its compounds leads to their modification. In changed forms, its compounds may be facilitated an upward accumulation in the food chain (Chen et al., 2009; Liu et al., 2009), magnified, and through seafood intake by humans certainly likely to result in ailments of either acute or chronic (Barwick and Maher, 2003).

Oxidization of arsenite to arsenate is reported to occur in many bacteria (Osborne and Ehrlich, 1976; Phillips and Taylor, 1976; Gihring et al., 2001; Langner et al., 2001). Results from this study ascertain the ability of the ARB used in the experiments to grow in high As concentrations. This is a promising evidence for their application in clean-up of As contaminated sites. Prevalence of quite a number of isolates in disperate species/genera tolerating and growing in high As concentrations. This is similar to observation by Summers and Silver (1978) and Silver et al. (2002), all heterotrophs examined in this study, apparently possess the inducible arsenic reductases which enable their rapid growth following adaptation to toxic milieu.

Resistance to Hg and/or other toxic substances is reported from many heterotrophic bacterial species (Osborn and Ehrlich, 1976; Silver and Phung, 1996; Nies, 1999; Ramaiah and De, 2003; De and Ramaiah, 2006; De et al., 2007 and 2008). In addition, in toxic milieu, marine microflora is shown to undergo selection. The array of microbes reducing arsenate or oxidizing arsenite (Weeger et al., 1999) do detoxify both these forms of As.

From the observations of this study, it is suggested that rapid biotransformation of As by all 13 ARB strains is possible and such high tolerance is

effective in altering arsenite in polluted environments. The oxidation of arsenite to arsenate by these ARB represents their potential detoxification and, can be useful in bioremediation applications (Oremland et al., 2004). Similarly, *Thiomonas* sp. is reported to have the ability to oxidize arsenite (Casiot et al., 2003). Also reported to oxidize arsenite are strains in *Pseudomonas*, *Xanthomonas* and *Achromobacter* (Turner, 1949; Campos et al., 2010). It is seen in this study strains belonging to different genera are growing in media containing arsenite and detoxify it. The results presented here demonstrate they could represent the prevalence of many candidate/species of bacteria and can be explored for arsenic remediation in polluted sites laden with As.

Efficient detoxification arsenite, a highly toxic metalloid, from natural systems is of great relevance. Being a primary pollutant in drinking water its removal is vital (Zouboulis and Katsoyiannis, 2004; Chiban et al., 2012). Strains isolated in this study from estuarine (brackish) waters, as Rosen (2002) and Saltikov et al. (2003) propose, are of significance in arsenic detoxification. Since the isolates examined in this study are from natural systems, they are more acceptable and safe for As and other toxicants-detoxification.

The ARB isolates from the study area appear to be a vital set for examining their As biotransformation potential. ARB fromthis study suggests do show a biotransformation potential for prospecting them in toxic waste bioremediation. Further, studies on their detailed metabolic pathways and genetic constitution responsible for mediating detoxification is necessary to ascertain their bioremediation efficiency. Some aspects of genes involved in As detoxification pathway are described in chapter 8.

6.1 Introduction

Some heavy metals are in fact, essential as trace elements for many metabolic activities (Chowdhury and Chandra, 1987; Prasuna and Varaand, 2012). At high concentrations however, they can bedeleterious to most life forms. They tend to induce production of complex compounds that are often detrimental to the cellular functions. Since toxic metalloids and metals persist in natural systems as a result of their release from industrial processes or other anthropogenic activities, they are reported to possess tolerance and grow in their milieu (Mukhopadhyay et al., 2002; Rosen, 2002). Pathways such as efflux, complexation, or reduction (Stolz et al., 2002; Silver and Phung, 2005) are reported.Spain (2003) observed that the use of heavy metal ions as terminal electron acceptors in anaerobic respiration is well known.

Persistenceof toxicants bears extensive, prolonged and ill effects many life forms in aquatic/terrestrial habitats. Moreover, they are not easily or quickly removed. Thus the toxicmetals accumulate and get biomagnified in the process (De et al., 2003). Ultimately they end up inhumans producing ailments which can be acute or chronic (Forstner and Wittmann, 1979). Indeed, De Flora et al. (1994) reported numerous mutations in DNA as well.

Although the role of arsenate-reducing or, arsenite-oxidizing Bacteria and Archaea is realized to be important, detailed information on sub-surface mobility of As and responsible processes by native population structure in estuarine ecosystems is lacking. In this study, arsenic resistant bacterial strains isolated from estuarine, marine and midstream locations were identified based on 16S rDNA based characteristics. Sequencing data of these isolates are used to construct phylogenetic trees to establish their evolutionary relationship to other bacteria.

6.2 Materials and Methods

6.2.1 Selection of Arsenic Resistant Bacterial Isolates

Details of isolation of ARB are presented in chapter 3. Briefly, many isolates growing in NA with 50 ppm As were grown in media containing up to 1000 ppm As. All those isolates capable of tolerating ≥100 ppm Aswere termed as arsenic resistant bacteria (ARB). All these isolates were subjected to ARDRA profiling. After careful selection

of unique ARDRA profiles as many as 123 select were taken up for 16S rDNA sequencing.

6.2.2 Extraction of Genomic DNA

From all 500 ARB isolates, the DNA was extracted using DNeasy extraction kit using the protocol provided by Qiagen. The pure cultures of bacteria grown overnight in 5 ml NB. Nutrient broth containing 25% seawater was used for the samples from estuarine while NB with distilled water was used for the midstream samples. The 24 h old culture was spun at 1000 rpm for 1 min, and supernatant discarded. Pellets resuspended in 160 µl of enzyme lysis buffer and 40 µl of lysozyme, and held at 37°C for 30 min. Then, proteinase K (20 µl) and RNase (4 µl) were added and held for 2 min at RT. To these tubes, 200 µl of AL buffer was individually added and ensuing mixture kept at 56°C for 10 min. After adding 200 µl of ethanol, the above mixture was pippeted into DNeasy mini spin column placed in a 2 ml collection tube and centrifuged at 3000 rpm for 1 min. The supernatant was discarded, the mini spin-column placed into a collection tube and 500 µl of AW1 buffer was added. The assembly was spun at 8000 rpm for 1 min. The fitrate was discarded. This process was repeated using buffer AW2 at 14000 rpm. The DNA binds during centrifugation to DNeasy membranes while all other components pass through. The mini spin column then placed into a fresh 1.5 ml centrifuge tube to elute DNA following incubation with 200 µl of AE buffer and centrifuging at 8000 rpm for 1 min. The eluted DNA samples were quantified using the Nanodrop and to check the quality of the DNA.

All the DNA extracts were electrophoresed through agarose gel to confirm the integrity. Further, 260/280 ratios were checked to find out if the desired range of 1.7-2.0 as to ensure purity and to note the contamination, if any. DNA extraction was repeated from a few samples when the quantity/purity was not satisfactory for further analyses.

6.2.3 PCR Amplification of 16S rRNA Gene

16S rRNA gene from all 500 DNA samples was amplified using the PCR. The universal primers: forward primer 27f (5' - AGAGTTTGATC(AC)TGGCTCAG - 3') and reverse primer 1492r (5' - CTACGGCTACCTTGTTACGA - 3') were used. PCR

reactions were performed in a final volume of 25 µl using a thermal cycler Veriti (Applied Biosystems, USA). Each reaction tube with 0.5 µl the template DNA, 2.5 µl Taq buffer, 2.5 µl each of the four dNTPs, 1µl each of reverse and forward primers, 0.25 µl of Taq polymerase and 17.25 µl of autoclaved deionised water. The PCR parameters were: 94°C for 4 min for initial denaturation followed by 35 cycles each at 94°C for 1 min denaturation, 1 min of annealing at 55°C and one min of extension at 72°C. Negative control having the reaction mixture without the tempelate DNA was also run along with the samples for ascertaining reagent and sample purity and to ensure propoer sampling/analyses.

The resulting PCR amplicons were run through 1% agarose gel using 1kb molecular ladder ranging from 300 bp to 10000 bp. The PCR products were subjected to purification using Axygen purification kit (Axygen) as per the manufacturer's instructions. All properly amplified PCR products were purified and it was found that the ribosomal DNA for all the samples had been amplified were ~1500 bp in size.

6.2.4 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

In order to evaluate the restriction pattern of 16S rDNA, all 500 DNA samples were subjected to *Msp*I and *Hha*I restriction analyses. For this, 5 µl16S rDNA PCR amplicon digested at 37°C adding 5 Units of endonucleases namely *Msp*I (5'…C↓C GG…3') and *Hha*I (5'…GC G↓C…3') in restriction buffer (Fermentas) in 15 µl volume for 3 h. A 100 bp DNA marker ranging from 100 bp to 3000 bp was used. Restriction digests consisted of 10 µl mixture and 5 µl amplified PCR amplicon. Up to 5 µl of amplified PCR products were digested with 10 µl of ARDRA mastermix, which is composed of water (7 µl), 1X tango buffer (1 µl) and restriction enzymes (1 µl each). The mixture was incubated at 37°C for 3 h. Restriction digests mixed with 3 µl of gel loading dye and ran on 2% agarose gels at 150 V for 2 hor more. After staining with ethidium bromide (0.5 µl/ml) polymorphism was looked for. Restriction fragments were measured from all gels to note the differences in their sizes.

6.2.5 Purification of PCR Products

In order for ensuring good quality DNA for sequencing, Axyprep PCR purification kit was used for PCR clean-up. To achieve this, three volumes of PCR-A buffer was added to one volume of PCR amplicon (e.g. 75 µl of PCR-A for 25 µl amplicon) and

was vortexed briefly to mix the contents. A PCR column placed in a 2 ml eppendorf tube and the above solution pipetted into the PCR column to spin at 14000 rpm for 1 min. After discarding the filtrate, 700 µl of buffer W2 pipetted into the column and spun again for a min at 14000 rpm. Again filtrate discarded and 400 µl of buffer W2 pipetted into the column spin once more at 14000 rpm for one min. The PCR column transferred into an eppendorf tube and 25-30µl of the eluent (prewarmed at 65°C) was carefully added to the centre of the membrane in the column. The tubes then allowed for 5 min at RT for efficient elution of DNA. The tubes were then centrifuged for one min at 14000 rpm for collecting purified PCR product and held at -20°C until sequencing.

6.2.6 Sequencing of 16S rDNA

Sequencing was performed with 15 to 50 ng of the PCR amplicon adding one pmol each forward (27f- AGAGTTTGATC(AC)TGGCTCAG) and reverse primer (1492r-CTACGGCTACCTTGTTACGA) using an ABI 3130 Genetic Analyzer following dideoxy chain termination technique. Resulting 16S rDNA sequencing results were compared to those in National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), for their matches using BLAST programs and the identification confirmed.

6.2.7 Phylogenetic Analysis

Sequences obtained by dideoxy chain termination method were compared by searching for their homologs in NCBI database using the BLAST programs. Phyologenetic trees were constructed using Neighbor joining method using Mega 4 by default settings of the program. For constructing phylogenetic tree EzTaxon database was used which gives type strain identification.

6.3 Results

As detailed above, 500 ARB collected from different locations in Mandovi-Zuari estuarine complex were examined. The genomic DNA from all 500 isolates was extracted and purity ensured by checking the extracts for A_{260nm}/A_{280nm} ratio within 1.7 - 2.0. The extracted DNA was amplified for 16S rRNA gene using 27f (forward primer) and 1492r (reverse primer). The products thus obtained were electrophoresed

on 1% agarose gel, and it was found that the ribosomal DNA for all the samples had been amplified were ~1500 bp in size (Figure 6.1a, b, c, d, e, f, g and h). All 500 amplified PCR products loaded on 1% agarose gel, lane 1, 13 and 25 with 1Kb DNA marker (300 bp to 10000 bp) and, lane 2 to 12 and lane 14 to 24 with PCR amplified products while lane 24 in the third row is negative control.

The restriction profiles of PCR amplified 16S ribosomal DNA (ARDRA) of all 500 ARB was used to analyse the RFLP. For this, the 16S rRNA fragments amplified and digested by *Msp*I and *Hha*I were analysed by agarose gel electrophoresis. In all, the ARDRA generated 123 different profiles from the 16S rRNA gene resetriction digestion of 500 cultures. Different restriction profiles generated for 500 samples are shown in Figures 6.2a, b, c, d, e, f, g and h. As can be seen, the ARDRA profiles of 16S rDNA samples shown in Figure 6.2a (lanes 2 to 12) lanes 14-24 (first row) and lanes 2-6 (second row) are identical. The ARDRA profile of the DNA samples in lanes 7-11 and lanes 14 to 15 differ from the first profiles thus yielding 2 different patterns, which are different from as many as 39 DNA extracts from bacterial isolates capable of growing in the seawater nutrient agar with As. Lanes 16-22 in second row and lanes 10-12; lanes 14-21 represent one type of ARDRA profile. Lanes 23-24, in the second row as well as lanes 2-9 and lanes 22-23 in the third row represent a pattern different from others (thus, four different ARDRA patters are shown in this gel). Lane 1, 13 and 25 are for 100 bp DNA marker (ranging from 100 bp to 3000 bp).

Figure 6.2b shows five more different ARDRA profiles. Six different ARDRA pattern are shown in Figure 6.2c. Figure 6.2d depicts seven different ARDRA patterns. Similarly 11 different patterns are shown in Figure 6.2e. Varied diverse profiles are seen on gel (Figure 6.2g) with 24 ARDRA patterns from a total 65 isolates. Similarly, 15 different patterns are shown Figure 6.2h from total of 45 PCR amplified products of different bacterial isolates. ARDRA profiles were not compared from gel to gel but within gel and hence total of 123 different ARDRA patterns were observed. These 123 different ARDRA patterns were the main basis for choosing the DNA samples for sequencing.

Twenty-seven Gram -positive and -negative ARB belonging to various species and genera identified. From their 16S rRNA gene sequences of the ARB, it was

possible to recognize 12 different genera and 27 different species with 99% similarity. Of the 123 ARDRA based groups, as many as 63 belonged to the genus *Bacillus*, 14 to *Rhodococcus* and 13 to *Psychrobacter* sp. The sequencing was performed for a total of 123 amplified PCR products distinguished by ARDRA profiles. The following phylogenetic similarities were observed among the ARB species (total number of strains in parantheses). A total of 12 different bacterial groups were identified (Table 6.1). The16S rRNA gene sequences based phylogenetic analysis represented 27 different genera/species namely belonging to *Psychrobacter* sp. (9 isolates), *Psychrobacter celer* (4), *Bacillus* sp. (31), *Bacillus aquimaris* (5), *Bacillus baekryungensis* (16), *Bacillus cereus* (1), *Bacillus horikoshii* (4), *Bacillus hwajinpoensis* (1), *Bacillus marisflavi* (2), *Bacillus subtilis* (2), *Bacillus tequilensis* (1), *Pantoea agglomerans* (1), *Pantoeadispersa* (2), *Staphylococcus arlettae* (3), *Dietzia* sp. (8), *Kocuria* sp. (2), *Kocuria flava* (1), *Erwinia* sp. (2), *Rhodococcus* sp. (9), *Rhodococcus erythropolis* (4), *Rhodococcus equi* (1), *Pseudomonas* sp. (1), *Pseudomonas putida* (5), *Acinetobacter* sp. (5), *Acinetobacter johnsonii* (1), *Paenibacillus* sp. (1) and *Planococcus maritimus* (1).

The phylogenetic trees (Figs 6.3a, b, c) clearly imply high homology between ARB from different isolates which have clustered in the same clade. Phylogenetically diverse ARB are present in water and sediment samples of estuarine regions off Goa. The phylogenetic tress constructed indicates that the ARB are distributed in three different Phylum namely Actinobacteria (21.0%), Proteobacteria (24.2%) and Firmicutes (54.8%) respectively. Genera, *Kocuria*, *Rhodococcus* and *Dietzia* belonging to Phylum Actinobacteria (Figure 6.3a), *Pantoea*, *Pseudomonas*, *Acinetobacter* and *Psychrobacter* belong to PhylumProteobacteria (Figure 6.3b) and, *Bacillus*, *Planococcus*, *Paenibacillus* and *Staphylococcus* to Phylum Firmicutes (Figure 6.3c).

6.4 Discussion

In high concentrations, it is a well known fact that intracellular toxic molecules are the reaction products of heavy metal ions (Nies, 1999). Heavy metal ions ought to be inside the cell for toxicity to show up. For bacterial growth and enzymatic functions some heavy metals are made use of, as mentioned earlier. Therefore, uptake processes do occur at molecular levels allowing cytoplasmic entry of ions (Spain, 2003). Among the two known processes is a quick, not requiring energy/ATP and nonspecific one regulated by chemi-osmotic membrane gradient. The other process is a slower, energy dependent and highly substrate-specific one (Issazadeh et al., 2013). The former being highly energy efficient toxic metal ion influx, in particular when such ions and their compounds or ions are present in high concentrations.

To cause toxic effects inside the cell as Nies and Silver (1995) propose microbes are to be susceptible. In order to thwart and continue to remain metabolically active when metal-stress is prevalent many microbes do possess different mechanisms. They are metal ion-efflux, complexation, accumulation, and/or reduction of toxic metal ions (Nies, 1999). These are quite important metal tolerance mechanisms in microbes. Khan et al. (2009) found that certain microbes in metal-contaminated habitats, perform certain special respiration processes. As oxidation of metal ions determine their solubility, some investigators (Rajendran et al., 2003; Wuana and Okieimen, 2011; Akhtar et al., 2013) demonstrated the use of those microbes capable of oxidizing or reducing toxic metals allowing remediation of polluted locations.

For As to be toxic, it ought to be bioavailable. Its intake in microbes is by analogous phosphate transporters and its expunging by efflux pump (Nies and Silver, 1995). Microorganisms do possess numerous strategies to deal with As toxicity. Among the well understood one is the presence of an array of genes in *ars* cluster (Zheng et al., 2013; Fernandes et al., 2014). Both Gram positive and negative bacteria make use of similar biochemical modes producing gene/s in either plasmid or chromosome (Ji and Silver, 1992b and 1995). The number of genes, as Silver and Phung (1996) proposed, can vary and even their functions may differ.

The DNA extracted from the 500 bacterial isolates from estuarine regions of Goa showed a 260/280 ratio between the desired range of 1.7 and 2.0 assuring proteins and/or other cellular components were minimal or absent. Such high quality genomic DNA was used to specifically amplify the 16S ribosomal RNA gene by using universal primers for these genes and running a polymerase chain reaction. On electrophoresing the products thus obtained the amplified product from each sample banded at ~1500 bp as observed by comparing with the molecular ladder after ethidium bromide staining.

The PCR amplicons were examined for ARDRA with the mixture of two restriction enzymes namely *Msp*I and *Hha*I. The ARDRA profile for 500 isolates generates 123 different patterns. Of the 123 different ARDRA pattern/clusters, 63, 14 and 13 belonged to*Bacillus*, *Rhodococcus* and *Psychrobacter* respectively. Eight found to belong to *Dietzia* and six were found to belong to *Pseudomonas* and *Acinetobacter* sp. while others were found in less numbers belonging to *Pantoea*, *Staphylococcus*, *Kocuria*, *Erwinia*, *Paenibacillus* and *Planococcus*. The prevalence of relatively large number of strains in different genera tolerating and growing in higher concentration of arsenic was thus evident.

Previous reports showed the presence of As tolerant/resistant strains of *Vibrio*, *Pseudomonas*, *Staphylococcus* and *Acinetobacter* species form natural and arsenic contaminated sites (Anderson and Cook, 2004; Saltikov and Olson, 2002; Pepi et at., 2007; Fan et al., 2008 and Chang et al., 2010). During this study, I have identified ARB isolates in *Psychrobacter*, *Bacillus*, *Pantoea*, *Staphylococcus*, *Dietzia*, *Rhodococcus*, *Pseudomonas*, *Acinetobacter*, *Kocuria*, *Erwinia*, *Paenibacillus* and *Planococcus* genera from Mandovi and Zuari region. In a previous study, Nagvenkar and Ramaiah (2010) had observed some estuarine strains had abilities to grow in higher concentrations of arsenic upto 1000 ppm.

In the overall, results from this study imply the prevalence of numerous ARB species in estuarine waters off Goa where the As Concentrations are far lower. It can therefore be proposed that resistance mechanism in such bacteria is regardless of As concentration levels.

The As tolerance/resistance in bacterial isolates is acquired apparently via energy dependent efflux of arsenate or arsenite by *ars* genes-system mediation (Ji and Silver, 1992a, b and Cervantes et al., 1994). Present results imply that these new reports of ARB would be useful in providing an insight on molecular mechanisms and, are good candidates for bioremediation of As-polluted sites. However, detailed information on ecosystem laden with As and other toxic materials based on bacterial As tolerance/resistance obtained via advanced analyses would prove worthwhile.

A plentitude of ARB and microorganisms isolated from different ecosystems is available (Turner, 1949; Turner, 1954; Turner and Legge, 1954; Osborne and Enrlich, 1976; Phillips and Taylor, 1976; Ilyaletdonov and Abdrashitova, 1981; Gihring et al., 2001; Saltikov and Olson 2002; Anderson and Cook, 2004; Rokbani et al., 2010). Among these are isolates tolerant/resistant from very low or no As contaminated (Oliveira et al., 2009) and arsenic contaminated environmental samples (Chopra et al., 2007). Saluja et al. (2011) isolated *Bacillus* sp. from heavy metal contaminated environments resistant to 160 mM $Na_2As_2O_5$. Pennanen et al. (1996) proposed that in field sites with prolonged contamination, the microbial populations could easily develop tolerance to stress due to metals and metalloids.

The isolates collected and analysed for this study possessed very high As resistance compared to many marine strains examined by other studies reported so far. These and similar ones are of genuine interest both for revalidating contemporary opinions on resistance to As resistance and for elucidating the importance of As resistance in native microflora.

High As concentration/contamination do exert selective pressures and a generally low diversity *in situ* (Jackson et al., 2005; Achour et al., 2007). However, sampling locations used in our study receive discharges containing iron and manganese from mining areas (Nair et al., 2003) leading to selection of certain species already well adapted to high As contaminations.

Notably, from this study it can be suggested that restriction digestion of the amplified rRNA gene enables differentiation and identification of bacterial strains, unambiguously. The careful differentiation of ARDRA fragments is useful to emphasize here that there was no missing of any other possible genus or genera in 500

DNA extracts. This fact is reflected in the species obtained during this study based on 16S rRNA gene sequencing. Not a single ARDRA band was chosen which was a repetitive of selection. This was also further confirmed by sequencing the DNA from isolates with the identical ARDRA profiles. Therefore, it can be suggested that a careful choice of restriction enzymes and optical electrophoretic conditions are useful in reducing the sequencing efforts.

Due to prevalence of quite a number of strains in as many as a dozen different genera capable of tolerating and growing in higher concntration of arsenic, it is possible from this study to emphasize that such naturally As tolerant/resistant bacteria are superior candidates of choice in Asdetoxification efforts.

7.1 Introduction

The global contaminant arsenic (As), exists mainly as arsenate: AsO_4^{3-} or $HAsO_4^{2-}$, as arsenite: AsO^{2-}or H_2AsO^{3-}. Both of these inorganic forms are toxic to life processes with arsenite being several times more toxic than arsenate (Cullen and Reimer, 1989). Toxicity of arsenate arises from it being analogous to PO_4^{3-}. Due to its interference with phosphate transport intracellularly it hampers the whole of cell metabolic processes. To thwart arsenate entry, certain microbes possess genetic elements which facilitate neutralization of As toxicity effects either by exclusion or extrusion (Gihring et al., 2003). Studies on *Escherichia coli* operons of *ars* (Carlin et al., 1995), and of *Pseudomonas aeruginosa* (Cai et al., 1998) and *Staphylococcus* spp (Silver et al., 1981; Rosenstein et al., 1992) have affirmed that *ars* operons consist of *ars*R, *ars*Band *ars*C. Further, *E. coli* plasmids R773 (Chen et al., 1986) and R46 (Bruhn et al., 1996) and, plasmid pKW301of *Acidiphilium multivorum* (Suzuki et al., 1998) have five genes: *ars*RDABC.

Being both structural and functional entities of the cell, proteins are pivotal to the living world. The protein encoded *ars*RDABC operon helps in As detoxification. The aresenite binding to ArsR leads to repressor protein-dissociation from promoter DNA (Shi et al., 1996). Detailed account of ArsA role in the functioning of extrusion pump ArsAB is provided by Zhou et al. (2000). Additionally, Martin et al. (2001) and Messens et al. (2002) have described various aspects of ArsC, the arsenate reductase that reduces AsO_4^{3-} to AsO_2^{-}thus aiding in deciphering As detoxification process. Also Lin et al., (2006) have reported a metallochaperone,ArsD, in *E. coli* that is ascribed to attach arsenite to ArsA for extrusion.

Inspite of the knowledge that As is a noted toxic metalloid and a human carcinogen, studies on its formation of ligands or molecular complexation are lacking. Expression of the proteins in three strains tolerating higher concentration (1000 ppm) of As was examined in this study. In addition to examining the growth dependant expression of proteins, identification of peptides/proteins responsible for As resistance, the types of As/metal modulations proteins that became prominent was evaluated through proteomic approach.

7.2 Materials and Methods

Quantitative estimations of protein concentrations and expression of proteins that are As/metal binding were made. Three bacterial strains growing in 1000 ppm As were used for studying protein profile. The cultures of *Staphylococcus arlettae* (KJ719326),*Planococcus maritimus* (KJ719380) and *Bacillus baekryungensis*(KJ719328) were grown in the nutrient broth containing different concentrations of arsenic (10, 50, 100, 200, 300, 500 and 1000 ppm) with flasks with no added arsenic nutrient broth (controls).

7.2.1 Protein Extraction

Five ml of 24 h old cultures were aliquoted and spun at 5000 rpm at 4ºC for 10 min. After discarding the supernatant, the cell-pellets resuspended in 150 µl of lysis-B reagent containing EDTA-free protease inhibitor cocktail tablets (Roche, Germany) by vortexing until the cell suspension is homogeneous. After one min, the mixture was spun at 13000 rpm for 5 min to pellet any insoluble proteins. Soluble protein portion in the supernatant was carefully removed for analyses. For analyzing the insoluble protein fractions, 150 µl of lysis-B reagent with EDTA-free protease inhibitor cocktail was added to this pellet for bringing it into solution.

7.2.2 Protein Estimation

The Bradford method (Bradford, 1976)was employed to measure protein concentrations. The protein was estimated following the manufacturer's protocol using bovine serum albumin (BSA; w/v 1 mg ml^{-1}) provided in protein estimation kit. Protein concentration estimated spectrophotometrically (UV-1800, Shimadzu) at 595 nm wavelength. Three replicate samples were used for this measurement.

7.2.3 1-D Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis (PAGE) was done according to Laemmli (1970). This method allows determination of relative molecular mass of the protein with 16% polyacrylamide resolving gel and 8% stacking gel. After boiling for 5 min in a buffer of 0.5 M Tris-HCl; 2% (w/v), SDS; 10% (w/v), glycerol; 0.025% (w/v), bromophenol blue and 50 µl mercaptoethanol, the samples were loaded on to the gel.

Equal concentrations of each soluble as well as insoluble fraction were loaded. The SDS-PAGE which was run using broad range (205 to 3.5 KDa) and medium range (97.4 to 14.3 KDa) protein markers (perfect protein marker, GeNei). Electrophoresis was carried out at 90 V in SDS-tris-glycine-buffer in a dual slab-electrophoretic cell. The gels were kept overnight in a fixative solution and stained using silver nitrate and in finally distilled water prior to scanning for documentation. For profiling the proteins, protein extraction was done every 2 h until 24 h and SDS-PAGE electrophoresis performed. Equal volumes of all extracts from 2 h to 24 h were loaded for unambiguous profiling of different proteins expressed in the absence or presence of As.

7.2.4 2-D Gel Electrophoresis

Two-dimensional gelelectrophoresis on protein extracts of three bacteria *Staphylococcus arlettae*, *Planococcus maritimus* and *Bacillus baekryungensis* was done. Briefly, these ARB tolerating 1000 ppm As were grown in NB containing 50 ppm and 100 ppm As. Controls without As were also run. Prior to iso-electric focusing (IEF), the salts, detergents and other interfering substances from protein samples were removed using Perfect-FOCUS kit following the manufacture's protocol. To 1-100 µg protein solutions 300 µl UPPA-I was added and mixed well. This was held at 4-5ºC for 15 min. Following the addition of 300 µl of UPPA-II, the solution was spun at 12000 rpm for 5 min, and the supernatant was discarded. Then 40 µl of FOCUS-Wash was added mixture was spun for 5 min and the wash was discarded. 25 µl of pure water was added and vortex. After adding 1 ml OrgoSol buffer, pre-chilled at -20ºC and 5 µl SEED, the above mixture was kept for 30 min at -20ºC. This was centrifuged at 14000 rpm for 5 min to form a tight pellet and, the supernatant drained out. White pellet in the tube was allowed to air dry until it becomes translucent. Then, protein pellet suspended in an apt vol of 2-D buffer and load on IEF gel.

Iso-electric focusing (IEF) of protein samples was carried out. An IPG strip of pH 3-10, 7 cm long was rehydrated overnight at room temperature with equal concentration (100 µg) each of soluble as well as insoluble protein samples was mixed with 2-D buffer and the gels run per manufacturer's protocol. Finally the gel

images were taken by densitometer, using Quantity One software (BIO-RAD) and proteins were analysed using PDQuest software.

7.2.5 Mass Spectrometry Using Q-ToF

The2-D gel electrophoresis was employed for examining protein profiles. The main aim was to analyze the protein profiles that help in understanding As resistance mechanism. Following the 2-D GE, selected protein spots recognised by Q-ToF mass spectrometry. The protein samples from *Bacillus baekryungensis*,that were loaded for iso-electric focusing (IEF) following ‘‘in gel rehydration’’ were examined by Q-ToF (Agilent Technologies 6538 UHD Acuurate-Mass Q-TOF LC/MS).

Protein spots of interest in the in-gel digestion, were excised from polyacrylamide gel using spot picker and placed in an autoclaved 1.5 ml eppendof tube. They were washed twice with milliQ water by vortexing vigorously. Aliquot of 100 µl of freshly prepared solution (1:1; v/v) of sodium thiosulphate (100 mM) and potassium ferricyanide (30 mM) was added and kept for 10 min at RT or until color disappeared completely. Gel pieces were further destained and dehydrated for 5 to 10 min with 50 mM Ammonium bicarbonate (NH_4HCO_3)-50 per cent Acetonitrile (1:1 v/v). All standard procedures were then followed before subjecting to mass spectrometric analyses.

For in-solution digestion, the proteins extract (50 µg) in cell lysate were precipitated using pre-chilled acetone in 1:10 ratio. The lysate was kept overnight at -80ºC after adding acetone to the lysate. The proteins that got precipitated were centrifuged at 12000 rpm for 10 min or till a tight pellet is formed and air dried. The resulting pellet resuspended in 500 µl of 6M urea (w/v) by pipetting up and down to ensure proper mixing. Thirty microlitres of the above mixture were transferred to 0.5 ml tube and 3 µl 200 mM DTTadded. This was kept at RT for 1 h. It was further incubated for 30 min in dark at room temperature after adding 8 µl of 200 mM IAA. Eight microlitres of 200 mM DTT were added to this and incubated further for one h at RT. Then, 250 µl of 50 mM Tris-HCL was added to reduce urea concentration to ~0.6 M. To this, 20 µg (1 µl) of Trypsin solution was added. The mixture was gently tapped and kept overnight at 37°C. 10 µl of this digested was used for the mass

spectrometric analysis. To the remaining digest, formic acid was added to adjust pH around 3-4 and stored at -20^{o}C.

7.3 Results

The proteins that upregulated in a select few ARB isolates grown with and without arsenic were examined in this study. There were noticeable changes in the concentrations as a detoxification response in all isolates subjected various concentrations of arsenic. Figure 7.1 shows SDS-PAGE profile of *Staphylococcus arlettae* isolate grown with and without As. The SDS-PAGE revealed that fragments corresponding to 29 KDa and 32 KDa apparently suppressed when grown in nutrient medium with 500 ppm As in both *Staphylococcus arlettae* (Figure 7.1) and *Planococcus maritimus* (Figure 7.2) when exposed to arsenic respectively. The SDS-PAGE protein profile of *Bacillus baekryungensis* (Figure 7.3) of soluble, and insoluble fractions at different (0, 10, 50, 100, 200, 300, 500 and 1000 ppm) concentrations of arsenic using markers (205 to 3.5 KDa and 97.4 to 14.3 KDa) did show difference. Most notably, the protein profiles indicated the show up of a new moiety (46 KDa) in the test condition, *Bacillus baekryungensis* at 1000 ppm concentration of arsenic which was absent in case of the control (Figure 7.3). Since the results from 1-D were not always easy to distinguish or consistent, 2-D gel electrophoresis was carried out to confirm changes if any in protein expression in the presence and absence of As.

The SDS-PAGE profile of *Staphylococcus arlettae* isolated at different time intervals grown with (50 and 100 ppm) and without As (Figure 7.4) suggests more expression of more number of protein in medium with 50 ppm As at eight hours, 18 h and 20 h. In the presence of 100 ppm As, more proteins were expressed at 24 h. Only minimal/minorvariations discernible in protein profiles of 2 h to 6 h at different As concentrations. The isolate *Planococcus maritimus* produced more proteins at 24 h with 50 and 100 ppm (Figure 7.5). In the case of *Bacillus baekryungensis*, not much variation in protein profile at 2 h to 6 h was evident (Figure 7.6) and very few proteins were expressed. While at 8 h proteins expressed were more at 50 ppm As (Figure 7.6). Very faint or no proteins were expressed at 100 ppm As in 16 h and the protein profile was same for 50 ppm and 100 ppm at 24 h. In overall, more proteins were expressed in As treated (50 ppm and 100 ppm) media than that without arsenic. Such profile

indicates that over expression of some proteins may impart isolates for resistance to very high concentrations of arsenic. The corresponding 2-D profile for *Staphylococcus arlettae*, *Planococcus maritimus* and *Bacillus baekryungensis* are included (Figure 7.7, 7.8 and 7.9).

The isolate *Staphylococcus arlettae* exposed to arsenic showed appearance of more distinct dark spots (~20 KDa) which are seen faint in gel with protein sample without added arsenic (Figure 7.7 A, C and E). In contrast, the gel profiles of insoluble protein samples showed similar pattern in all different conditions treated. Three of spots vary considerably in intensity (~14.3 kDa) from only faintly visible in figure 7.7 B, however, in figure 7.7 F (with same amount of protein load) it stands out far more clearly and darker. In the 2-D gel with an isoelectric focusing range of protein load of 100 µg and pI 3-10 of more than 200 spots were visualized by silver staining. Figure 7.7 shows the 2-D profile of *Staphylococcus arlettae*grown with 50, 100 ppm As and with no arsenic. On comparing 2-D gel profile in the absence of As (with 159 protein spots), 80 and 53 spots were found when grown in 50 ppm and 100 ppm As respectively (Figure 7.7 A, C and E). In the case of insoluble proteins, a total of 36 spots were detected with 100 ppm As and 27 spots with 50 ppm As (Figure 7.7 F and D). In the absence of arsenic, 41 spots were detected (Figure 7.7 B).

In the 2-D gel with protein load of 100 µg and pI 3-10, more than 200 spots were visualized by silver staining. Figure 7.8 shows the 2-D profile of *Planococcus maritimus* grown with 50, 100 ppm As and with no arsenic. On comparing 2-D gel profile in the absence of As (with 103 protein spots), 187 and 176 spots were found when grown in 50 ppm and 100 ppm As respectively (Figure 7.8 A, C and E). In the case of insoluble proteins, a total of 224 spots were detected with 100 ppm As and 32 spots with 50 ppm As (Figure 7.8 F and D). In the absence of arsenic, 95 spots were detected (Figure 7.8 B). when exposed to 50 ppm As, the isolate *P. maritimus* showed appearance of more spots than observed in control between 29 KDa to 66 KDa (Figure 7.8 A and C). Also, a spot at ~14 KDa and ~20 KDa in control (Figure 7.8 B and D) is totally absent at 50 ppm As.

Figure 7.9 shows results obtained with a protein sample from bacterial isolate, *Bacillus baekryungensis*. Among the protein spots detected, 81 were present without arsenic stress (Figure 7.9 A). As many as 26 and 16 protein spots are detected in the

treatments with 50 ppm and 100 ppm As respectively. A clear spot in the silver stained image (Figure 7.9 D) corresponding to ~<14 kDa (Figure 7.9 D) in 50 ppm As was apparently suppressed or, disappeared completely (Figure 7.9 B and F) in either, when grown in nutrient medium with no added arsenic and 100 ppm As respectively.

This preliminary study has thrown light on whether or not and how the resistant isolate responds to arsenic. Mass spectrometric analyses of in gel digestion, protein spots showed mostly presence of keratin hence, in solution digestion mass spectrometric analysis were carried out. The most prominent over expressed or suppressed proteins are marked as arrow on each gel.

The 2-D gel profile of arsenic resistant bacterial isolates whether uninduced or induced with arsenic yielded many proteins that were upregulated. The proteolytic masses obtained were then evaluated using Spectrum Mill software (Agilent, USA). By mass spectrometry, a total of 119, 101 and 16 proteins were identified in *Bacillus baekryungensis* in control (0 ppm As), 50 ppm and 100 ppm arsenic respectively (Table 7.1, 7.2 and 7.3) by in-solution digestion. As many as 35 common proteins were present in both 0 ppm and 50 ppm As. Proteins common in both 50 ppm and 100 ppm As treatments were not apparent.

The pI of most proteins was in the range of 4 to 7. Proteins of different functional classes were seen in all cultures subjected to As-stress. Some were homologous translational proteins, synthesis of various amino acids. Some proteins such as elongation factor G, 50S ribosomal protein, Cold shock protein were induced in *B. baekryungensis* in the presence of arsenite.

Proteins namely,50S ribosomal protein L15, ATP synthase subunit alpha 2, Cold shock protein CspD, Putative cysteine protease YraA and Putative nitrogen fixation protein YutI were found to be present at two different concentrations (50 ppm and 100 ppm) of arsenic as well as in test culture without added arsenic which might play some role in imparting resistance to the isolates certainly in the case of those isolates tolerating to 50 and 100 ppm arsenite. By comparing control and As grown samples, it was found that only 5 proteins were common in all conditions. The S3 (a 30S ribosome moiety): rpsc,rplO: (a 50S ribosome moiety, L15), Cold shock protein:

cspD, Putative cysteine protease: yraA, FeS cluster assembly protein: sufD, GTP cyclohydrolase-2: ribA, Inositol 2-dehydrogenase: iolG, Membrane protein insertase: yidC, Nonribosomal peptide synthetase 14: NRPS14, Pseudoazurin: bcp, Solute carrier family 46 member 3: SLC46A3, Subtilin biosynthesis protein: spaB, Translation initiation factor 1A: eIF1A, ATP synthase subunit alpha 2: atpA2, Putative nitrogen fixation protein: yutI and Ubiquitin carboxyl-terminal hydrolase 16: usp16 were upregulated in the presence of 100 ppm (Table 7.3). All proteins identified by MS/MS analysis with their protein pI, possible involvement in biological functions/processes and molecular functions are described in brief in Table (7.1, 7.2 and 7.3).

7.4 Discussion

The main aim of protein analyses was to distinguish the protein profiles which will ultimately help in understanding unexplored mechanisms of arsenic resistance as well as to get an idea of the protein(s) which could be considered for targeting while developing newer or alternate bacterial resistance mechanisms. Therefore, the study included whole cell lysate proteins from *Bacillusbaekryungensis* which will cover all proteins present in the cells. In this study, the focus was on bacterial strains growing in very high As concentrations which were several times more than those reported by Anderson and Cook (2004) and Bhat (2007).

The ability *Staphylococcus arlettae*, *Planococcus maritimus* and *Bacillus baekryungensis* strainsto resist up to 1000 ppm As, augurs well for them to be most valuable bacterial isolates. This may be attributable to *ars* as well as other resistance mechanisms.The soluble As binding peptide fractions in *Bacillus baekraungensis* imply that some of proteins identified do bind As and thus showing resistance to As. These observations confirm presence of genetic/physiological mechanisms in this ARB. It may be that these are not necessarily exclusive in gene expression of As detoxifying genes.Analysing the over expression of such peptides/protein moieties are useful for As bioremediation and in recombinant bacterial constructions.

Mukhopadhyay and Rosen (2002) reported that the family of arsenate reductase known as thioredoxin (Trx) clade and is linked with *ars*Carsenate reductase gene. Ji and Silver (1992b) stated that ArsC (12-15 KDa) mediatingarsenate reductase is a soluble enzyme coupling oxidation of thiols. Similar protein, thioredoxin (~11 KDa to ~34 KDa) is also reported in this study at 50 ppm As as well as in control, without As. Recently, Lu et al. (2007) reported that Trx made up of NADPH, TrxR. The Trx and glutathione are reported to be made up of NADPH and glutathione reductase.

Another relatively uncommon protein ArsM (arsenite *S*-adenosylmethionine methyltransferase) catalyses the formation of a number of methylated intermediates from As(III) with trimethylarsine as the end product. This protein is reported from *Rhodopseudomonas palustris* by Qin et al. (2006). The structure of arsenite*S*-adenosylmethionine methyltransferase (SAM) was described by Ajees et al. (2012) by

X-ray crystallography having molecular weight of 29.6 KDa. Recently, the arsenite *S*-adenosylmethionine methyltransferase (ArsM) has been inserted into the chromosome of *Pseudomonas putida* KT2440 for potential bioremediation of environmental arsenic (Chen et al., 2013). Ajees et al. (2012) reported that aresenite binding changes in conformation of arsenite binding domain. In this study too such proteins were observed to be expressed in the medium with 50 ppm As and without As.

Storz et al. (1989) and Horsburgh et al. (2001) reported Alkyl hydroperoxide reductase and thioredoxin reductase as oxidative stress proteins. 1-pyrroline-5-carboxylate dehydrogenase 2 enzymes know to be inhibited by arsenite (Mechanism unknown). Protein, Ubiquitin carboxyl-terminal hydrolase detected at 100 ppm As at pI 5.38 is known to be resistant to arsenite (Zhou et al., 2009). Xie et al. (2014) reported that Superoxide dismutase (SOD) activity of *Bacillus subtilis* and *Bacillus thuringiensis* was expressed by arsenite and arsenate. In this study superoxide dismutase protein is detected at 100 ppm As at pI 5.2.

Since proteins involved in various metabolic pathways were induced in the presence of arsenic. This is suggestive that As might be causing alteration to overall cell physiology as well as metabolism. Several proteins essential for cell-envelope synthesis were up-regulated. This observation is useful to putforth that functioning and organization could be different in cells exposed to arsenite, reflecting cell-responses contributing to the detoxification of this metalloid.

The mechanisms by which microorganism exhibited resistance include exclusion of metal ions, extra cellular precipitation, complexing with metal ions at the surface of bacteria, transformations mediated by enzymes,oxidation/reduction mediated sequestration or de novo synthesis of extracellular- and/or metal binding-proteins. Factors such as toxic-metal species, redox potential, soil particulates, pH and organics (Patel et al., 2007)could also regulate/impact As toxicity/biotransformation/resistance. This first attempt to provide the data for highly ARB from estuarine bacterial community especially from tropical ecosystems is variously relevant.

8.1 Introduction

In the recent years, one major concern is release of arsenical compounds in particular arsenite and arsenate, into the environment. Their toxicity and/or carcinogenicity in some forms, arsenites in particular, to a variety of organisms and human beings is a major concern. Arsenate redox is mediated either enzymatically or through respiration (Silver and Phung, 2005; Chang et al., 2008; Yoon et al., 2008). Different resistance pathways for As species in Bacteria are reported by Mukhopadhyay et al. (2002) and Rosen (2002).

The *ars* operons encoded either chromosomally or by plasmids, three (*ars*RBC) or five (*ars*RDABC) genes (Silver and Phung, 1996) are well known and widespread. Ji and Silver (1992a); Ahmann et al. (1994); Cervantes et al. (1994); Santini et al. (2000); Jackson et al. (2001) and Lee et al. (2001) reported on the use of As and its species as either acceptors or donors of electrons, also on their possessing disparate As detoxification modes. Monitoring As pollution level is aided by detection and description of *ars* genes (Stocher et al., 2003;Ji and Silver 1992a, b; Cervantes et al., 1994). Many researchers have worked on different *ars* genotypes to relate them to As resistanct phenotype (Saltikov and Olson, 2002; Anderson and Cook, 2004; Jackson et al., 2005; Sun et al., 2004). Molecular detection and genotyping *ars* genes is useful to discern the diversity of bacterial flora (Cervantes et al., 1994; Diorio et al., 1995; Oremland et al., 2004).

The ARB isolates from near coastal marine waters representing all the 27 genera/species identified through 16S rRNA gene sequencing were examined for a few chromosomally encoded *ars* genes in this study. This was done to check for the presence of *ars*, *aox* and *Acr*3genes associated with As resistance in a set of ARB to describe the innate detoxification mode/s of As biotransformation.

8.2 Materials and Methods

8.2.1 PCR Amplification of *ars* (*ars*R,-B,-C, -A, -D, -AB and -H) Genes

As many as 27 representative isolates (Table 8.1) were examined for the presence of *ars* (*ars*R, -B, -C, -A, -D, -AB and -H), *aox* (*aox*B and *aox*R) and *Acr*3 genes. Genomic DNA extraction and sequencing of the isolates is described in Chapter 6.

PCR amplification of these seven, well known arsenic resistant (*ars*) genes was done using the PCR primers listed in Table 8.2. All these *ars* primers used have an annealing temperatureof approximately 53ºC to 60ºC (Chang et al., 2008). PCR amplification carried out using 25 µl with 1 µl DNA of concentration within a range of 25 - 40 µg DNA μl^{-1} and 10 pmol each of forward and reverse primers. PCR was conducted using a thermal cycler (Veriti, Applied Biosystes, USA). The gene/s amplification was done following a standard protocol with 5 min hot-start at 95ºC initially, 1 min 94ºC denaturation (35 cycles), annealing at 53-57ºC for 1 min, finally 1 cycle of 2 min extension at 72ºC. PCR was ended by a 7 min extension at 72ºC for *ars* genes. PCR amplifications of all examined genes was detected through gel electrophoresis using 1% agarose gel. The PCR amplification of *ars*B (using different set of primer) and *Acr*3 was performed using primers as given in Table 8.2 and by following Achour et al. (2007). PCR primers of Achour et al. (2007) were used to confirm some of these results.

8.2.2 PCR Amplification of *aox*B and *aox*R Genes

The primer sets for *aox* gene PCR are presented in Table 8.2 and their amplification was done following Cai et al. (2009). The PCR of *aox*R was carried out as described by Chang et al. (2010).PCR amplifications were examined through gel electrophoresis using 1% agarose gel.

8.2.3 Cloning of *ars*B Gene

Cloning kit (TOPO TA, Invitrogen, USA) was used for *ars*B gene cloning according to the standard protocol. The PCR amplicons were purified and ligated into TOPO and transformed in *E.coli* competent cells (one shot TOP10). The transformed cells were spread plated on Luria Bertani (LB) agar containing ampicillin and X-gal and grown for16 h at 37°C following manufacturer's protocol. Atleast 10 transformed (white) colonies picked up and sequenced following the standard sequencing procedure.

8.2.4 Sequencing of *ars*, *aox* and *Acr*3 Genes

Sequencing of PCR products was performed with 15-50 ng PCR products and one pmol of each forward and reverse primers. The sequencing was done using ABI

sequencing instrument following the dideoxy chain termination method. The samples were sequenced on a ABI 3130 Genetic Analyzer (ABI, USA) and all raw sequences were manually screened for low quality regions. After end trimming and removal of low quality/chimeric regions, sequencing results were compared with sequences from NCBI database (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), for their matches using BLASTX programs for confirmation.

8.3 Results

PCR detection of *ars* genes in the chromosomal DNA of 27 representative bacterial isolates was done in this study. Notably, the isolates differed in harbouring As modulating genes. For example, the *ars*B of ~700 bp (Figure 8.1a) encoding efflux pump was observed in six of 27 representative ARB strains in as many different genera identified by 16S rRNA gene sequencing. These are GN34, *Acinetobacter* sp. (KJ719350); GN1, *Psychrobacter*sp. (KJ719317); GN35, *Pseudomonas* sp. (KJ719351); GN10, *Staphylococcusarletae* (KJ719326); GN2, *Bacillus subtilis* (KJ719318) and GN31, *Pseudomonas putida* (KJ719347) encoding ArsB efflux pump (KM673296), arsenic efflux pump protein (KM820844), ArsB efflux pump (KM820845), arsenical pump membrane protein (KM820846), arsenical pump membrane protein (KM820847) and arsenical pump membrane family protein (KM820848) respectively.

The isolates differed with respect to the *ars*B gene they carried from two differently used primers. For example, with the primers of Achour et al. (2007), the *ars*B gene was observed in six ARB strains. These are GN84, *Bacillus aquimaris* (KJ719399); GN1, *Psychrobacter* sp.(KJ719317); GN2, *Bacillus subtilis* (KJ719318); GN25, *Bacillus cereus* (KJ719341); GN10, *Staphylococcus arlettae* (KJ719326) and GN96, *Bacillus* sp.(KJ719411) encoding arylsulfatase (KP161070 to KP161073) and arsenite-antimonite efflux pump (KP161074 to KP161075) respectively (Figure 8.1b). With both sets of PCR primers isolates *Psychrobacter* sp., *Bacillus subtilis* and *Staphylococcus arlettae* were positive for *ars*B gene.

Presence of the *aox* (*aox*B) genes within the chromosome was detected in only one isolate GN96, *Bacillus* sp. (KJ719411) encoding NADH oxidase (KP100806). The fragment length of *aox*B is ~350 bp (Figure 8.2). The *ars*D gene is encoded by

GN25, *Bacillus cereus* (KJ719341) showing the presence of chemotaxis protein CheY (KP100804) at ~350 bp (Figure 8.2). *Acr*3 gene fragment ~350 bp is found to be encoded (Figure 8.2) by GN25, *Bacillus cereus* (KJ71934) asABC transporter(KP100805).

Phylogenetic tree was constructed for *ars*B gene detected in the strains (Figure 8.3). It is useful to recognize that the *ars*B fragments from GN1, *Acinetobacter* sp. (KM673296),GN2, *Psychrobacter* sp. (KM820844), GN3, *Pseudomonas* sp. (KM820845)and GN6, *Pseudomonas putida* (KM820848) were found to cluster with sequences to *Staphylococcus epidermidis* (WP_031272610.1), *Shigella flexneri* (WP_032322508.1) and *Psychrobacter* sp.JCM 18902 (GAF59143.1). All these clustered in the same clade. The sequences from GN1, *Acinetobacter* sp. and GN3, *Pseudomonas* sp. clustered in the same node. The fragments from GN5,*Bacillus subtilis*(KM820847) and GN4, *Staphylococcus arlettae* (KM820846) were found to cluster with sequences of *Escherichia coli* (WP_032249039.1) and *Escherichia coli* (WP_032298753.1). A very high homology between GN4, GN5 and *E. coli* clustering in the same node is useful to note the identical evolutionary process of this gene.

Phylogenetic tree was constructed for *ars*B gene detected in the strains (Figure 8.4). It is seen that no much homology is found between fragments from *ars*B genes. The GN11, *Bacillus aquimaris* (KP161070); GN12, *Psychrobacter sp.* (KP161071); GN13, *Bacillus subtilis* (KP161072); GN14, *Bacillus cereus* (KP161073); GN15, *Staphylococcus arlettae* (KP161074) and GN16, *Bacillus sp.* (KP161075) were found to cluster with sequences to Uncultured bacterium (ACA14310.1) in the same node.

Phylogenetic tree was constructed for *ars*D gene detected in the strain *Bacillus cereus* (Figure 8.5). It is useful to recognize that the *ars*D fragments from GN7, *Bacillus cereus* (KJ719341) encoding *ars*D gene, chemotaxis protein CheY (KP100804) were found to cluster with sequencers to *Pseudomonas mosselii* (WP_028689077.1), *Pseudomonas mosselii* (AIN61540.1),*Pseudomonasputida* (WP_012316605.1) and *Pseudomonas* sp.(WP_027917512.1). All these clustered in the same clade. The sequences from GN7 and *Pseudomonas* sp.(WP_027917512.1) clustered in the same node.

Phylogenetic tree for *Acr*3 gene was constructed (Figure 8.6). It is evident to recognize that the *Acr*3 fragments from GN8, *Bacillus cereus*,asABC

transporter(KP100805) were found not to cluster with any sequences. It is useful to recognize from phylogenetic tree that *aox*B gene detected in the strain GN9, *Bacillus* sp.(Figure 8.7)encoding NADP oxidase (KP100806) were found to cluster with sequences to *Paenibacillus lactis* (WP_007128835.1), *Bacillus* sp. (WP_028404116.1), *Paenibacillus* sp. (ETT41226.1), *Clostridium* sp. (KGK85949.1) and *Paenibacillus* sp. (GAK41357.1) All these clustered in the same clade. A very high homology between GN9, *Bacillus* sp. and *Bacillus aquimaris* (WP_032087021.1) clustering in the same node is useful to note the identical evolutionary process of this gene.

Eight of the 10 randomly picked out transformants/clones were positive for the presence of *ars*B gene. All these positive *ars*B clones had homology with arylsulfatase. The accessions numbers for each of positive clones are as follows. Clone 1 (KP090137), Clone 2 to clone 8 (KP100797 to KP100803).

8.4 Discussion

The As mobilization across soil and groundwater is facilitated by arsenate reduction/methylation by in situ microflora (Oremland and Stolz, 2003). Arsenic resistant *ars* genes, arsenite oxidising *aox* genes and arsenic transported *Acrs*3 genes examined from 27 different arsenic resistance bacterial strains suggest strains specific responses. For instance, the function of *ars*R, which encoding for regulator seem to be very specific.so also are the functions of*ars*B, *aox*B and *aox*R. The last two performing oxidation of arsenite to arsenate and *Acrs*3 the arsenic transport. Some of the bacterial isolates possessed *ars* genes which encode various functions. High tolerance/resistance of As in some isolates I examined could be ascribed to an existance of many *ars* operon types on their chromosomes (*sensu* Ordonez et al., 2005) or transposon (Tuffin et al., 2006). Restoration of As-laced habitats by applying such microbes is known (Carbonell et al., 1998; Dedysh et al., 1998; Ibrahim et al., 2006; Weber et al., 2006; Chang et al., 2007).

In this study, the *ars*D gene encoded by *Bacillus cereus* (KJ719341) showing the presence of chemotaxis protein (CheY) is useful to suggest the abilty of many ARB to sense the toxic moieties. Similar to this study, Muller et al. (2007) isolated an heterotrophic bacterium, *Herminiimonas arsenicoxydans* from a wastewater treatment unit and is the first fully characterized arsenic-metabolizing microorganism known to possess unsuspected mechanisms for coping with arsenic.Apart from arsenic oxidation/reduction, oxidative stress resistance and As(III) extrusion. It is therefore of key significance in the bioremediation of contaminated environments, leading to the sequestration of this toxic metalloid As.

Detection of *aox*B in the chromosome of *Bacillus* sp. (KJ719411; NADH oxidase; KP100806), is pertinent to suggest as Lynn et al. (2000) report that activation of NADH oxidase is brought about by arsenite leading to the formation of superoxide. This superoxide is detrimental as it damages the DNA. It is quite likely that such mechanisms do operate in certain bacteria. The *Acr*3 gene is encoded by *Bacillus cereus,* asABC transporter(KP100805). These ABC transporters are involved in resistance (Tamas and Wysocke, 2001) to arsenic and antimony-containing compounds. Rosen (1999) found that extrusion of arsenite in yeasts aided by Acr3p.

As Yang et al. (2012) point out, such efflux/extrusion processes are governed by protein families that also confer drug resistance in some microbes.

The *ars* genes are normally located as clusters either on plasmids or chromosomes. However as exceptions they may occur as single units (Diorio et al., 1995; Cai et al., 1998; Sato and Kobayashi, 1998; Suzuki et al., 1998; Butcher et al., 2000; Butcher and Rawlings, 2002; Maury et al., 2003). Bacterial strains possessing *ars* operons control As mobility or, consequently its detoxification through anyone of the possible array of speciation. Thus, any ARB bearing *ars* genes are vital and prospective candidates As bioremediation.

Detection of *aox* genes in Mandovi-Zuari estuarine waters, is useful to suggest its utility in arsenite oxidation. Philips and Taylor (1976) demonstrated that arsenite oxidation of *Alcaligenes faecalis* is brought about by an enzyme and/or an electron acceptor which is formed when grown in media with As(III). Whereas Osborne and Ehrlich (1976) show that a strain of *Alcaligenes* sp. acquired its arsenite-oxidizing enzyme through growth-dependent induction. In this study, ability of arsenite oxidation (the trait enabled by *aox*B gene) was identified in only one strain,*Bacillus* sp. (Figure 8.2).It is to be noted that in most ARB all genes essential for thwarting As toxicity were not present. This may be due to divergence in the *ars* operons as has also been noted earlier by some researchers (Jackson and Dugas, 2003 and Achour et al., 2007). Therefore, it is worthwhile to explore possible diversity of As resistance genes.

Migration of more toxic arsenite across sediment and aquifer is a serious concern. Microbial arsenic resistance is a common characteristic not only in contaminated areas but also in arsenic-free environments (Jackson et al., 2003). As an example Nagvenkar and Ramaiah, (2010) isolated bacterial strains from low As contaminated coastal region and reported many strains of ARB tolerating high concentrations (1000 ppm) of arsenite and also found their arsenite detoxifying potential to be very high.

Previously, Anderson and Cook (2004); Pepi et al. (2007); Fan et al. (2008) and Chang et al. (2010) reported arsenic resistant strains from the genera *Staphylococcus*, *Acinetobacter*, *Vibrio* and *Pseudomonas* from natural and arsenic

contaminated sites. In this study, ARB isolates in the genera *Psychrobacter*, *Bacillus*, *Pantoea*, *Staphylococcus*, *Dietzia*, *Rhodococcus*, *Pseudomonas*, *Acinetobacter*, *Kocuria*, *Erwinia*, *Paenibacillus* and *Planococcus* were identified from estuarine waters ofMandovi and Zuari.

The results obtained in this study are certain evidences to the presence of numerous arsenic resistant genetic material in estuarine bacterial populations. Regardless of As concentration levels the resistance to As in many bacterial strains is formidable. Their ability to encode a few *ars* if not all the battery ofgenes brings forth the fact that such strains do perform As detoxification in the natural ecosystems. In any event, strains possessing a few to many *ars* operons seem to be conferred of arsenic resistance.

Studies on prevalence of flora capable of As tolerance are of pertinence for an understanding on As pollution and to realize their potential detoxification of As and other toxicants. It is evident from this study that sizable fraction of native bacteria growing in medium containing As is quite widespread. A broad range of bacterial types seem to carry out either reduction of arsenate or oxidization of arsenite. This aspect is clearly realized in this study.

As acknowledged variously, environmental effects of metal pollution are broad. Since various adverse effects due to a plethora of toxicants affect the coastal systems different experiments were conducted out to see whether marine ARB are adept enough in detoxifying arsenic and also to understand the *ars* gene constitution. The hypothesis putforth was whether native isolates capable of tolerating high As could detoxify it.

Water and sediments samples collected from coastal locations of River Mandovi reveal that bacterial fraction capable of growing in nutrient medium containing 15, 25 and 50 ppm arsenic is sizable. Thirteen randomly isolated environmental ARB strains capable of tolerating 1000 ppm arsenite were characterized biochemically. In addition, the effects of antibiotics were examined to check if certain alterations are due to As. Genomic DNA from a total of 500 isolates of ARB was extracted and, ARDRA other analyses were carried out. Twenty seven strains which represented all the genera of ARB isolated in this study were investigated for presence of *ars*, *aox* and *Acr*3 genes. Further, proteins expressed in *Bacillus baekryungensis* at different As concentrations were also analysed.

The following are the major features emanating from this study.

- The ARB have adapted to lower stretches of Mandovi-Zuari, exposed to low or moderate pollution. These systems do harbor ARB possessing mechanism(s) to deal with As toxicity. A total of 500 ARB (isolates tolerating ≥100 ppm arsenite) from different locations (midstream: 187, estuarine: 218 and marine: 95) were isolated on NA medium amended with As. Thirteen of these isolates tolerating 1000 ppm were submitted to an extensive biochemical characterization. Two out of thirteen isolates were found to be Enterobacteriaceae, two *Pseudomonas* sp., one *Corynebacterium* sp., one

Xanthomonas sp., one *Acinetobacter* sp., one *Flavimonas* sp., one *Micrococcus* sp., one *Bacillus* sp., one *Staphylococcus* sp., one *Rhodococcus* sp. and one *Planococcus* sp.

- Some of these isolates bore resistance to many antibiotics suggesting metal and antibiotic resistance go hand in hand. This observation brings to fore the need to evaluate the ecological significance of native flora capable of tolerating such high concentrations of As and/or other toxic metals.

- All environmental strains tolerant to 1000 ppm arsenic were tested to realize their potential to detoxify arsenic.From the quantification of arsenic removal by 13 ARB strains, it was observed that amount of As removed was substantial (>85% of initial concentration) within 120 h by all of them. The highest removal rate was by *Planococcus* sp. (13) and *Micrococcus* sp. (9) followed by Enterobacteriaceae(3), *Xanthomonas* sp. (5), *Rhodococcus* sp. (12), *Pseudomonas* sp. (2 and 7), *Staphylococcus* sp. (11), *Bacillus* sp. (10), *Corynebacterium* sp. (4), *Flavimonas* sp. (8) and *Acinetobacter* sp. (6).

- The DNA from all 500 ARB isolates which tolerated arsenic concentration ≥ 100 ppm, was extracted and studied for the amplified ribosomal DNA restriction (ARDRA) of PCR-amplified 16S ribosomal RNA. My ARDRA analysis generated 123 different profiles from the 16S rRNA gene restriction digestion of 500 cultures. The sequencing was performed for a total of 123 amplified PCR products selected on their basis of unique ARDRA profiles.

- Analyses of 16S rRNA gene sequences represent 12 different genera and 27 different species with 99% similarity.

- Sequencing data of these isolates were used to construct phylogenetic trees to establish their evolutionary relationship to other bacterial clades. Based on 16S rRNA gene sequencing data, species were found to belong to three Phylum: Actinobacteria, Firmicutes and Proteobacteria.

- Whole cell lysate proteins from *Bacillusbaekryungensis* through LCMS QToF analysis yielded respectively a total of 119, 101 and 16 proteins when grown

without As, with 50 ppm and 100 ppm As (as arsenite). By comparing control and As grown samples, it was found that only 5 proteins were similar.

- Analyses of *ars* genes from the 27 representative strains yielded interesting insights. Nine isolates namely *Acinetobacter* sp. (KJ719350), *Psychrobacter* sp. (KJ719317), *Pseudomonas* sp. (KJ719351), *Staphylococcusarletae* (KJ719326), *Bacillus subtilis* (KJ719318), *Pseudomonas putida* (KJ719347), *Bacillus aquimaris* (KJ719399), *Bacillus cereus* (KJ719341) and *Bacillus* sp.(KJ719411) possessed efflux pump system (Accession no. KM673296; KM820844 to KM820848; KP161070 to KP161075) encoded by the *ars*B (important in arsenite extrusion).The isolate *Bacillus* sp.(KJ719411)was found to encode *aox*B gene (Accession no. KP100806). Detection of *aox* genes in the estuarine isolates, responsible for arsenite oxidation would be importantin ecological functions pertaining arsenite oxidation. Further, *ars*D gene encoded by *Bacillus cereus*, achemotaxis protein CheY (KP100804) and *Acr*3 gene encoded by *Bacillus cereus*, asABC transporter(KP100805) were also identified.

Future Prospects

In principle, higher the abundance of ARB, more likely could be the As metalloid contamination in that environment. Enumeration of ARB, if carried out on a regular basis might be a vital aspect in the up-keep of environmental health.

More detailed studies on ARB from marine environment would give rise to novel insight on, for instance, presence of non-*ars* ARB strains. Such studies are needed for acquiring more information on arsenic resistance and therefore the possibility of obtaining a set of hither to unknown, robust candidates of ARB useful for As bioremediation. Absence of *ars* (*ars*R,-C, -A, -AB and -H) and *aox*R in the marine ARB strains of arsenic resistance/tolerance needs to be affirmed.

Importance of each proteins in the modulation of As are quite important. It is therefore worthwhile to check/look for enzymes or ionic processes conferring arsenic resistance.

Arsenic is a metalloid that causes harm to humans and environments. It is important to remove and reduce this pollutant from the environment through different methods such as physical, chemical, and biological. The use of bioremediation to remove arsenic from contaminated soils and aquifers could be an effective and economic way. This is because, a wide range of microorganisms have been found in this study to be successfully detoxifying As. For this, protocols for removing arsenic from the environment should meet basic technological criterion that include reliability, eco-safety, and safe from human-systems perspectives.

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Publications

- **Geeta S. Nagvenkar** and N. Ramaiah. (2010). Arsenite tolerance and biotransformation potential in estuarine bacteria.*Ecotoxicology*. l19, 604-613.

- **Nagvenkar, G.S.**, Ramaiah, N. (2009). Abundance of sewage-pollution indicator and human pathogenic bacteria in a tropical estuarine complex. *Environmental Monitoring and Assessment*. 155, 245-256.

Conference proceeding and presentations

- Poster presented at "International conference on Ballast Water Control and Management Programm" held at National Institute of Oceanography, Dona Paula, Goa from 5th to 7th February 2008.
  Title: Sewage-pollution indicator and human pathogenic bacteria from Mormugao Bay
  Authors: **Nagvenkar G.S**. and N. Ramaiah.

- Oral presentation at "ASEAN-India Marine Biotechnology workshop" held during 19th to 22nd March, 2013, organized by National Institute of Oceanography (CSIR), Goa India.
  Title: Arsenic Biotransformation potential in Estuarine Bacteria
  Authors: **Geeta S.Nagvenkar** and N. Ramaiah

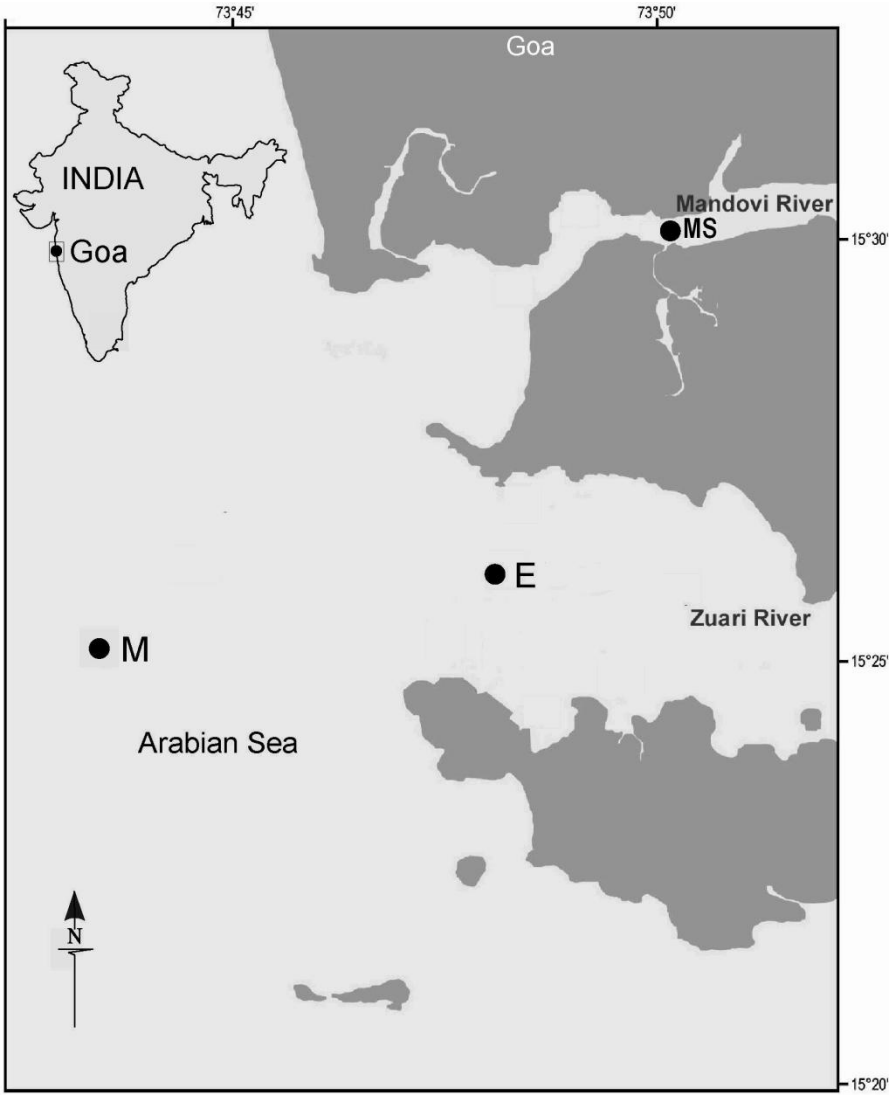


Figure 3.1 Map showing sampled locations. One location each in the 'midstream' (MS), estuary (E) and marine (M) zones were selected. In ~ 15-20 m vicinity of each of these stations, three samples of water were collected for enumerating and isolating arsenic tolerant bacteria.

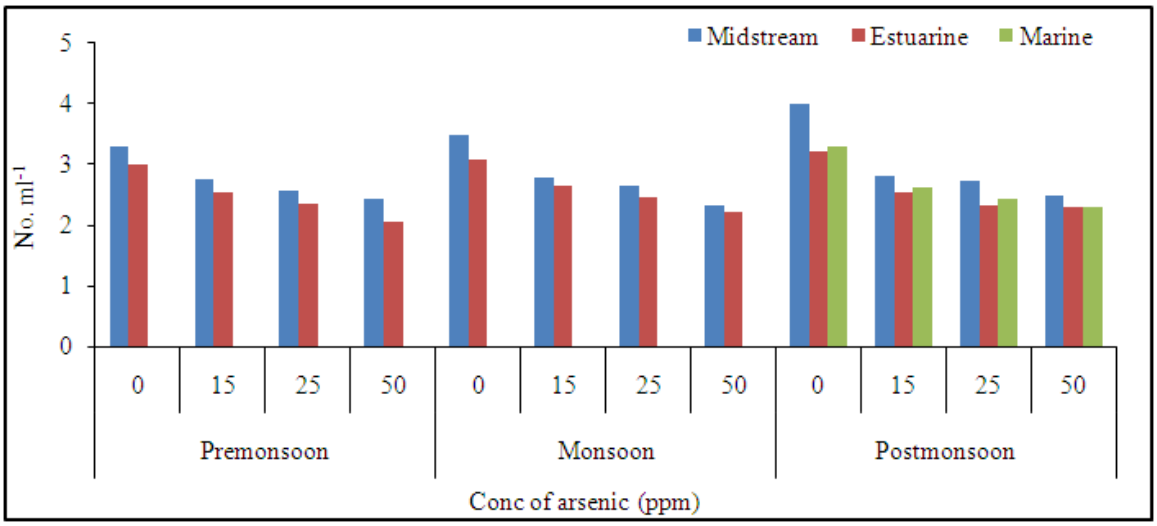
Table 3.1 Abundance of bacterial colony forming units (CFU) enumerated from 'midstream' (MS), estuarine (E) and marine (M) location using nutrient agar amended with arsenite

	Concentration of arsenite (ppm)			
	0	**15**	**25**	**50**
Water (CFU no. ml^{-1})				
Pre-monsoon				
MS	2000	560	366	267
E	1020	339	218	112
M	ND*	-	-	-
Monsoon				
MS	3000	606	433	212
E	1200	454	292	159
M	ND	-	-	-
Post-monsoon				
MS	9600	661	532	295
E	1600	349	210	193
M	2000	406	270	191
sediment (CFU no.$X10^5$ gm^{-1} dry wt)				
Pre-monsoon				
MS	77	5.6	3.5	2.6
E	58	5.7	4.3	3.6
M	ND	-	-	-
Monsoon				
MS	39	5.5	3.8	2.8
E	43	4.9	4.1	3.6
M	ND	-	-	-
Post-monsoon				
MS	195	7.6	6	4
E	102	9.3	8.7	6.6
M	71	17.2	12.2	9.6

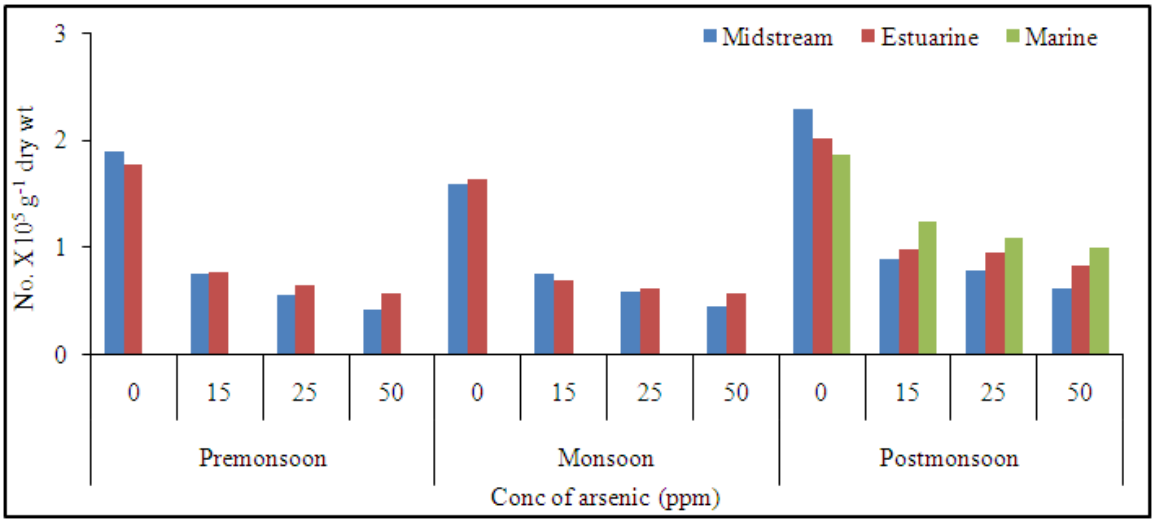
*- Not Sampled owing to rough monsoon weather

Table 3.2 Number of ARB growing in Nutrient agar amended with different concentrations of arsenite

Sampling zone (No. of isolates tested)	**As Concentration (ppm)**				
	100	**200**	**300**	**500**	**1000**
Midstream (187)	187	166	155	135	39
Estuarine (218)	218	185	170	137	68
Marine (95)	95	73	62	50	20



(a)



(b)

Figure 3.2. Bacterial counts (log scale) in (a) water and (b) sediment samples examined during pre-monsoon, monsoon and post-moonsoon periods.

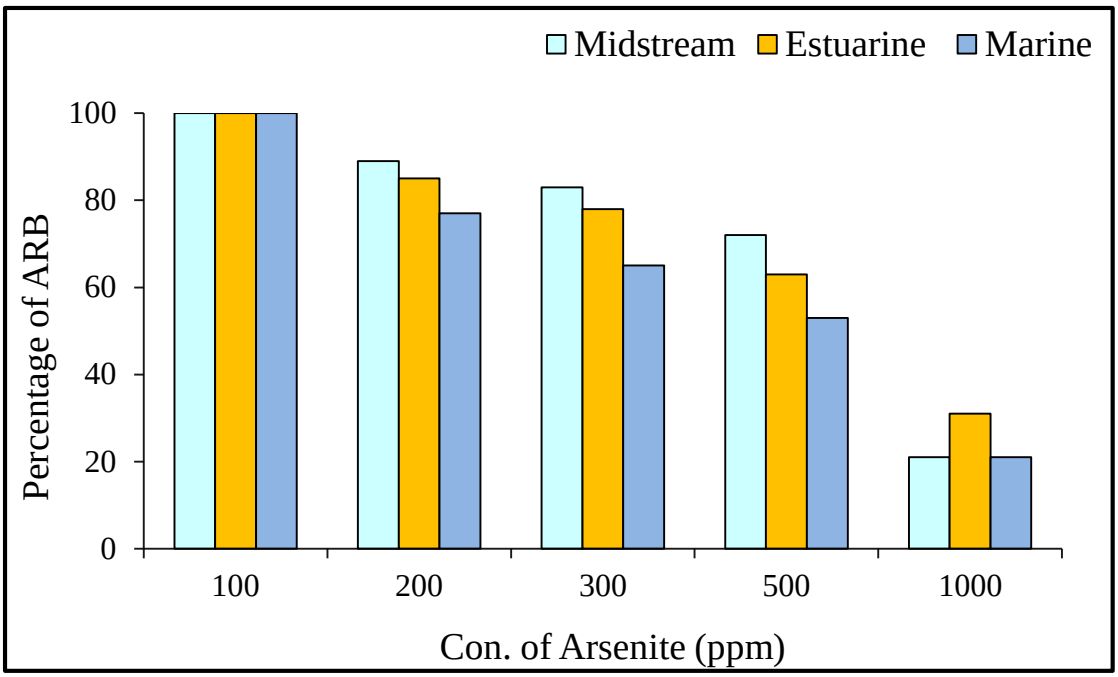


Figure 3.3 Percentage of arsenic resistant bacteria (ARB) growing in different arsenite concentrations.

Table 4.1 Various characteristics (morphological and biochemical) of isolates grown in nutrient medium with 1000 ppm As

Characteristic	**ARB Isolate#**												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
Colour	CW	C	C	WT	O	C	W	DY	WO	C	C	O	O
Shape	SR	SR	SR	R	SR	LR	SR	LR	C	SR	C	SR	C
Gram stain	-	-	-	+	-	-	-	-	+	+	+	+	+
Motility	+	+	+	-	+	+	+	+	-	-	-	-	-
Indole	-	-	-	-	-	-	-	-	-	-	-	-	-
MR	-	-	-	-	-	-	-	-	-	-	-	-	-
VP	-	-	-	-	-	-	-	-	-	-	-	-	-
SC	+	+	+	+	+	+	+	+	+	+	+	+	+
Urease	+	+	+	+	+	-	+	+	+	+	+	-	+
H_2S	-	-	-	-	-	-	-	-	-	-	-	-	-
PA	-	-	-	-	-	-	-	-	-	-	-	-	-
NO_3^{-2} reduction	+	-	-	-	+	-	+	+	-	-	+	-	-
TSI	-	-	AB&S	-	AB	AB	AB	AB	-	AB	-	-	-
Oxidase	-	-	-	-	-	-	+	-	-	-	-	-	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+
ONPG	-	-	-	-	+	-	-	-	-	-	+	-	-
Gelatinase	-	-	-	+	+	+	-	+	-	+	-	-	+
Starch	-	-	+	-	-	+	-	-	-	+	-	-	-
Carbohydrate	F	O	F	F	O	O	O	O	O	O	O	O	O
Litmus milk	-	-	-	-	+	+	-	-	+	-	-	-	-
Esculin	+	+	+	+	+	+	+	+	-	-	+	+	-

(continued)

Utilization of:													
Lactose	-	-	-	-	-	-	+	-	-	+	-	-	-
Xylose	-	-	-	-	-	-	+	-	-	+	-	-	+
Maltose	-	-	-	-	-	+	+	-	-	-	+	-	-
Fructose	+	+	+	+	+	+	-	+	+	+	-	-	+
Dextrose	-	-	-	-	-	+	+	-	-	-	-	-	-
Galactose	-	-	-	-	-	-	+	-	-	-	-	+	-
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-
Trehalose	-	-	-	-	-	+	-	-	-	-	-	-	-
Melibiose	-	-	-	-	-	-	+	-	-	-	-	-	-
Sucrose	-	-	-	-	-	+	-	-	-	+	-	-	-
L-Arabinose	-	-	-	-	-	-	+	-	-	-	-	+	+
Mannose	-	-	-	-	-	-	+	-	-	-	-	-	-
Inulin	-	-	-	-	-	+	-	-	-	-	-	-	-
Sodium gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycerol	-	+	+	+	+	+	-	+	+	-	-	+	+
Salicin	-	-	-	-	-	+	-	-	-	-	-	-	-
Glucosamine	-	-	-	-	-	-	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-
Sorbitol	+	+	+	+	+	-	-	+	-	+	-	-	-
Mannitol	+	+	+	+	+	-	-	-	+	+	+	-	+
Adonitol	+	-	-	-	-	-	-	-	-	-	-	-	-
α-methyle-D-Glucoside	-	-	-	-	-	-	-	-	-	-	-	-	-
Ribose	-	-	-	-	-	-	-	-	-	-	-	+	-
Rhamnose	-	-	-	-	-	-	-	-	-	+	-	+	-
Cellobiose	-	-	-	-	-	-	+	-	-	-	-	-	-

(continued)

Melezitose	-	-	-	-	-	-	-	-	-	-	-	-	-
α-methyle-D-Mannoside	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabinose	-	-	-	-	-	-	+	-	-	-	-	-	-
Malonate	-	-	-	-	-	-	-	-	-	-	-	-	-
Sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-

Key: +: positive; –: negative; CW: Cream white; C: Cream; WT: White turbid; O: Orange; W: White; DY: Dirty yellow; WO: Whitish orange; MR: Methyl red; VP: Voges Proskauer; SC: Simmon citrate; PA: Phenylalanine deaminase; NO_3^{-2} reduction: nitrate reduction; TSI: Triple Sugar Iron; SR: Straight rod; R: Rod; LR: Long rod; C: Cocci; O: Oxidative; F: Fermentative; AB: Acidic butt; AB&S: Acidic butt and slant.

Bacterial isolates: 1 and 3 (Enterobacteriaceae); 2 and 7 (*Pseudomonas* sp.); 4 (*Corynebacterium* sp.); 5 (*Xanthomonas* sp.); 6 (*Acinetobacter* sp.); 8 (*Flavimonas* sp.); 9 (*Micrococcus* sp.); 10 (*Bacillus* sp., Accession No. KJ719328); 11 (*Staphylococcus* sp., Accession No. KJ719326); 12 (*Rhodocccus* sp., Accession No. KJ719354) and, 13 (*Planococcus* sp., Accession No. KJ719380). Several attempts to extract the genomic DNA of reliable quality from isolates 1 to 9 failed. Hence, no NCBI Accession numbers for them.

Table 4.2 Response of ARB to antibiotics measured in terms of inhibition zone in diameter

Antibiotics tested	Tested conc (µg/disc)	Sensitivity limit (mm)	Isolates of ARB												
			1	2	3	4	5	6	7	8	9	10	11	12	13
			Zone of inhibition diameter (mm)												
Penicillin -G	10 U	29	19(R)	15(R)	12(R)	20(R)	37(S)	25(R)	NZ	37(S)	27(R)	18(R)	35(S)	15(R)	35(S)
Streptomycin	25	15	26(S)	23(S)	27(S)	22(S)	NZ	19(S)	NZ	25(S)	15(S)	27(S)	24(S)	20(S)	23(S)
Tetracycline	30	19	20(I)	30(S)	21(I)	29(S)	22(I)	30(S)	38(S)	30(S)	25(S)	22(I)	20(I)	22(S)	32(S)
Chloramphenicol	30	18	21(S)	21(S)	19(I)	20(I)	17(R)	21(S)	NZ	30(S)	24(S)	21(S)	17(R)	17(R)	21(S)
Kanamycin	30	18	15(I)	19(S)	15(I)	16(I)	14(I)	18(S)	24(S)	25(S)	22(S)	15(I)	19(I)	18(S)	24(S)
Gentamycin	30	15	14(I)	27(S)	24(S)	28(S)	25(S)	38(S)	35(S)	21(S)	15(S)	26(S)	14(I)	14(S)	25(S)
Oxytetracycline	30	19	24(S)	26(S)	23(S)	21(S)	15(R)	21(S)	29(S)	31(S)	20(S)	25(S)	15(R)	16(R)	21(S)
Neomycin	30	17	18(S)	18(S)	17(S)	21(S)	NZ	25(S)	NZ	29(S)	20(S)	19(S)	NZ	18(S)	18(S)

Key: R- Resistant; S-Sensitive; I-Intermediate; NZ- no zone of inhibition.
Bacterial isolates: 1 and 3 (Enterobacteriaceae); 2 and 7 (*Pseudomonas* sp.); 4 (*Corynebacterium* sp.); 5 (*Xanthomonas* sp.); 6 (*Acinetobacter* sp.); 8 (*Flavimonas* sp.); 9 (*Micrococcus* sp.); 10 (*Bacillus* sp., Accession No. KJ719328); 11 (*Staphylococcus* sp., Accession No. KJ719326), 12 (*Rhodocccus* sp., Accession No. KJ719354) and, 13 (*Planococcus* sp., Accession No. KJ719380).

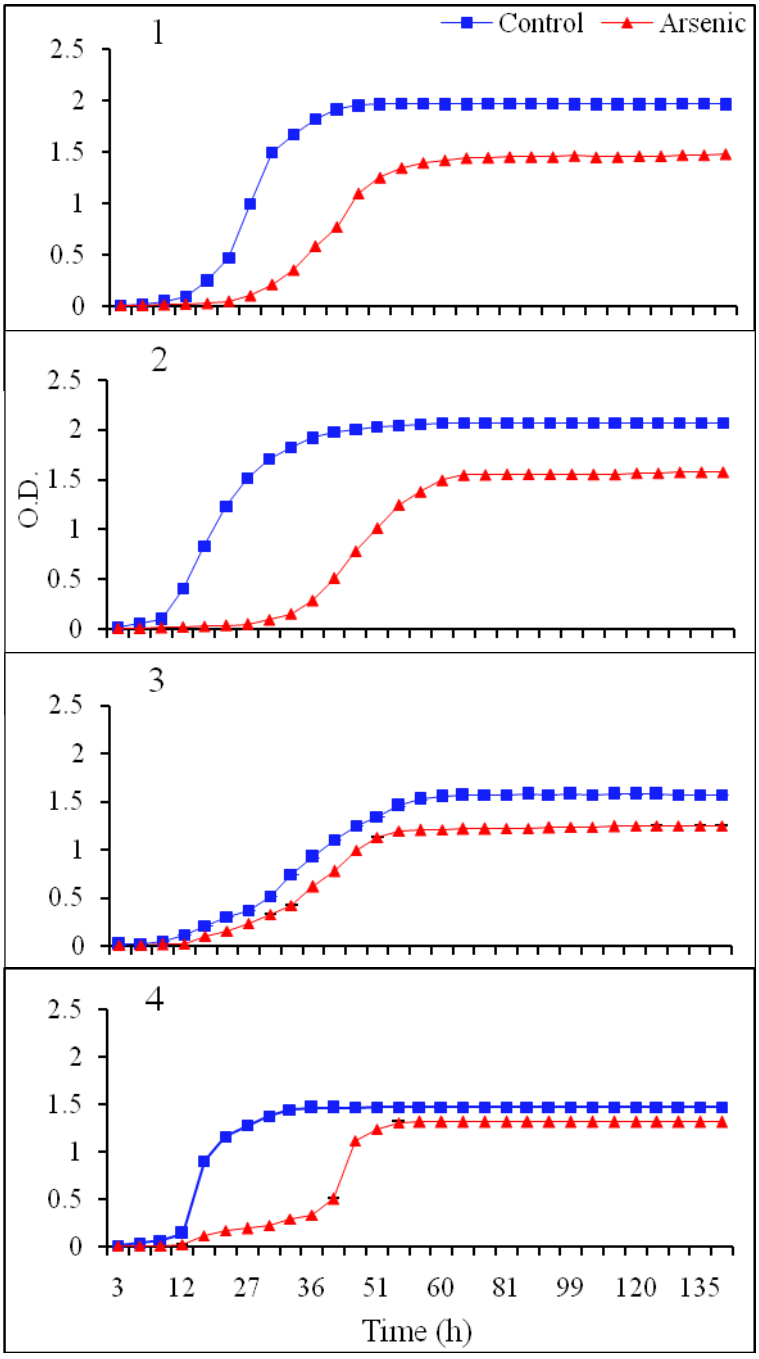


Figure 4.1a Growth (OD_{600}) of different isolates in nutrient broth (NB) without arsenic (squares) and in NB amended with 200 ppm arsenic (triangles). Isolates 1 and 3 belong to Enterobacteriaceae; 2 to *Pseudomonas* sp. and 4 to *Corynebacterium* sp.

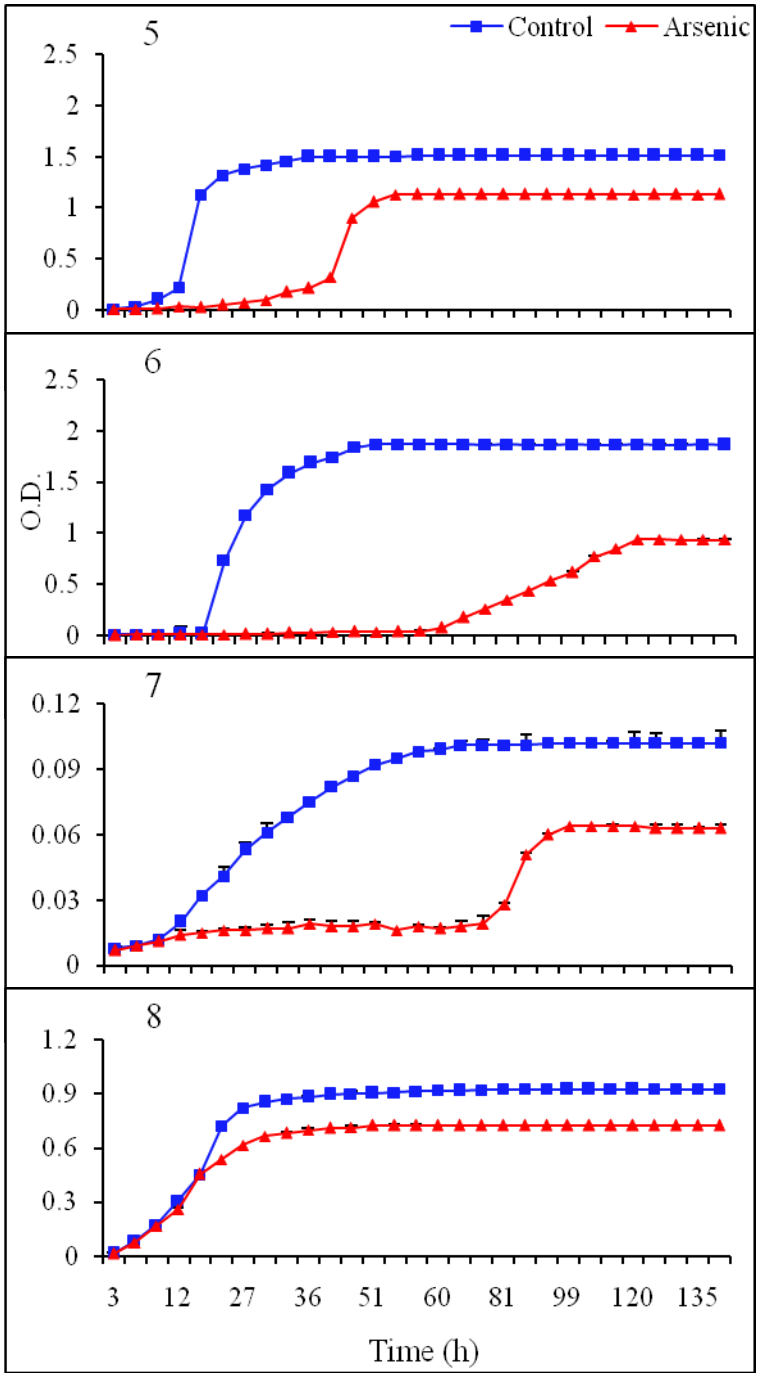


Figure 4.1b Growth (OD_{600}) of four isolates in nutrient broth (NB) without arsenic (squares) and in NB amended with 200 ppm arsenic (triangles). Isolates 5 belong to *Xanthomonas* sp.; 6 to *Acinetobacter* sp.; 7 to *Pseudomonas* sp. and 8 to *Flavimonas* sp.

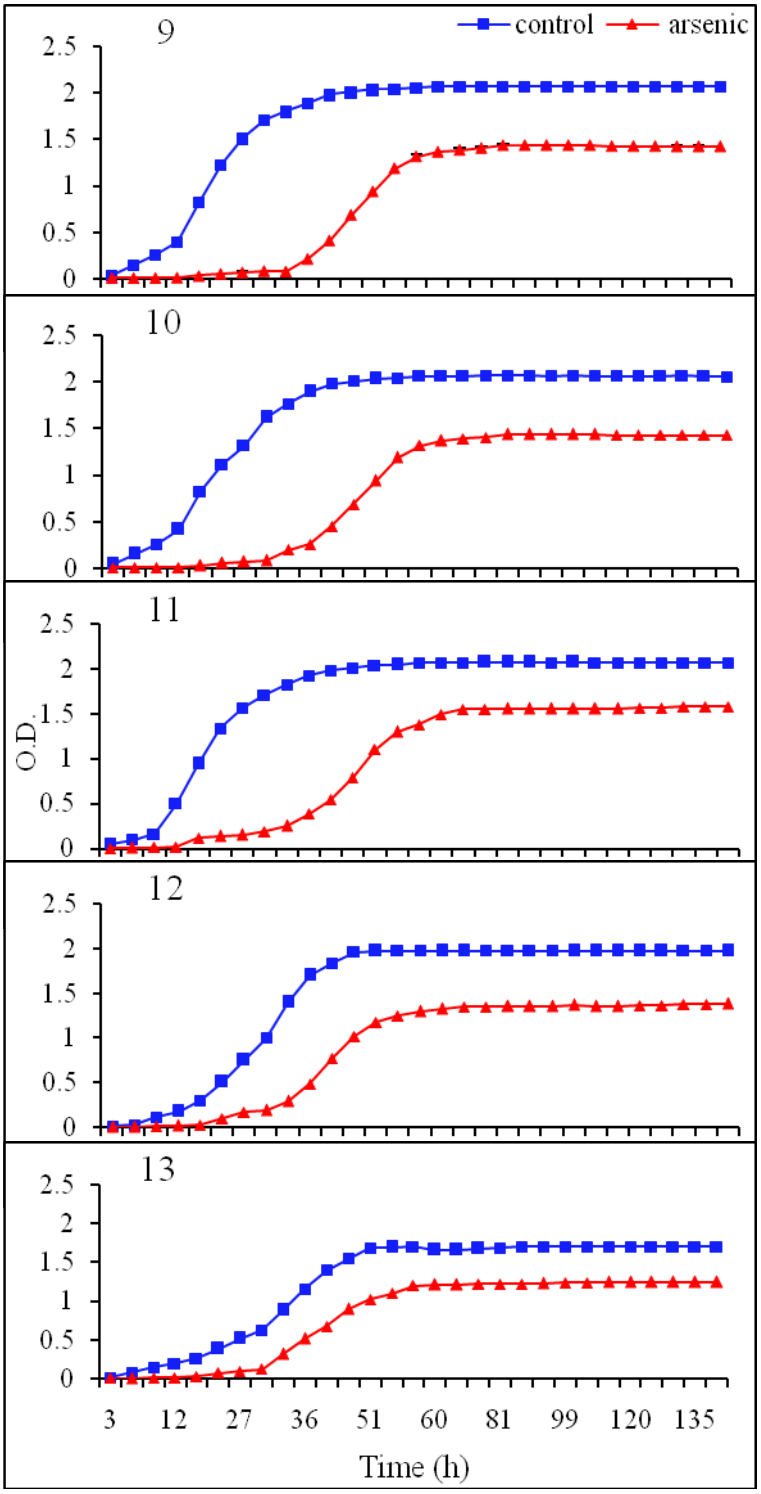
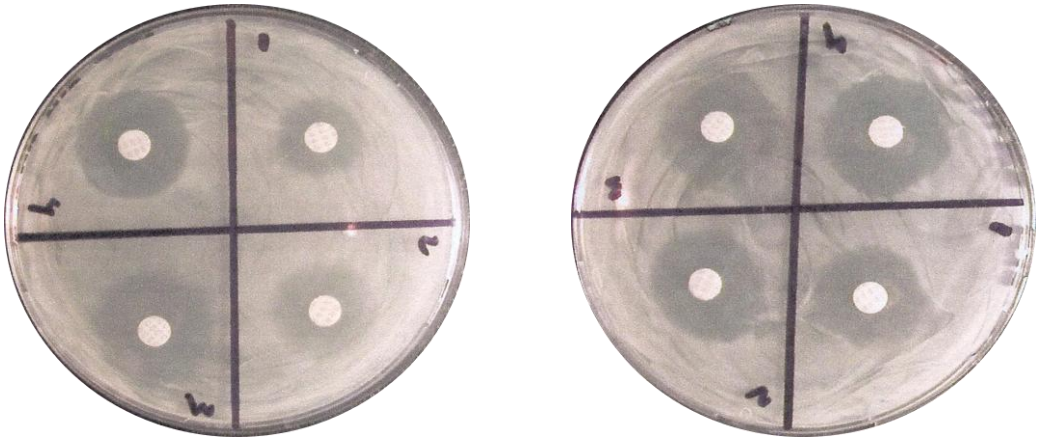


Figure 4.1c Growth (OD_{600}) of isolates, 9 belong to *Micrococcus* sp.; 10 to *Bacillus* sp., (Acc No. KJ719328); 11 to *Staphylococcus* sp. (Acc No. KJ719326); 12 to *Rhodocccus* sp. (Acc No. KJ719354) and 13 to *Planococcus* sp. (Acc No. KJ719380) in NB without As and with 200 ppm As.

NOTE: Several attempts to extract the genomic DNA of reliable quality from isolates 1 to 9 failed. Hence, no NCBI Accession numbers for them.



(a) (b)

Figure 4.2 (a) and (b) Representative plates showing antibiotic sensitivity assay done for *Rhodocccus* sp. (12); Accession No. KJ719354. All zones of inhibition are suggestive of resistance/sensitivity to all antibiotics (Penicillin-G, Streptomycin, Tetracycline, Chloramphenicol, Kanamycin, Gentamycin, Oxytetracycline and Neomycin) shown in this picture. Pictures for all results on ARB not included in the thesis.

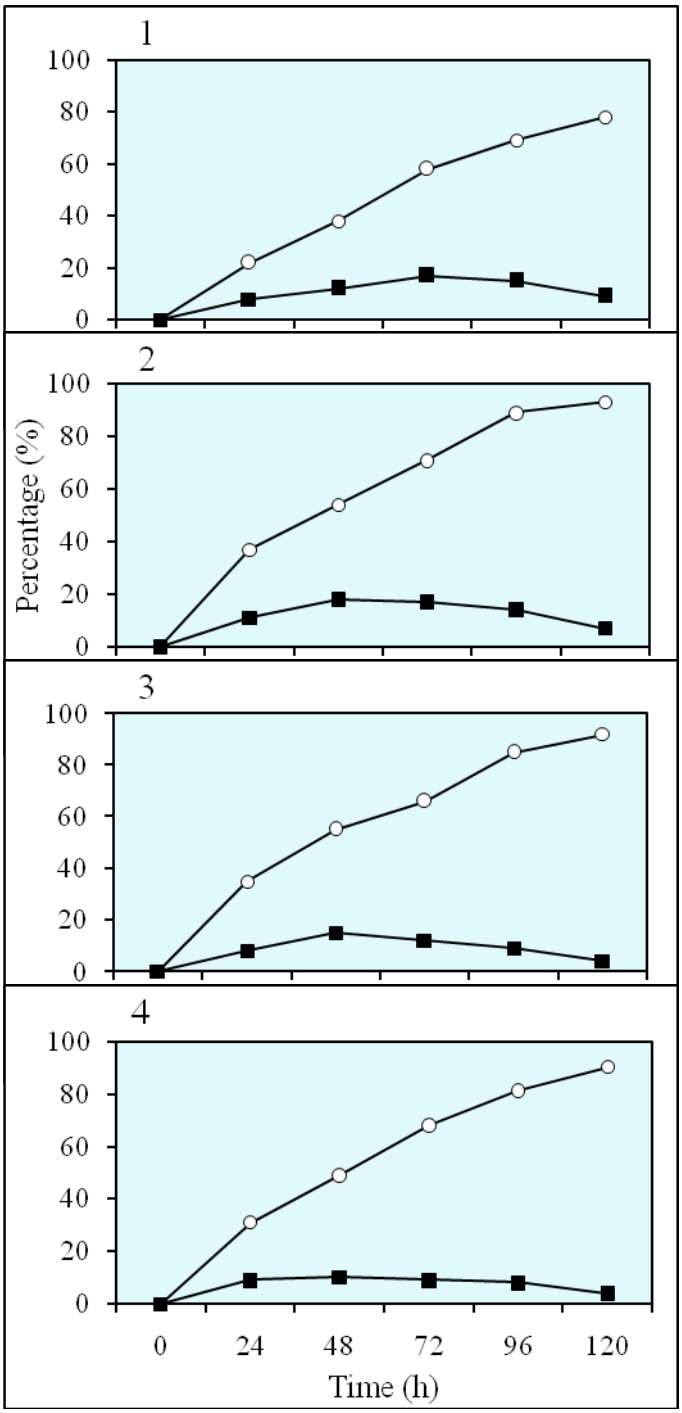


Figure 5.1 Arsenite biotransforation (%) from the growth medium by strains of Enterobacteriaceae (1 and 3), *Pseudomonas* sp. (2) and *Corynebacterium* sp. (4). The arsenite concentration in uninoculated medium and seawater blanks with 10 ppm remained unchanged throughout the experimental duration.

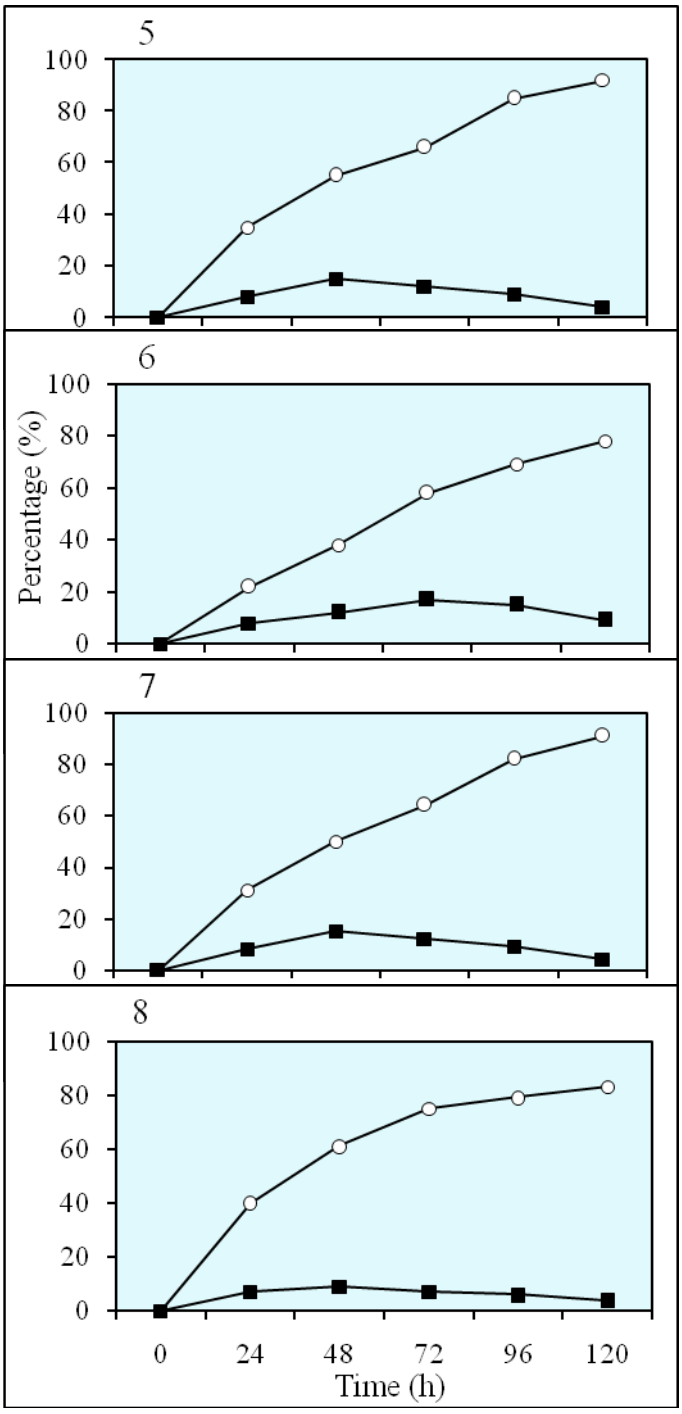


Figure 5.2 Percent of arsenic biotransformed from the growth medium by bacterial isolates *Xanthomonas* sp. (5), *Acinetobacter* sp. (6), *Pseudomonas* sp. (7) and *Flavimonas* sp. (8). The arsenite concentration in uninoculated medium and seawater blanks with 10 ppm remained unchanged throughout the experimental duration.

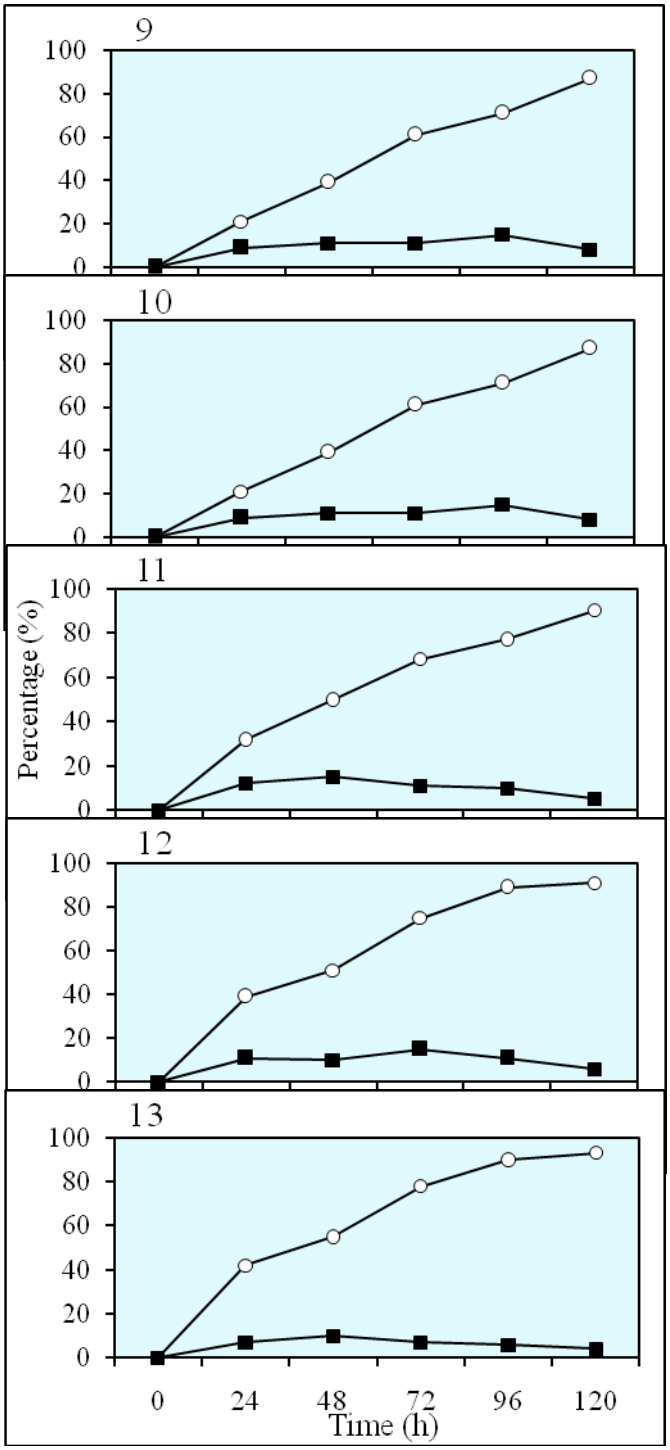
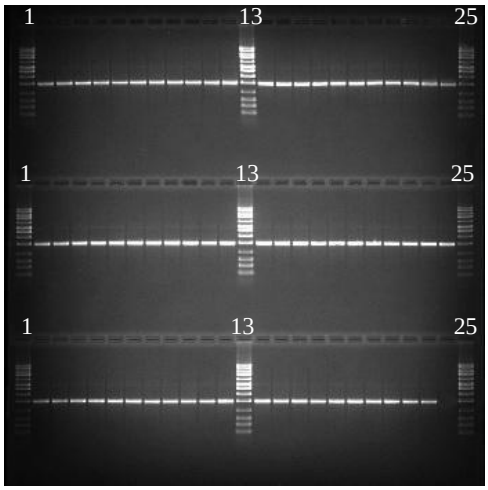
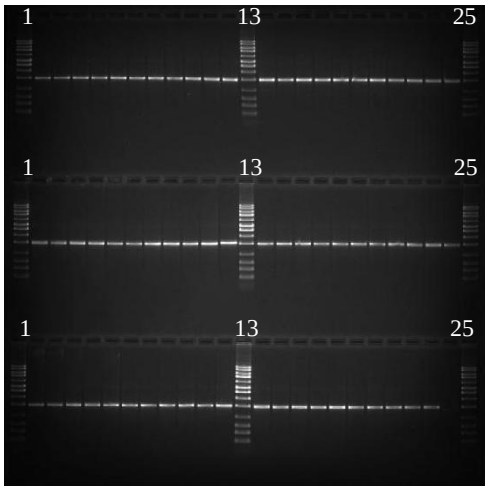


Figure 5.3 Percent of arsenic biotransformed by bacterial isolates *Micrococcus* sp. (9), *Bacillus* sp. (10), *Staphylococcus* sp. (11), *Rhodocccus* sp. (12) and *Planococcus* sp. (13). The arsenite concentration in uninoculated medium and seawater blanks with 10 ppm remained unchanged throughout the experimental duration.

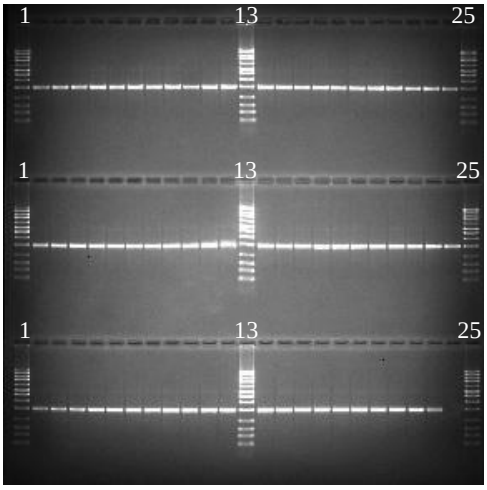
NOTE: Several attempts to extract the genomic DNA of reliable quality from isolates 1 to 9 failed. Hence, no NCBI Accession numbers for them.



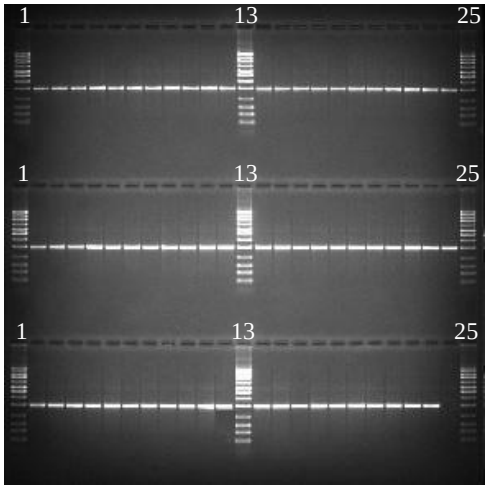
(a)



(b)

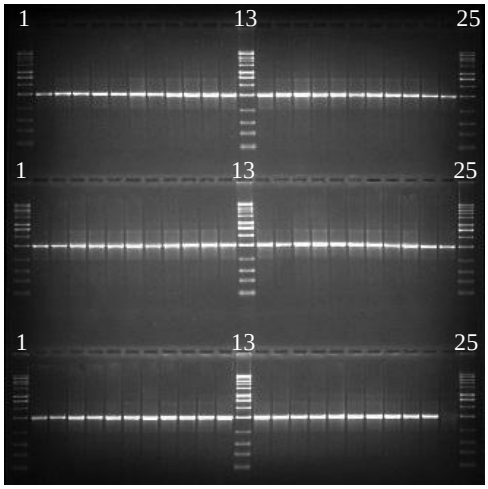


(c)

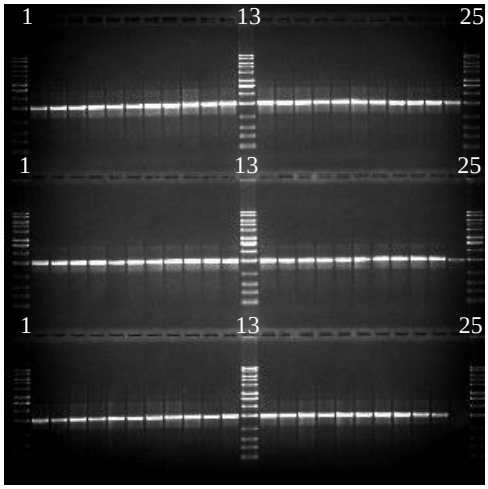


(d)

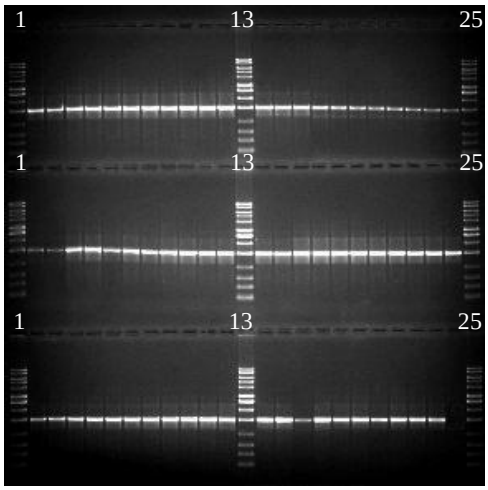
Figure 6.1a, b, c and d PCR amplified 16S rRNA gene from 260 (1-259) ARB. Lane 1, 13 and 25 with 1 Kb marker and lanes 2-12 and lanes 14-24 depict the PCR amplicons of ~1500bp 16S rRNA gene. Lane 24 in the third row is negative control.



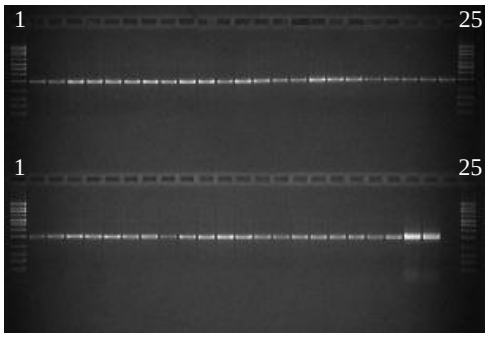
(e)



(f)

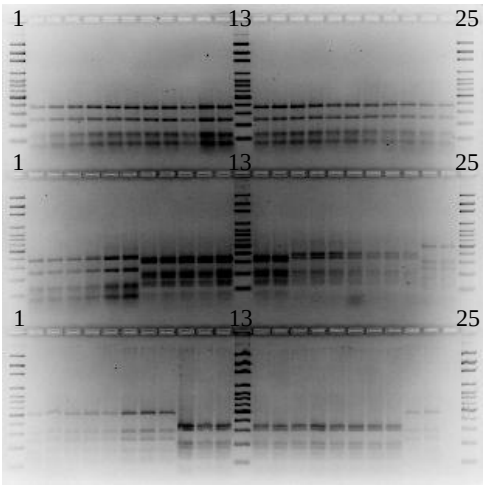


(g)

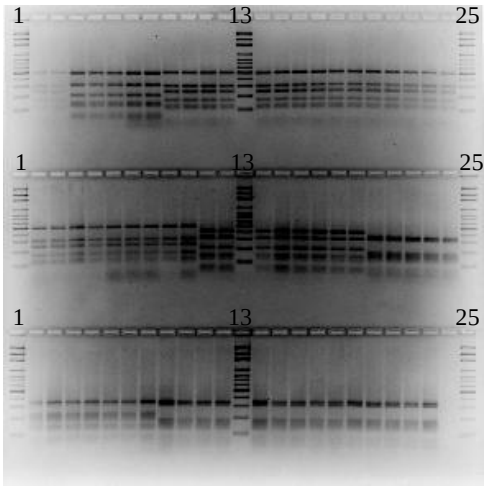


(h)

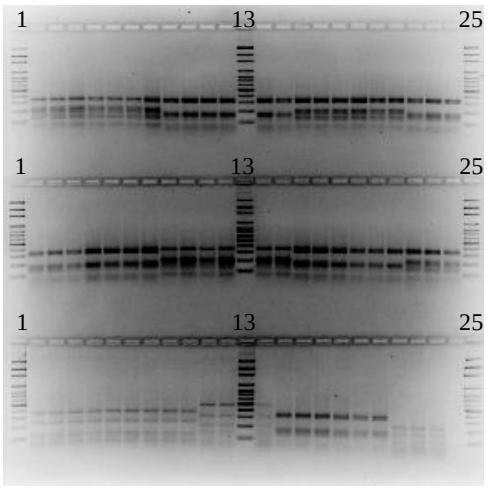
Figure 6.1e, f, g and h PCR amplified 16S rRNA gene from 240 (260-500) ARB. Lane 1, 13 and 25 with 1 Kb marker and lanes 2-12 and lanes 14-24 depict the PCR amplicons of ~1500bp 16S rRNA gene. Lane 24 in the third row is negative control. While lane 1 and 25 in gel h, is marker and lane 2 to 24 is PCR amplified rRNA gene and, lane 24 in second row is negative control.



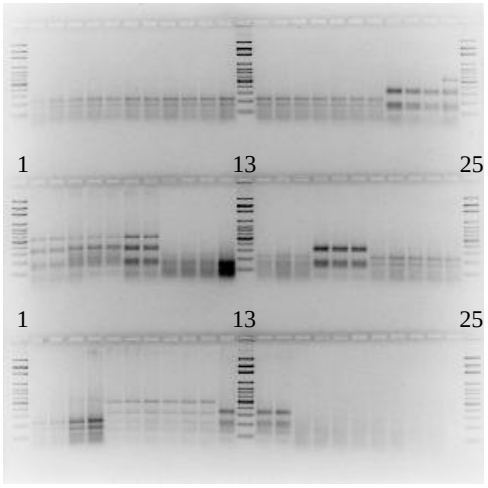
(a)



(b)

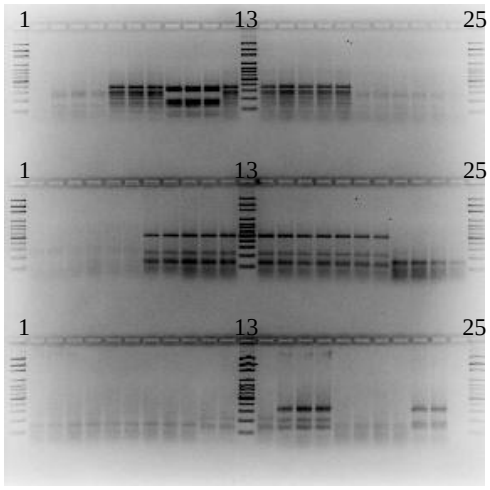


(c)

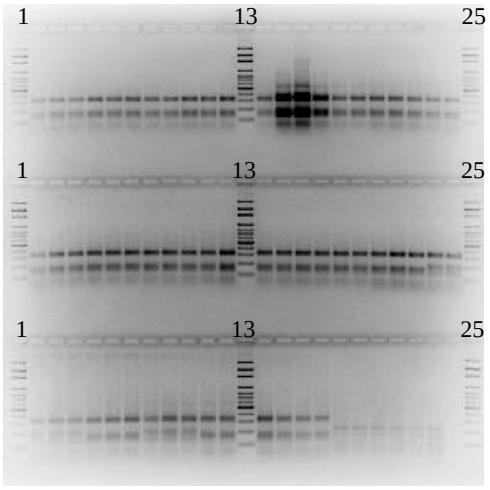


(d)

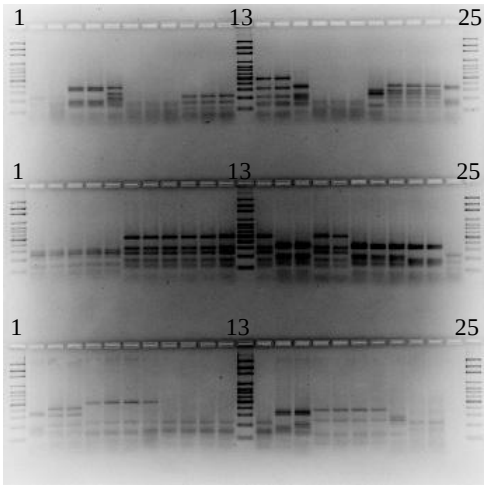
Figure 6.2 a, b, c and d ARDRA pattern derived through *Msp*I and *HhaI* restriction profile of 16S rRNA gene for 260 ARB. Lane 1, 13 and 25 with 100 bp DNA marker (100 bp-3000 bp); lane 2 to 12 and 14 to 24 represent different ARDRA patterns; Lane 24 in the third row is negative control.



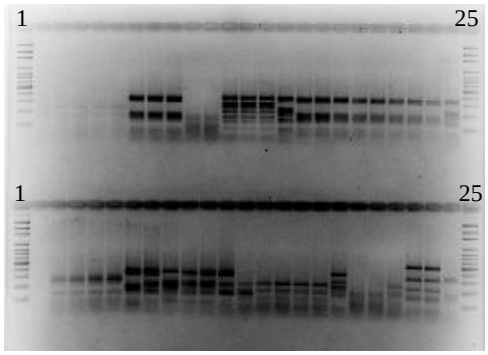
(e)



(f)



(g)



(h)

Figure 6.2 e, f, g and h ARDRA pattern derived through *Msp*I and *HhaI* restriction profile of 16S rRNA gene for 240 ARB. Lane 1, 13 and 25 with 100 bp DNA marker (100 bp-3000 bp); lane 2 to 12 and 14 to 24 represent different ARDRA patterns; Lane 24 in the third row is negative control.

Table 6.1 16S rRNA gene sequences based identification of ARB bearing ≥ 96% similarity with NCBI accession numbers

Sr No.	Genus/Group	No. of isolates	Isolates code, Accession No. (% similarity)
1	*Psychrobacter* sp.	9	GN1, KJ719317 (99); GN4, KJ719320 (99); GN5, KJ719321 (99); GN6, KJ719322 (99); GN7, KJ719323 (99); GN8, KJ719324 (99); GN60, KJ719375 (98); GN87, KJ719402 (98) and GN89, KJ719404 (98)
2	*Psychrobacter celer*	4	GN82, KJ719397 (99); GN83, KJ719398 (99); GN113, KJ719428 (99) and GN118, KJ719433 (99)
3	*Bacillus* sp.	32	GN14, KJ719330 (99); GN15, KJ719331(99); GN16, KJ719332 (99); GN17, KJ719333 (96); GN19, KJ719335(99); GN20, KJ719336 (97); GN23, KJ719339 (99); GN45, KJ719360 (99); GN46, KJ719361 (99); GN55, KJ719370 (99); GN61, KJ719376 (99); GN62, KJ719377 (99); GN75, KJ719390 (99); GN80, KJ719395 (99); GN85, KJ719400 (99); GN86, KJ719401 (99); GN90, KJ719405 (99); GN91, KJ719406 (99); GN96, KJ719411 (99); GN97, KJ719412 (99), GN99, KJ719414 (99); GN100, KJ719415 (99); GN102, KJ719417 (99); GN103, KJ719418 (99); GN104, KJ719419 (99); GN105, KJ719420 (99); GN109, KJ719424 (99); GN114, KJ719429 (99); GN115, KJ719430 (99); GN120, KJ719435 (99); GN123, KJ719438 (99) and GN124, KJ719439 (99)
4	*Bacillus aquimaris*	4	GN84, KJ719399 (99); GN95, KJ719410 (99); GN119, KJ719434 (99) and GN121, KJ719436 (99
5	*Bacillus baekryungensis*	16	GN12, KJ719328 (99); GN42, KJ719357 (99); GN43, KJ719358 (99); GN44, KJ719359 (99); GN47, KJ719362 (99); GN49, KJ719364 (99); GN76, KJ719391 (99); GN92, KJ719407 (99); GN93, KJ719408 (99); GN94, KJ719409 (99); GN98, KJ719413 (99), GN108, KJ719423 (99); GN111, KJ719426 (99); GN112, KJ719427 (99); GN117, KJ719432 (99) and GN122, KJ719437 (99)
6	*Bacillus cereus*	1	GN25, KJ719341 (99)
7	*Bacillus horikoshii*	4	GN18, KJ719334 (98); GN21, KJ719337 (99); GN48, KJ719363 (99) and GN74, KJ719389 (99)
8	*Bacillus hwajinpoensis*	1	GN106, KJ719421 (99)
9	*Bacillus marisflavi*	2	GN28, KJ719344 (100) and GN73, KJ719388 (99)
10	*Bacillus subtilis*	2	GN2, KJ719318 (99) and GN3,KJ719319 (99)
11	*Bacillus tequilensis*	1	GN54, KJ719369 (99)

(Continued)

12	*Pantoea agglomerans*	1	GN22, KJ719338 (97)
13	*Pantoea dispersa*	2	GN9, KJ719325 (99) and GN13, KJ719329 (99)
14	*Staphylococcus arlettae*	3	GN10, KJ719326 (99); GN11, KJ719327 (99) and GN63, KJ719378 (99)
15	*Dietzia* sp.	8	GN24, KJ719340 (99); GN50, KJ719365 (99); GN53, KJ719368 (99); GN67, KJ719382 (99); GN68, KJ719383 (99); GN72, KJ719387 (99); GN78, KJ719393 (99) and GN107, KJ719422 (99)
16	*Kocuria* sp.	2	GN36, KJ719352 (99) and GN37, KJ719353 (99)
17	*Kocuria flava*	1	GN110, KJ719425 (99)
18	*Erwinia* sp.	2	GN88, KJ719403 (99) and GN101, KJ719416 (99)
19	*Rhodococcus* sp.	9	GN26, KJ719342 (100); GN27, KJ719343 (99); GN29, KJ719345 (99); GN41, KJ719356 (99); GN51, KJ719366 (99); GN52, KJ719367 (99); GN56, KJ719371 (99); GN69, KJ719384 (99) and GN79, KJ719394 (99)
20	*Rhodococcus erythropolis*	4	GN39, KJ719354 (99); GN40, KJ719355 (99); GN64, KJ719379 (99) and GN77, KJ719392 (99)
21	*Rhodococcus equi*	1	GN30, KJ719346 (99)
22	*Pseudomonas* sp.	1	GN35, KJ719351 (100)
23	*Pseudomonas putida*	6	GN31, KJ719347 (100); GN32, KJ719348 (99); GN57, KJ719372 (99); GN59, KJ719374 (99); GN66, KJ719381 (99) and GN81, KJ719396 (99)
24	*Acinetobacter* sp.	4	GN34, KJ719350 (99); GN58, KJ719373 (99); GN70, KJ719385 (99) and GN71, KJ719386 (99)
25	*Acinetobacter johnsonii*	1	GN33, KJ719349 (99)
26	*Paenibacillus* sp.	1	GN116, KJ719431 (99)
27	*Planococcus maritimus*	1	GN65, KJ719380 (99)

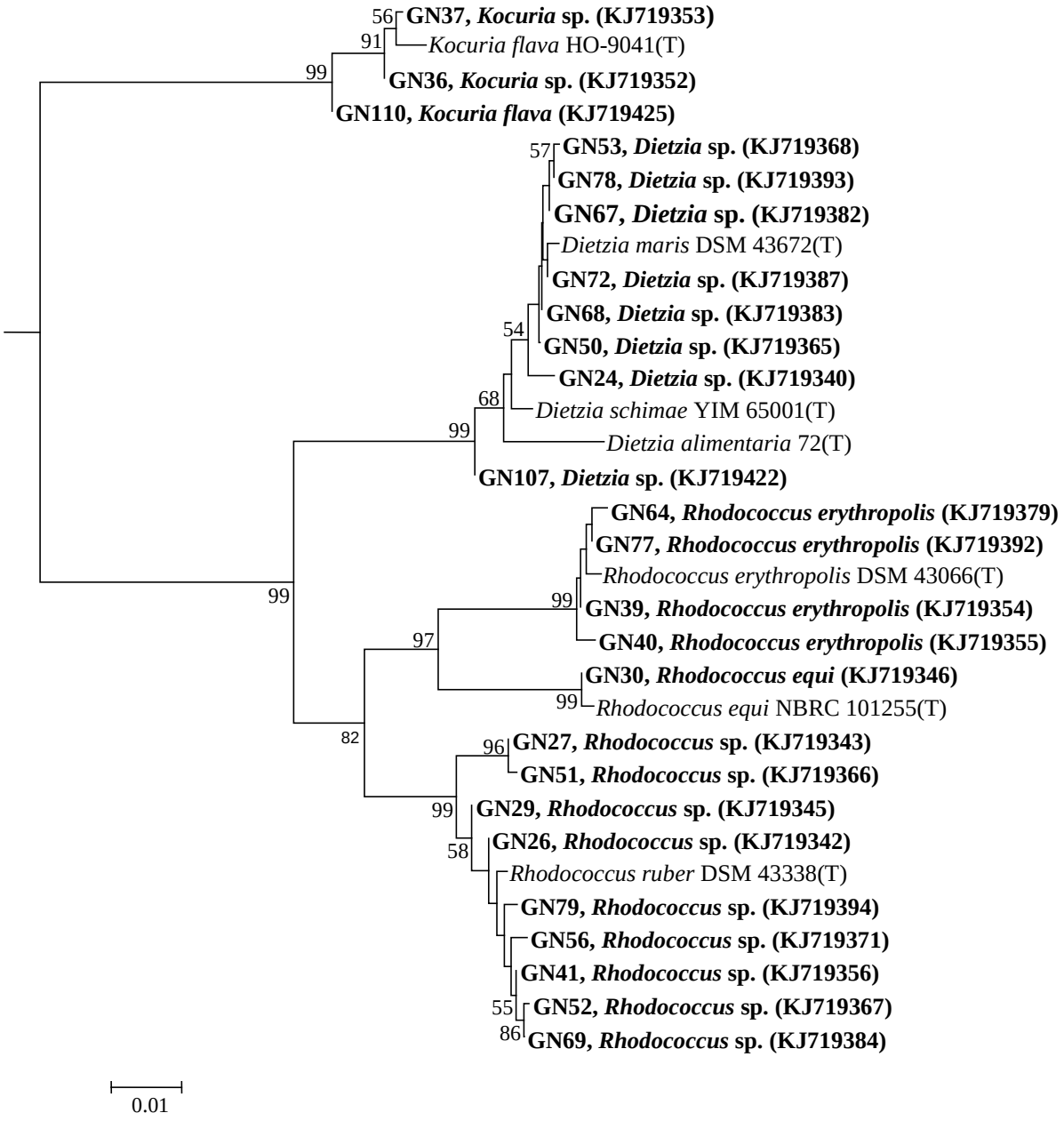


Figure 6.3a Phylogenetic tree (constructed using NJ method using Mega 4) of ARB isolates identified, by 16S rRNA genes sequences belonging to Phylum, Actinomycetes. Sequences in bold are from this study. The percentages of boot strap support are shown on the branches. The scale bar 0.01 indicates 1% nucleotide sequence substitution.

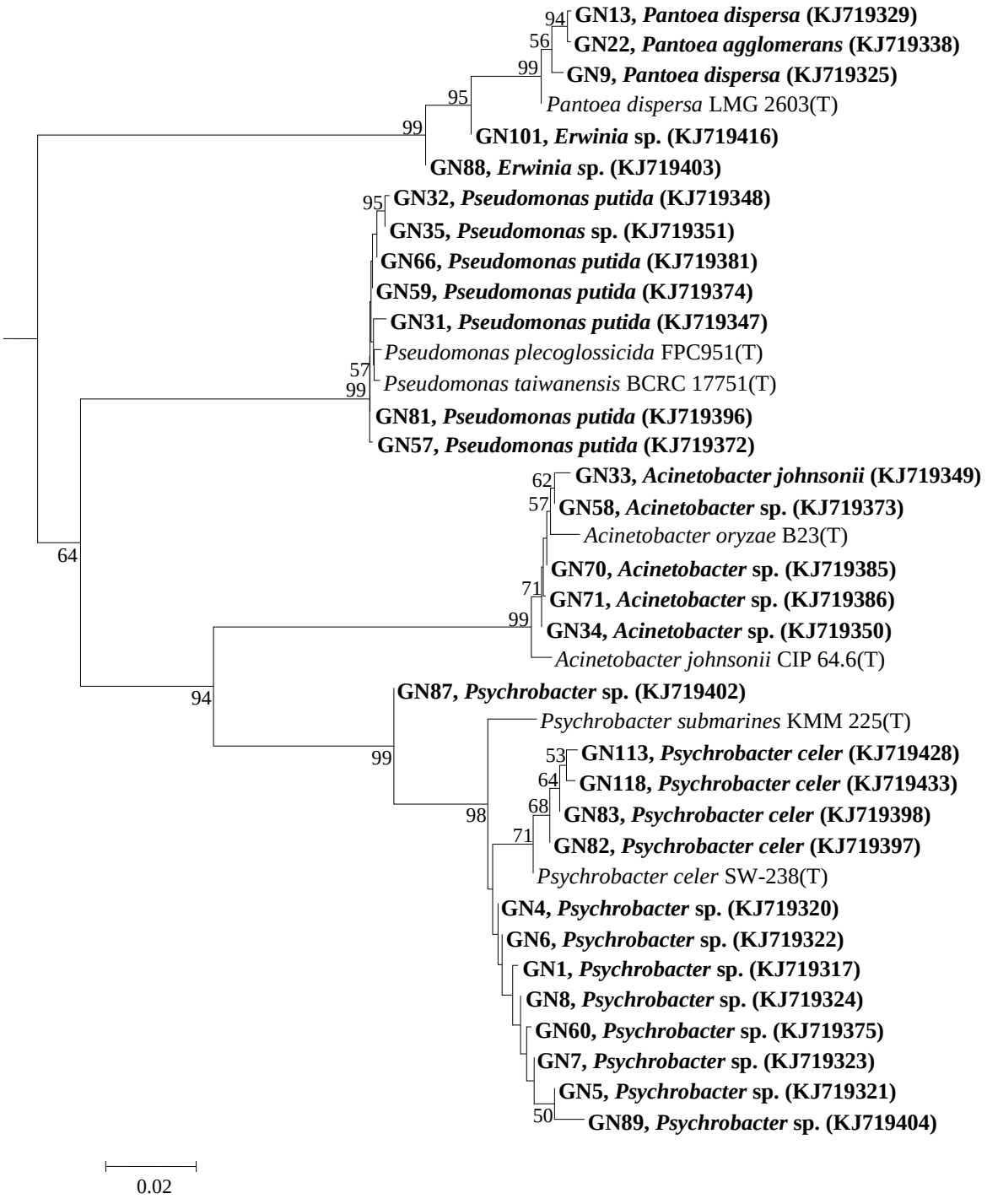


Figure 6.3b Phylogenetic tree of ARB isolates identified, by 16S rRNA genes sequences belonging to Phylum, Proteobacteria. Sequences in bold are from this study. The percentages of boot strap support are shown on the branches. The scale bar 0.02 denotes 2% nucleotide sequence substitution.

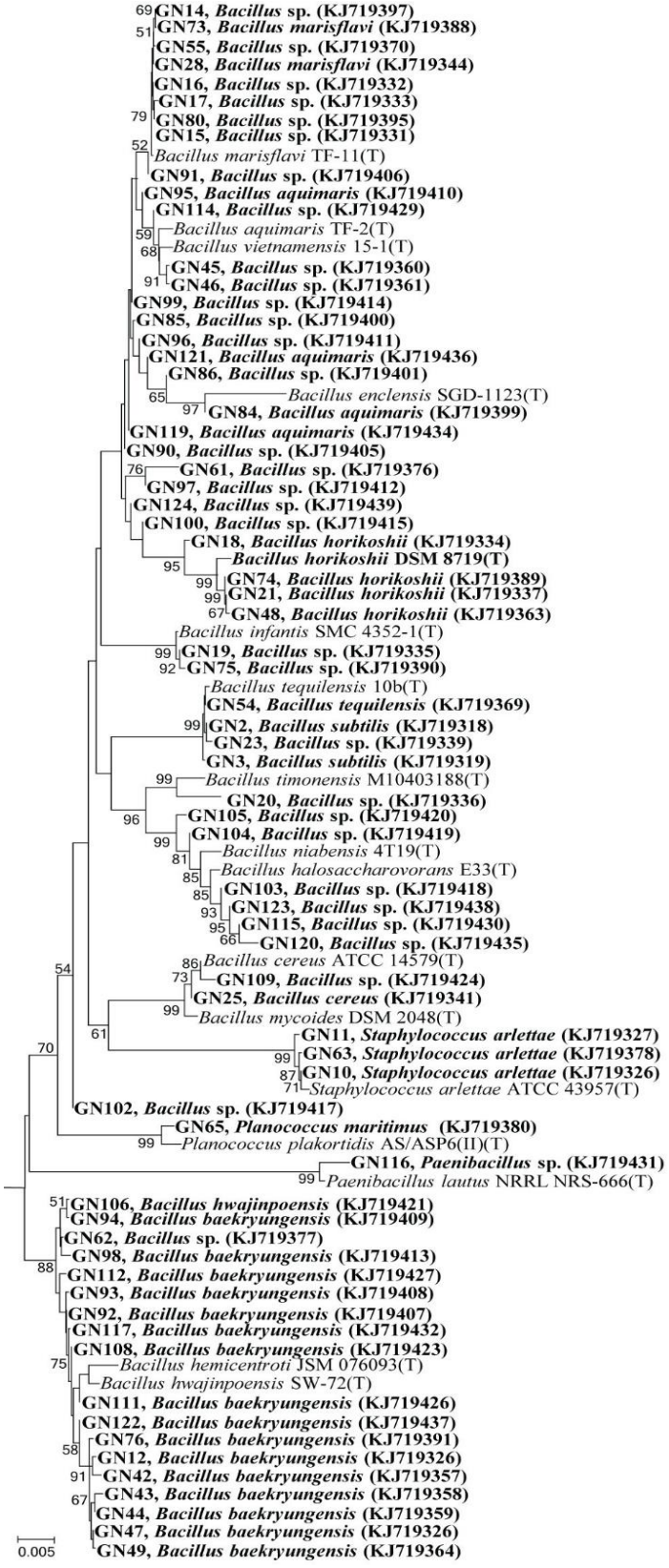
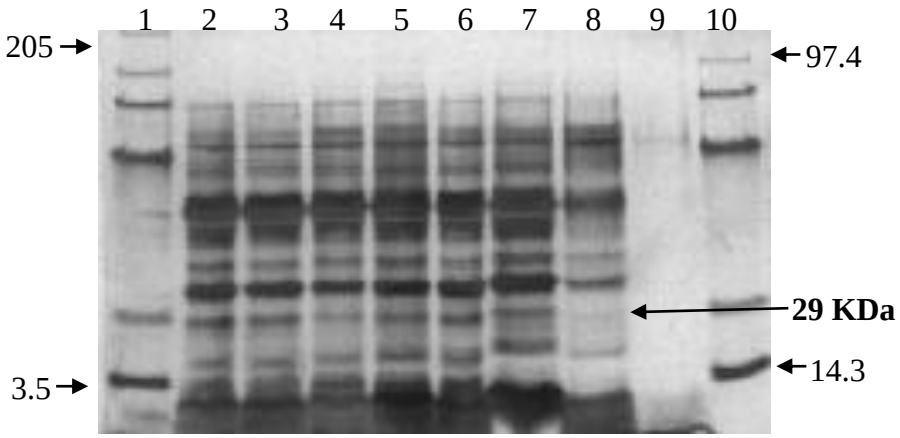
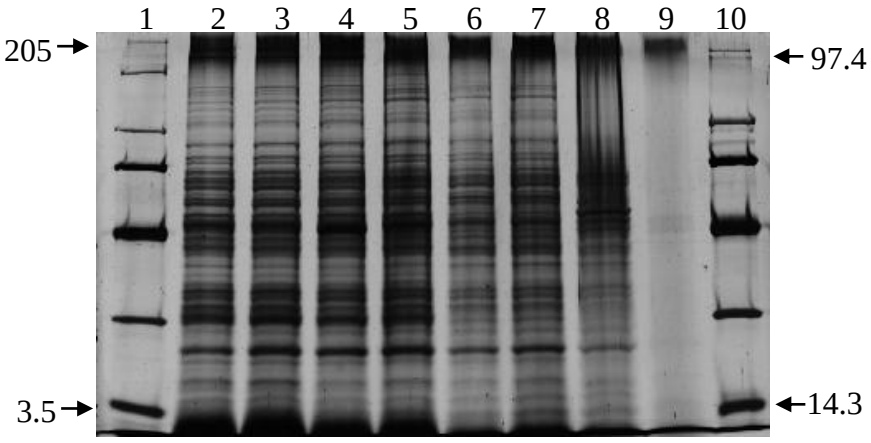


Figure 6.3c Phylogenetic tree of ARB isolates identified, by 16S rRNA genes sequences belonging to Phylum, Fermicutes. Sequences in bold are from this study. The percentages of boot strap support are shown on the branches.

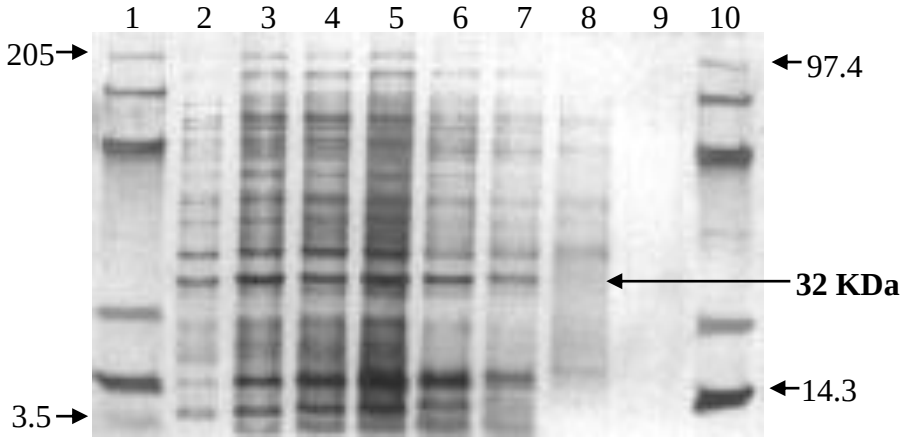


(a)

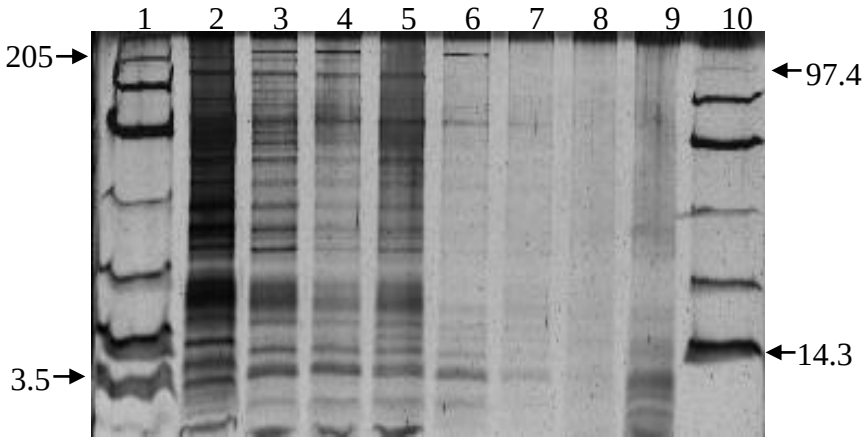


(b)

Figure 7.1 SDS-PAGE profiles of *Staphylococcus arlettae* (a) soluble, (b) insoluble proteins, at various concentrations of arsenic. Marker in lane 1 (205-3.5 KDa); 2-9, (0, 10, 50, 100, 200, 300, 500 and 1000 ppm arsenic); lane 10, marker (97.4-14.3 KDa).

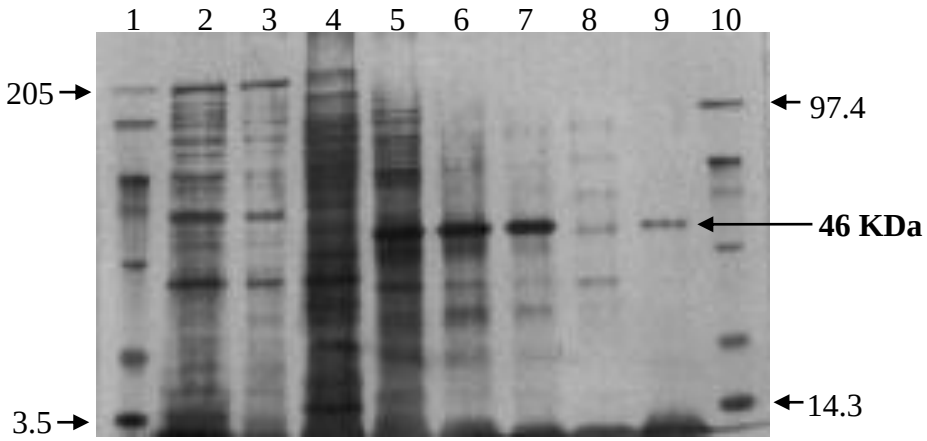


(a)

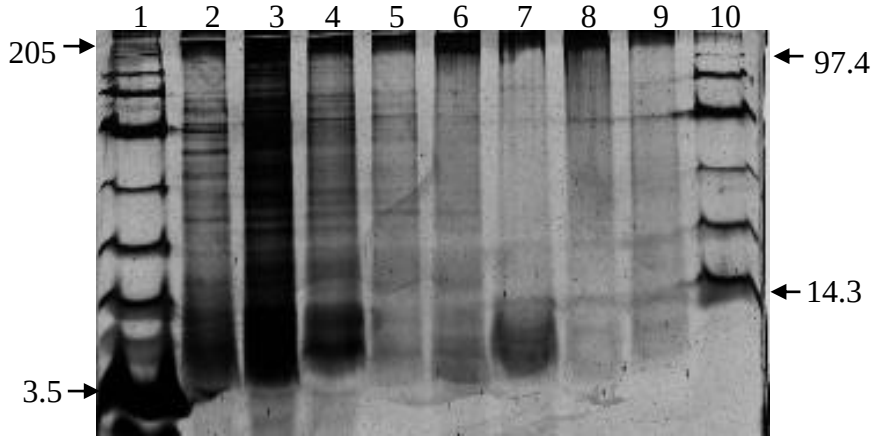


(b)

Figure 7.2 SDS-PAGE protein profiles of *Planococcus maritimus* (a) soluble, (b) insoluble, at various different concentrations of arsenic. Lane 1, marker (205-3.5 KDa); lanes 2-9, (0, 10, 50, 100, 200, 300, 500 and 1000 ppm arsenic); lane 10, marker (97.4-14.3 KDa).



(a)



(b)

Figure 7.3 SDS-PAGE protein profiles of *Bacillus baekryungensis* (a) soluble (b) insoluble at various different concentrations of arsenic. Lane 1, marker (205-3.5 KDa); lanes 2-9, (0, 10, 50, 100, 200, 300, 500 and 1000 ppm arsenic); lane 10, marker (97.4-14.3 KDa).

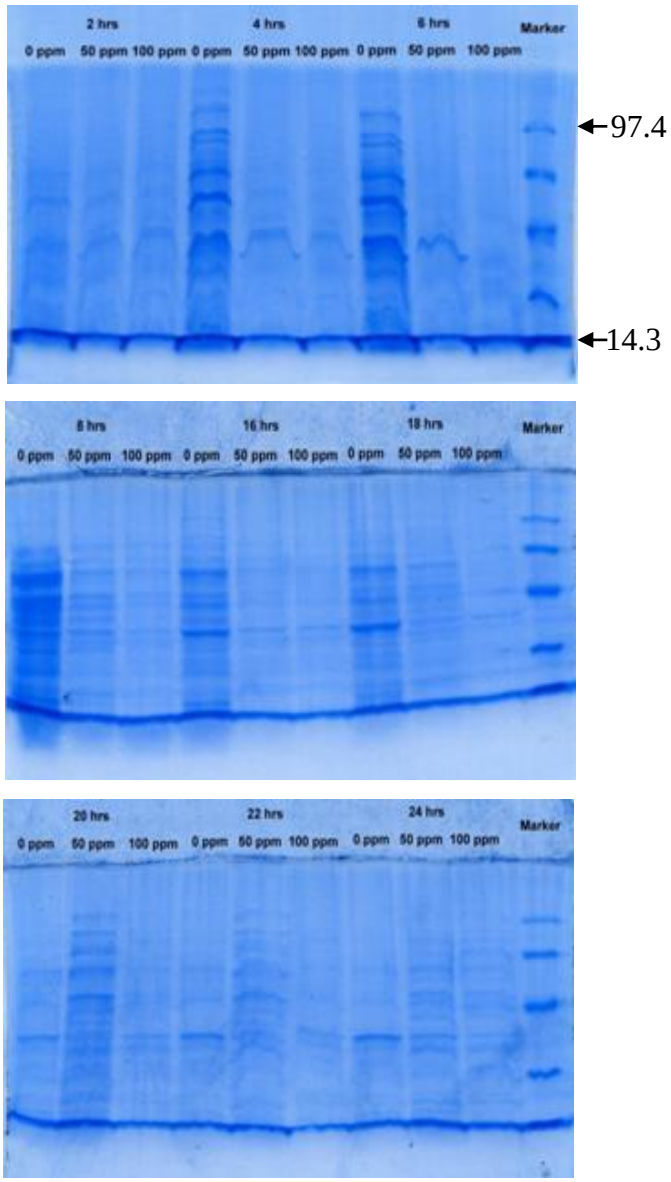


Figure 7.4 SDS-PAGE Proteins profiles of *Staphylococcus arlettae* at different concentrations (0, 50 and 100 ppm) of arsenic every two hours interval upto 24 h using marker ranging from 97.4 to 14.3 KDa.

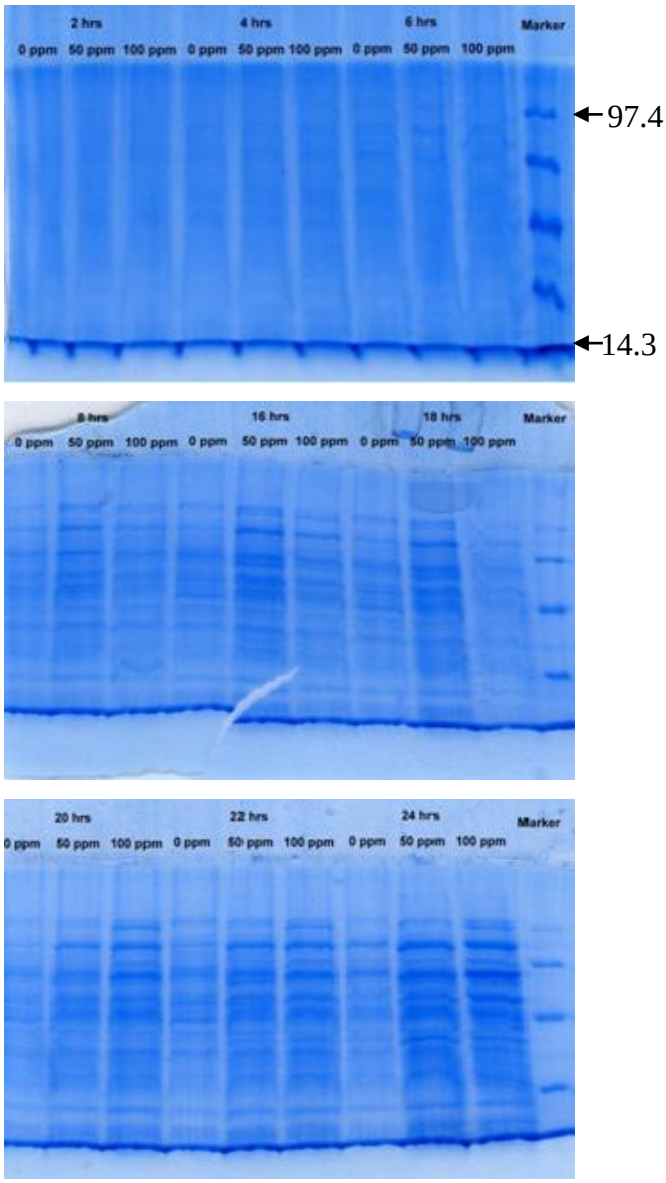


Figure 7.5 SDS-PAGE protein profiles of *Planococcus maritimus* at different concentrations (0, 50 and 100 ppm) of arsenic every two hours interval upto 24 h using marker ranging from 97.4 to 14.3 KDa.

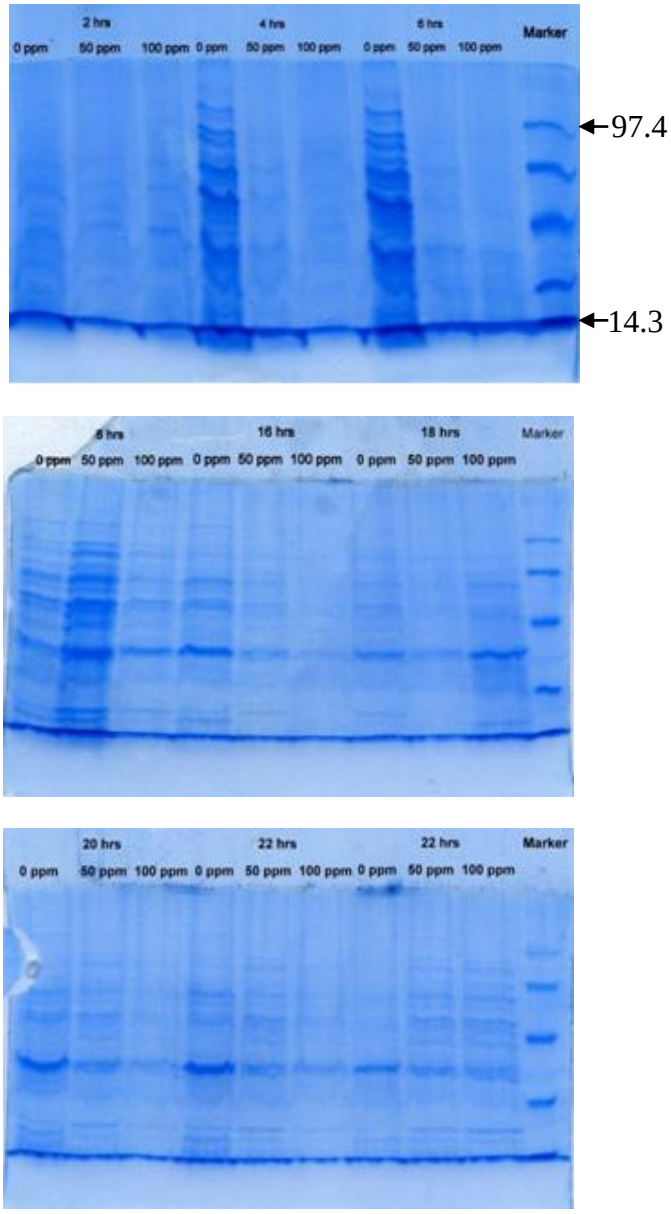


Figure 7.6 SDS-PAGE protein profiles of *Bacillus baekryungensis* at different concentrations (0, 50 and 100 ppm) of arsenic every two hours interval (2 h to 24 h) using marker ranging from 97.4 to 14.3 KDa.

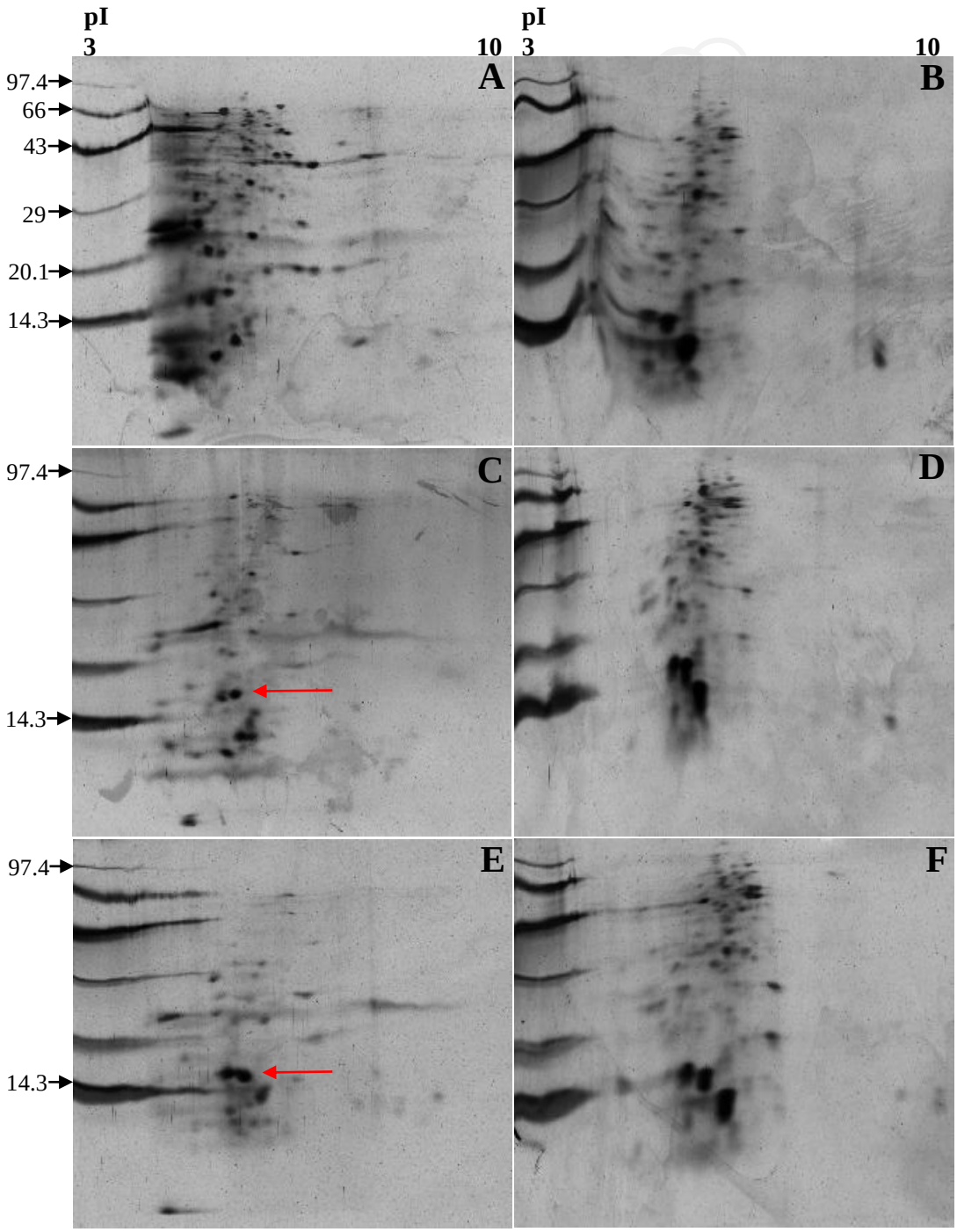


Figure 7.7 Two-dimentional (2-D) gel profile of *Staphylococcus arlettae* using a IPG strip of pH 3-10. (A) and (B) grown without arsenic, (C) and (D) with 50 ppm arsenic and; (E) and (F) with 100 ppm arsenic, soluble and insoluble respectively.

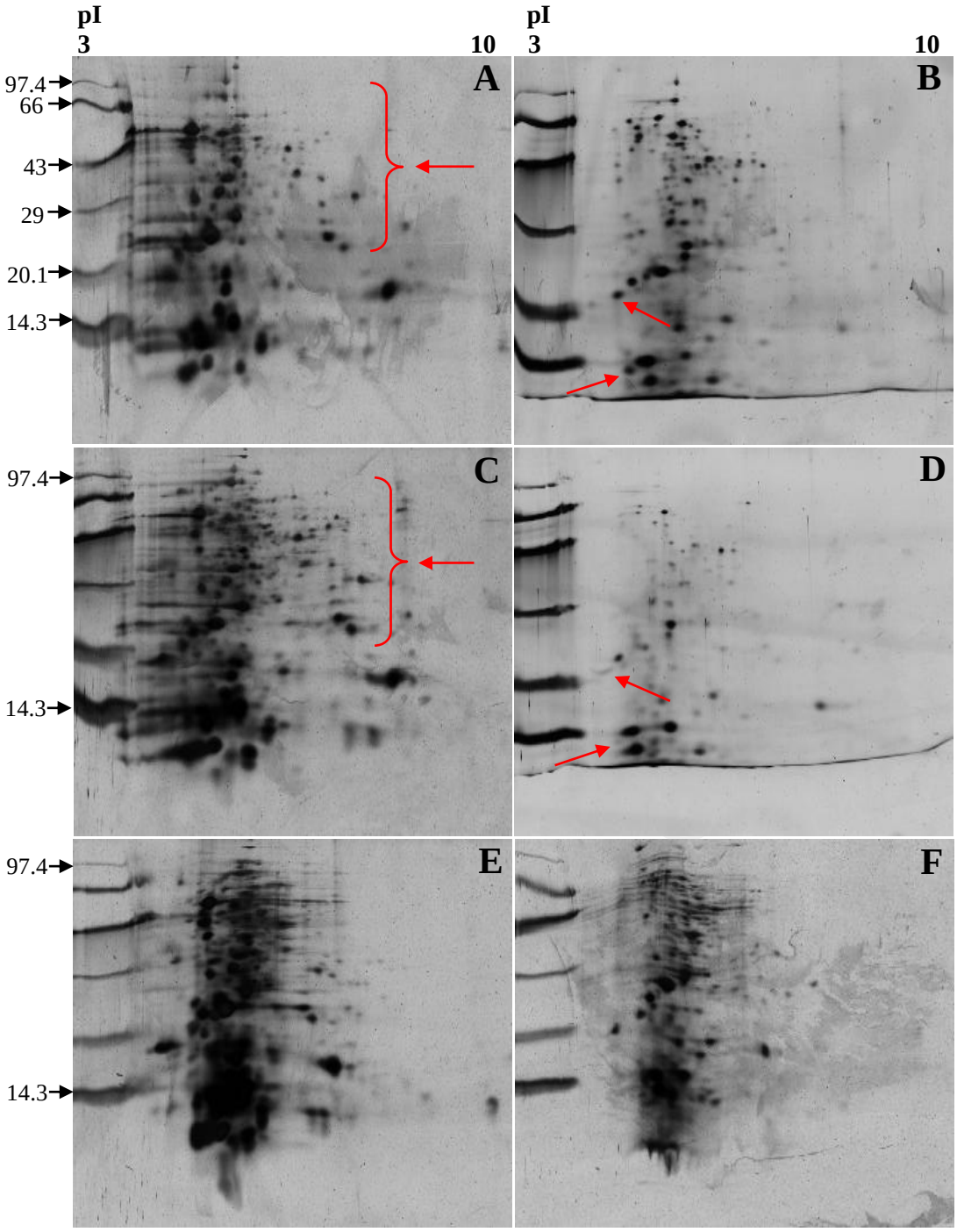


Figure 7.8 Two-dimentional (2-D) gel profile of *Planococcus maritimus* using a IPG strip of pH 3-10. (A) and (B) grown without arsenic, (C) and (D) with 50 ppm arsenic and, (E) and (F) with 100 ppm arsenic, soluble and insoluble respectively.

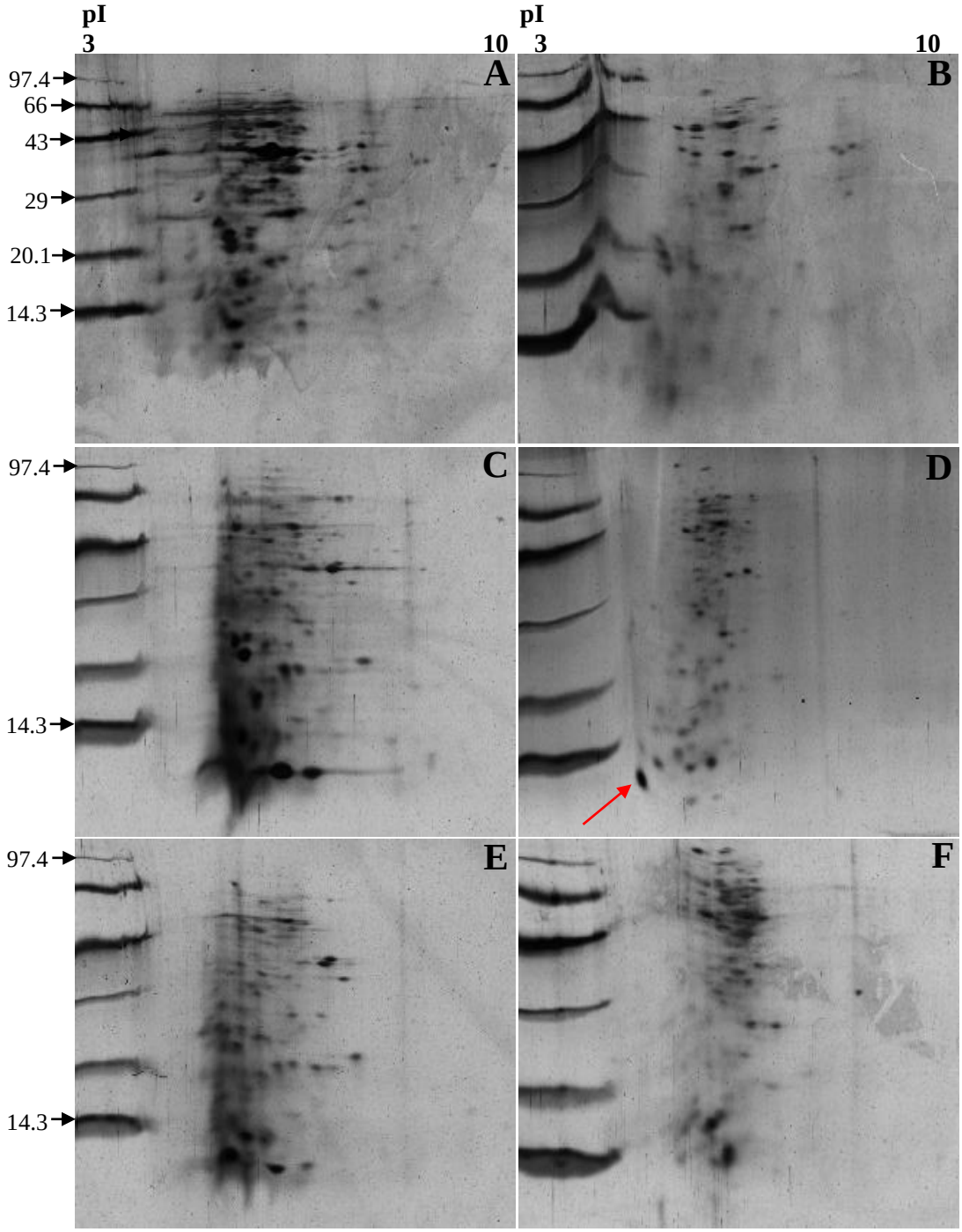


Figure 7.9 Two-dimentional (2-D) gel profile of *Bacillus baekryungensis* using a IPG strip of pH 3-10. (A) and (B) grown without arsenic, (C) and (D) with 50 ppm arsenic and; (E) and (F) with 100 ppm arsenic, soluble and insoluble respectively.

Table 7.1 Proteins expressed in *Bacillus baekryungensis* (0 ppm As) identified by MS/MS analysis

Sr No.	Protein identified	Gene name	Score	Protein pI	Protein MW (KDa)	Accession No.	Biological process/es	Molecular function/s
1	Alkyl hydroperoxide reductase subunit C	ahpC	23.97	4.48	20.80	P80239	response to oxidative stress	Peroxidase and peroxiredoxin activity
2	Superoxide dismutase [Mn]	sodA	23.62	5.2	22.49	P54375	response to stress, superoxide metabolic process	Metal ion complexing, superoxide dismutase function
3	Thioredoxin	trxA	22.3	4.51	11.51	P14949	cell redox homeostasis, electron transport chain, glycerol ether metabolism	electron carrier, protein disulfide oxidoreductase function
4	1-pyrroline-5-carboxylate dehydrogenase 2	ycgN	21.93	5.5	56.59	P94391	proline biosynthetic process, proline catabolic process to glutamate	1-pyrroline-5-carboxylate dehydrogenase functioning, oxidoreductase and conversion of aldehyde or oxo group of donors, NAD or NADP as acceptor
5	Uncharacterized protein YukE	yukE	21.08	4.38	10.99	C0SP85	-	-
6	Transcription elongation factor GreA	greA	20.48	4.75	17.36	A7Z726	regulation of DNA-dependent transcription, elongation, stress response, transcription, DNA-dependent	DNA binding

(continued)

7	Putative nitrogen fixation protein YutI	yutI	20.55	4.39	12.71	O32119	iron-sulfur cluster assembly, nitrogen fixation	iron ion binding, iron-sulfur cluster complexation
8	Probable fructose-bisphosphate aldolase	fbaA	20.22	5.19	30.57	P13243	fructose 1,6-bisphosphate metabolic process, glycolysis, formation of a cellular spore due to sporulation	Role in fructose-bisphosphate aldolase and zinc ion complexation
9	Uncharacterized protein YceE	yceE	20.07	4.58	21.12	O34384	Stress response	-
10	Ferredoxin	fer	20.01	3.77	9.10	P50727	electron transport chain	4 iron, iron ion and 4 sulfur cluster complexing, electron carrier function
11	Cold shock protein CspD	cspD	19.76	4.51	7.31	P51777	regulate transcription, stress response , transcription, DNA-dependent	DNA complexing
12	(R,R)-butanediol dehydrogenase	bdhA	19.26	4.99	37.51	O34788	-	(R,R)-butanediol dehydrogenase function, zinc ion and nucleotide complexing
13	Uncharacterized protein YqjE	yqjE	18.83	4.95	39.82	P54542	proteolysis	Metallopeptidase and metal ion complexing
14	Uncharacterized protein YcnI	ycnI	18.81	5.01	22.17	P94431	-	-
15	General stress protein 16U	yceD	18.52	4.49	20.75	P80875	response to stress	-

(continued)

16	Putative aldehyde dehydrogenase DhaS	dhaS	18.22	5.2	54.22	O34660	-	aldehyde dehydrogenase (NAD) activity
17	Cold shock protein CspB	cspB	17.64	4.54	7.37	P32081	regulate transcription, stress response, transcription, DNA-dependent	DNA binding
18	Chaperone protein DnaK	dnaK	16.95	4.76	66.06	P17820	protein folding and stress response	ATP complexing
19	Transketolase	tkt	16.34	4.99	72.40	P45694	-	metal ion complexing, transketolase function
20	UPF0447 protein YwfI	ywfI	16.16	5.15	29.56	P39645	-	-
21	10 kDa chaperonin	groS	15.6	4.8	10.18	A7Z206	protein folding	ATP binding
22	Putative cysteine protease YraA	yraA	15.18	4.92	18.57	O06006	proteolysis, response to stress	hydrolase and peptidase functions, acting on glycosyl bonds
23	Nucleoside diphosphate kinase	ndk	15.09	7.95	16.89	C5CGM3	CTP, GTP and UTP biosynthetic processes	ATP and metal ion complexing, nucleoside diphosphate kinase function
24	Protein IolS	iolS	15.07	5.5	35.17	P46336	-	oxidoreductase function
25	FMN-dependent NADH-azoreductase 2	azoR2	12.48	5.5	56.59	P94391	aromatic compound catabolism, response to toxin	oxidoreductase function, acting on nitrogenous compounds as electron donors
26	Oligoendopeptidase F homolog	yjbG	12.35	5.6	77.19	O31605	proteolysis	metalloendopeptidase function and zinc ion complexing
27	Uncharacterized protein YmcA	ymcA	12.24	5.25	16.22	O31779	-	-

(continued)

28	Manganese-binding lipoprotein MntA	mntA	11.91	6.16	33.47	O34385	cell adhesion, metal ion transport	metal ion binding
29	Glyoxal reductase	yvgN	11.37	5.23	31.72	O32210	-	methylglyoxal reductase (NADPH-dependent) function
30	Uncharacterized protein YjoA	yjoA	9.98	5.83	17.85	O34334	-	metal ion complexing
31	Protein kintoun	Ppi20	8.8	5.43	102.66	B4J4Y2	-	-
32	Alanine dehydrogenase	ald	8.74	5.28	39.74	Q08352	L-alanine and alanine catabolic processes,	alanine dehydrogenase function, metal ion and nucleotide binding
33	UPF0473 protein YrzB	yrzB	5.3	3.59	10.88	O34828	-	-
34	Acireductone dioxygenase	mtnD	16.21	4.61	20.94	A7Z3X7	L-methionine biosynthetic process from S-adenosylmethionine, L-methionine salvage from methylthioadenosine	acireductone dioxygenase (Ni2+-requiring) and acireductone dioxygenase [iron(II)-requiring] functions, iron ion binding, nickel cation binding
35	NADPH dehydrogenase	namA	14.41	5.28	37.70	P54550	stress response and response to toxin	FMN binding, NADPH dehydrogenase activity, reductase activity, trichloro-p-hydroquinone reductive dehalogenase activity
36	WD repeat-containing protein 48 homolog	AAEL012158	8.07	7.07	74.73	Q16MY0	-	-
37	Phosphoenolpyruvate synthase	ppsA	6.73	5.74	91.80	Q9ZMV4	gluconeogenesis, pyruvate metabolic process	ATP binding, metal ion binding, pyruvate, water dikinase activity

Table 7.2 Proteins expressed at 50 ppm As in *Bacillus baekryungensis* identified by MS/MS analysis

Sr No.	Protein identified	Gene name	Score	Protein pI	Protein MW (KDa)	Accession No.	Biological process/es	Molecular function/s
1	Putative cysteine protease YraA	yraA	26.86	4.92	18.57	O06006	proteolysis, response to stress	hydrolase and peptidase functions, acting on glycosyl bonds
2	Glyoxal reductase	yvgN	24.14	5.23	31.72	O32210	-	methylglyoxal reductase (NADPH-dependent) activity
3	Cold shock protein CspD	cspD	23.82	4.51	7.31	P51777	regulate transcription, stress response, transcription, DNA-dependent	DNA binding
4	Superoxide dismutase [Mn]	sodA	22.25	5.2	22.49	P54375	Stress response, superoxide metabolic process	metal ion complexing, superoxide dismutase functions
5	Probable NADH-dependent flavin oxidoreductase YqiG	yqiG	21.77	5.34	40.92	P54524	-	FMN complexing, oxidoreductase function
6	1-pyrroline-5-carboxylate dehydrogenase 2	ycgN	21.05	5.5	56.59	P94391	proline biosynthetic and catabolic process to glutamate	oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as electron acceptor
7	Uncharacterized protein YjoA	yjoA	20.86	5.83	17.85	O34334	-	metal ion complexing
8	Thioredoxin-like protein YdbP	ydbP	20.7	4.59	12.55	P96611	cell redox homeostasis, electron transport chain,	electron carrier and protein disulfide oxidoreductase function

(continued)

9	Alkyl hydroperoxide reductase subunit C	ahpC	20.65	4.48	20.80	P80239	oxidative stress response	peroxidase activity, peroxiredoxin activity
10	Thioredoxin reductase	trxB	19.86	5.18	34.63	P80880	Superoxide radicals removal	flavin adenine dinucleotide binding, thioredoxin-disulfide reductase function
11	Oligoendopeptidase F homolog	yjbG	19.47	5.6	77.19	O31605	proteolysis	metalloendopeptidase function, zinc ion complexing
12	Probable NADH-dependent butanol dehydrogenase 1	yugJ	19.46	5.35	42.90	O05239	-	metal ion complexing, oxidoreductase function
13	NADPH dehydrogenase	namA	19.31	5.28	37.70	P54550	response to toxin and stress	response to toxin, NADPH dehydrogenase activity, pentaerythritol trinitrate reductase activity,
14	Uncharacterized protein YuaE	yuaE	19.12	6.19	19.11	O32078	-	-
15	Manganese-binding lipoprotein MntA	mntA	18.86	6.16	33.47	O34385	cell adhesion, metal ion transport	metal ion complexing
16	Peptide methionine sulfoxide reductase MsrA	msrA	18.79	5.75	20.35	P54154	protein metabolic process	peptide-methionine-(S)-S-oxide reductase function
17	Thioredoxin	trxA	17.72	4.51	11.51	P14949	cell redox homeostasis, electron transport chain, glycerol ether metabolism	electron carrier and protein disulfide oxidoreductase function
18	UPF0435 protein YfkK	yfkK	16.24	4.84	8.14	O35019	-	-
19	Elongation factor G	fusA	16.12	5.05	77.37	B7GJ64	GTP catabolic process	GTP complexing, GTPase activity, translation elongation factor activity

(continued)

20	Uncharacterized protein YcnI	ycnI	14.87	5.01	22.17	P94431	-	-
21	Cold shock protein CspB	cspB	13.65	4.54	7.37	P32081	regulate transcription, stress response, transcription, DNA-dependent	DNA complexing
22	Transketolase	tkt	13.5	4.99	72.40	P45694	-	metal ion complexing, transketolase function
23	Tropomyosin A		13.04	4.59	32.27	O16127	-	-
24	Probable fructose-bisphosphate aldolase	fbaA	9.53	5.19	30.57	P13243	fructose 1,6-bisphosphate metabolic process, glycolysis, formation of a cellular spore due to sporulation	fructose-bisphosphate aldolase function, zinc ion complexing
25	Peptidase T	pepT	9.37	4.73	45.57	P55179	peptide metabolism, proteolysis	metallopeptidase activity, tripeptide aminopeptidase activity, zinc ion complexing
26	Thioredoxin-like protein YtpP	ytpP	9.08	4.5	12.92	O34357	glycerol ether metabolic process, cell redox homeostasis	electron carrier and protein disulfide oxidoreductase function
27	NADH-quinone oxidoreductase subunit D 2	nuoD2	7.31	5.31	44.07	A9G9T3	transport	NAD and quinone complexing, NADH dehydrogenase (quinone) function,
28	Putative NADP-dependent oxidoreductase YfmJ	yfmJ	6.99	4.94	36.78	O34812	aromatic compound catabolic process, response to toxin	nucleotide binding, oxidoreductase function, zinc ion binding

(continued)

29	Acireductone dioxygenase	mtnD	15.5	4.61	20.94	A7Z3X7	L-methionine biosynthetic process from S-adenosylmethionine, L-methionine salvage from methylthioadenosine	acireductone dioxygenase (Ni2+-requiring) and acireductone dioxygenase [iron(II)-requiring] function, iron ion and nickel cation complexing
30	Serine/threonine-protein phosphatase 5	PP5	11.2	5.93	62.49	Q84K11	intracellular signal transduction, lipid metabolic process, protein dephosphorylation	metal ion complexing, phospholipase C activity, phosphoprotein phosphatase function
31	Pirin-like protein YhaK	yhaK	8.1	6.4	26.11	P58115	-	-
32	Isoleucine--tRNA ligase	ileS	6.29	6.19	103.19	A5IY09	isoleucyl-tRNA aminoacylation, regulation of translational fidelity	ATP and metal ion complexing, aminoacyl-tRNA editing function
33	FMN-dependent NADH-azoreductase 2	azoR2	6.03	4.75	24.99	Q47ZU7	-	Oxidoreductase and catalytic activity, acting on nitrogenous compounds as electron donors
34	RanBP-type and C3HC4-type zinc finger-containing protein 1	Rbck1	5.91	5.84	59.51	Q62921	positive regulation of I-kappaB kinase/NF-kappaB cascade,	double-stranded DNA and zinc ion complexing, ubiquitin-protein ligase function
35	Inosine-5'-monophosphate dehydrogenase	guaB/ IMPDH	19.2	5.6	52.60	Q9KGN8	GMP biosynthetic process	IMP dehydrogenase function, metal ion complexing
36	UPF0061 protein Dshi_1157	Dshi_1157	6.82	5.3	52.49	A8LHV2	-	-

(continued)

37	Putative nitrogen fixation protein YutI	yutI	18.74	4.39	12.71	O32119	iron-sulfur cluster assembly, nitrogen fixation	iron-sulfur cluster and iron ion complexing
38	Tropinone reductase 2	TR2	7.56	5.87	28.77	P50163	tropane alkaloid biosynthetic process	nucleotide complexing, tropinone reductase function
39	DNA ligase	ligA	5.99	6.6	75.21	Q1IHJ4	DNA repair,	DNA and metal ion complexing, DNA ligase (NAD+) function

Table 7.3 Proteins expressed in *Bacillus baekryungensis* identified by MS/MS analysis at 100 ppm As

Sr No.	Protein identified	Gene name	Score	Protein pI	Protein MW (KDa)	Accession No.	Biological process/es	Molecular function/s
1	Putative cysteine protease YraA	yraA	18.76	4.92	18.57	O06006	proteolysis, response to stress	hydrolase and peptidase functions, acting on glycosyl bonds
2	Cold shock protein CspD	cspD	9.99	4.51	7.31	P51777	regulate transcription, stress response, transcription, DNA-dependent	DNA complexing
3	Membrane protein insertase YidC	yidC	7.55	5.3	60.40	Q6LW55	protein insertion into membrane, protein transport	-
4	50S ribosomal protein L15	rplO	8.01	10.41	19.82	Q04PV7	translation	rRNA binding, structural constituent of ribosome
5	ATP synthase subunit alpha 2	atpA2	6.96	4.92	56.85	A8ZNS4	ATP hydrolysis coupled proton transport, ATP synthesis coupled proton transport	ATP complexing, hydrogen ion transporting ATP synthase and proton-transporting ATPase function, rotational mechanism
6	Putative nitrogen fixation protein YutI	yutI	14.97	4.39	12.71	O32119	iron-sulfur cluster assembly, nitrogen fixation	iron ion and iron-sulfur cluster complexing
7	Inositol 2-dehydrogenase	iolG	9.93	5.44	37.22	B5Y2S5	inositol catabolic process	inositol 2-dehydrogenase activity, nucleotide binding

(continued)

8	Subtilin biosynthesis protein SpaB	spaB	9.19	5.88	1E+05	P39774	transport	-
9	FeS cluster assembly protein SufD	sufD	7.82	5.12	48.29	O32165	iron-sulfur cluster assembly	-
10	30S ribosomal protein S3	rpsC	7.82	5.12	48.29	O32165	translation	rRNA complexing, structural constituent of ribosome
11	Nonribosomal peptide synthetase 14	NRPS14	7.09	5.71	4E+05	Q4WAZ9	pathogenesis	isomerase, transferase and ligase activity, nucleotide and phosphopantetheine complexing,
12	GTP cyclohydrolase-2	ribA	6.14	6.44	22.35	Q889Q3	riboflavin biosynthetic process	GTP and metal ion complexing, GTP cyclohydrolase II function
13	Translation initiation factor 1A	eIF1A	5.82	10.25	12.36	A4FYM5	-	translation initiation factor activity
14	Ubiquitin carboxyl-terminal hydrolase 16	usp16	5.58	5.38	1E+05	Q6PAW2	cell division, histone deubiquitination, positive regulation of transcription, DNA-dependent, protein homotetramerization, transcription, DNA and ubiquitin-dependent protein catabolism	cysteine-type endopeptidase, transcription coactivator, ubiquitin thiolesterase and ubiquitin-specific protease functions, zinc ion complexing
15	Solute carrier family 46 member 3	SLC46A3	5.22	5.81	51.93	A5D7V7	transmembrane transport	-
16	Pseudoazurin	bcp	5.19	7.91	15.82	P19567	electron transport chain	copper ion complexing, electron carrier activity

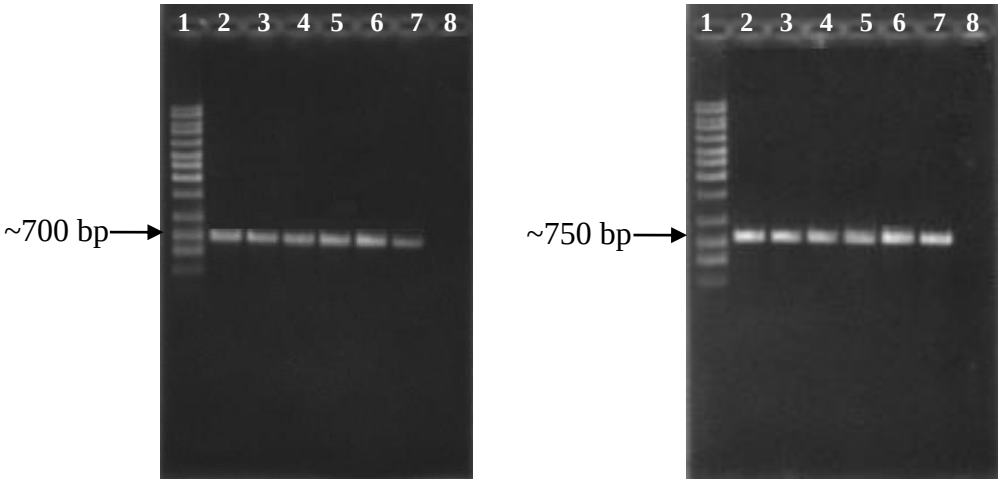
Table 8.1 Bacterial isolates screened for PCR amplification of *ars*, *aox* and *Acr*3 genes

Sr No.	Bacterial code	Bacterial isolates	Accession No.
1	GN1	*Psychrobacter* sp.	KJ719317
2	GN82	*Psychrobacter celer*	KJ719397
3	GN96	*Bacillus* sp.	KJ719411
4	GN84	*Bacillus aquimaris*	KJ719399
5	GN12	*Bacillus baekryungensis*	KJ719328
6	GN25	*Bacillus cereus*	KJ719341
7	GN18	*Bacillus horikoshii*	KJ719334
8	GN106	*Bacillus hwajinpoensis*	KJ719421
9	GN28	*Bacillus marisflavi*	KJ719344
10	GN2	*Bacillus subtilis*	KJ719318
11	GN54	*Bacillus tequilensis*	KJ719369
12	GN22	*Pantoea agglomerans*	KJ719338
13	GN13	*Pantoea dispersa*	KJ719329
14	GN10	*Staphylococcus arlettae*	KJ719326
15	GN24	*Dietzia* sp.	KJ719340
16	GN26	*Rhodococcus* sp.	KJ719342
17	GN39	*Rhodococcus erythropolis*	KJ719354
18	GN30	*Rhodococcus equi*	KJ719346
19	GN35	*Pseudomonas* sp.	KJ719351
20	GN31	*Pseudomonas putida*	KJ719347
21	GN34	*Acinetobacter* sp.	KJ719350
22	GN33	*Acinetobacter johnsonii*	KJ719349
23	GN36	*Kocuria* sp.	KJ719352
24	GN110	*Kocuria flavus*	KJ719425
25	GN88	*Erwinia* sp.	KJ719403
26	GN116	*Paenibacillus* sp.	KJ719431
27	GN65	*Planococcus maritimus*	KJ719380

Table 8.2 PCR primer sets used for amplification of chromosomal *ars*, *aox* and *Acr*3 genes for isolates resistant to As

Primers	Primer sequences	Annealing temperature
*ars*R	F 5´-ATCCAGCTCTTCAAAACC-3´	53
	R 5´-GTTTTTCAGCTTCATAC-3´	
*ars*B	F 5´-GTGGAATATCGTCTGGAATGCGAC-3´	57
	R 5´-GGTAATTTTCGGCCCCAAATCG-3´	
*ars*C	F 5´-TGCGGCACTTCGTGAAACAC-3´	57
	R 5´-AAGTATATCCAGAACCACTT-3´	
*ars*A	F 5´-ACCCACGCTTAGCAATATCATCGA-3´	55
	R 5´-TGAAAGTCTTCATATAGGTCTTCC-3´	
*ars*D	F 5´-ATGTGCTGCAGTACCGCCGT-3´	60
	R 5´-TATTACCACCACAGCAAC-3´	
*ars*AB	F 5´-AAAACTTCCATTTCCTGCGCGACG-3´	60
	R 5´-AAGTGAAAGAGAGACGTAGCGCCA-3´	
*ars*H	F 5´-ATGGACCAGTTCCCAGAC-3´	55
	R 5´-CTGATTGGGGATGGTGAACA-3´	
*aox*B	F 5'-GTSGGBTGYGGMTAYCABGYCTA-3'	59
	R 5'-TTGTASGCBGGNCGRTTRTGRAT-3'	
*aox*R	F 5´- AATCGCTCATCCAGCGACTTTCGC-3´	59
	R 5´- TTGCGTCCTCGCCAAGCGTACTGA-3´	
*ars*B*	F 5'-GGTGTGGAACATCGTCTGGAAYGCNAC-3'	57
	R 5'-CAGGCCGTACACCACCAGRTACATNCC-3'	
*Acr*3	F 5'-GCCATCGGCCTGATCGTNATGATGTAYCC-3'	57
	R 5'-CGGCGATGGCCAGCTCYAAYTTYTT-3'	

*Different set of primer was used for PCR amplification of *ars*B gene (Achour et al., 2007)



(a) (b)

Figure 8.1a and b PCR amplified product showing *ars*B gene from ARB. Lane 1 with 1 Kb DNA ladder (300 to 10000bp); lane 2 to 7 depict the PCR amplified at ~700bp and ~750 bp; lane 8 with PCR negative control.

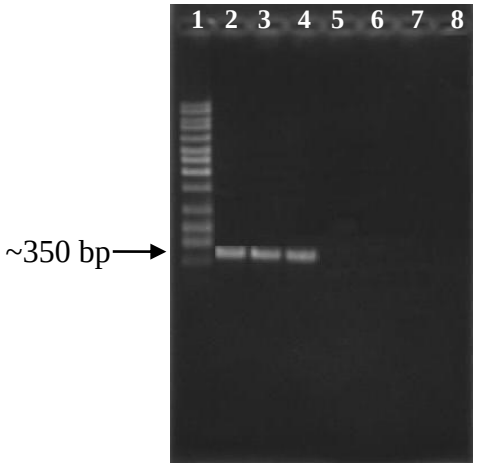


Figure 8.2 Agarose gel showing *aox*B, *ars*D, and *Acr*3 gene products amplified from genomic DNA of ARB. Lane 1 with 1 Kb DNA ladder (300 to 10000bp); lane 2: *aox*B; lane 3: *ars*D and lane 4: *Acr*3 amplified at ~350bp respectively; lane 5 to lane 8 with PCR negative control.

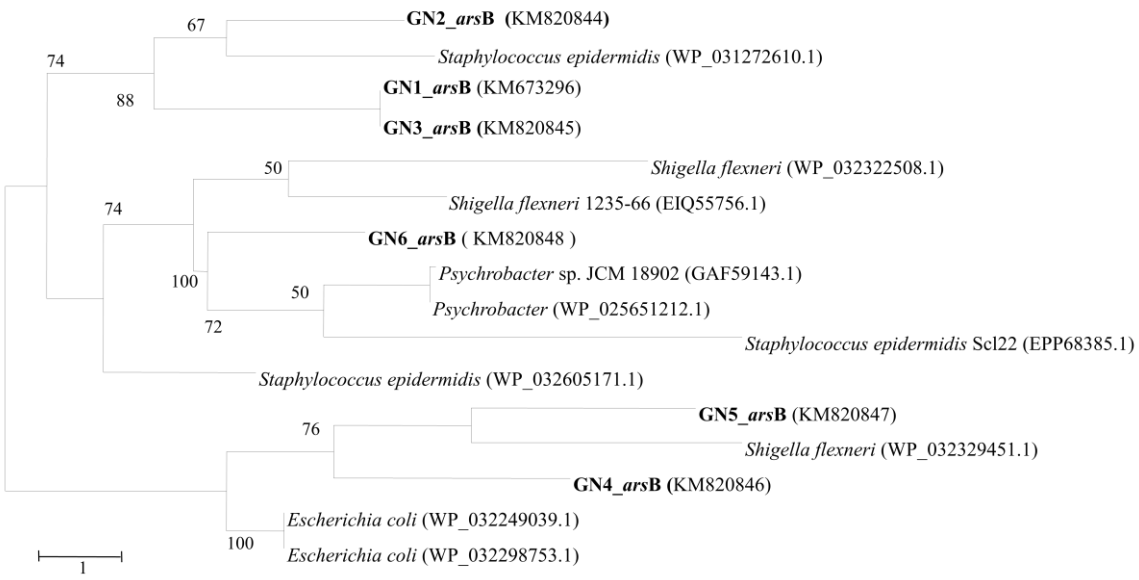


Figure 8.3 Phylogenetic tree constructed using Seaview for *ars*B gene in ARB with sequence homology of 100%. Sequences in bold are from this study. Percentages of boot strap support are shown on the branches. These values were calculated from 1000 bootstrap re-samplings.

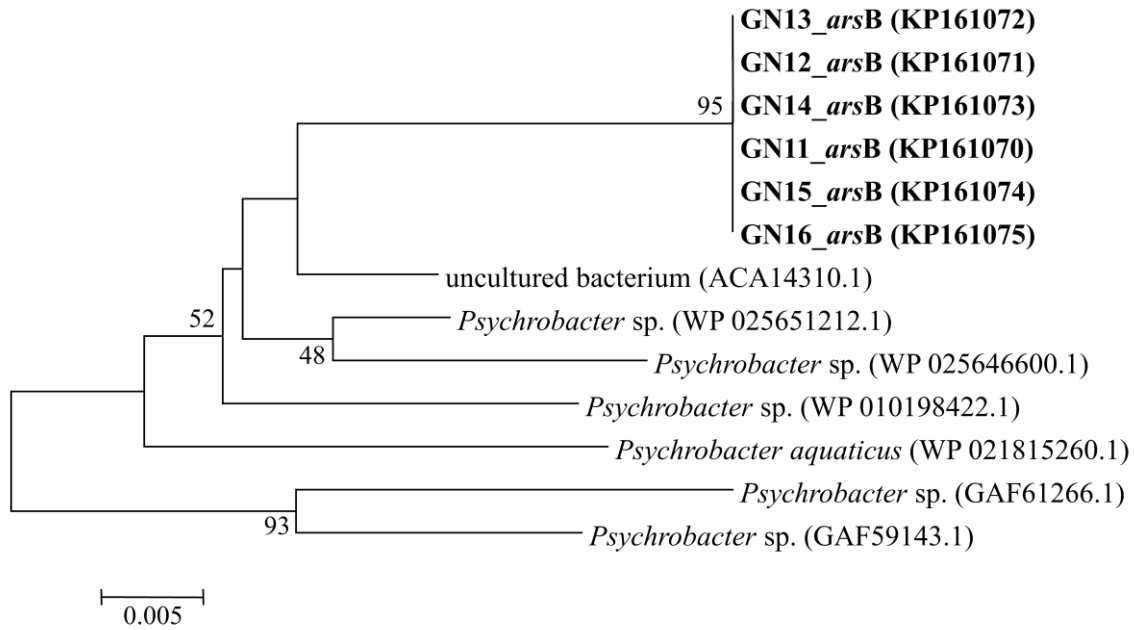


Figure 8.4 Phylogenetic tree of *ars*B (Achour et al., 2007) gene constructed using Neighbour joining method using Mega4 for ARB with sequence homology of 92%. Percentages of boot strap support are shown on the branches. These values were calculated from 1000 bootstrap re-samplings.

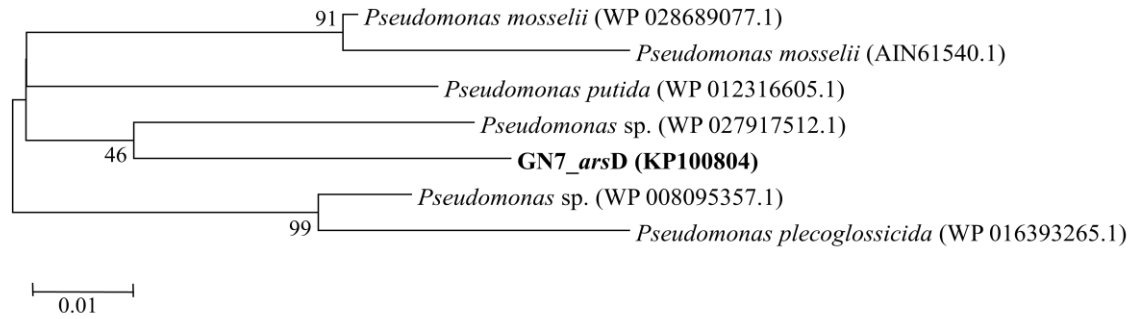


Figure 8.5 Phylogenetic tree (by Neighbour joining method using Mega4) of *ars*D gene in *Bacillus cereus* encoding *ars*D, chemotaxis protein CheY (KP100804) with sequence homology of 93%. Percentages of boot strap support are shown on the branches. These values were calculated from 1000 bootstrap re-samplings.

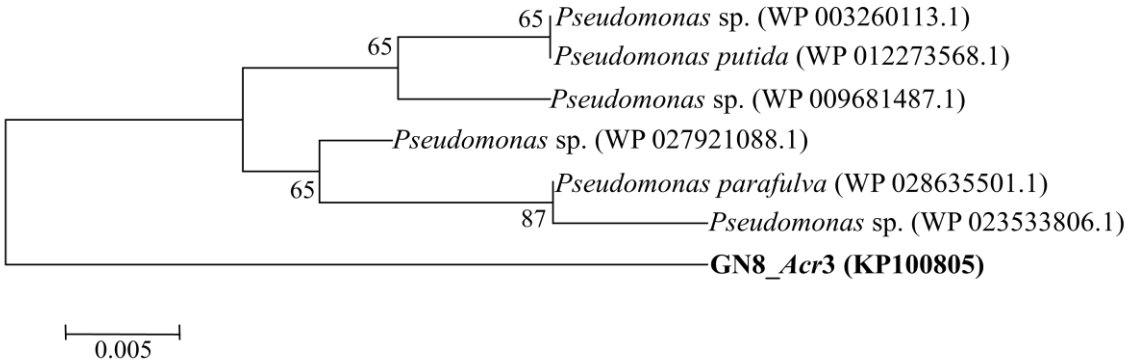


Figure 8.6 Phylogenetic tree (using Mega4) of *Acr*3 gene in ARB, *Bacillus cereus* encoding *Acr*3, as ABC transporter (KP100805) with sequence homology of 95%. Percentages of boot strap support are shown on the branches. These values were calculated from 1000 bootstrap re-samplings.

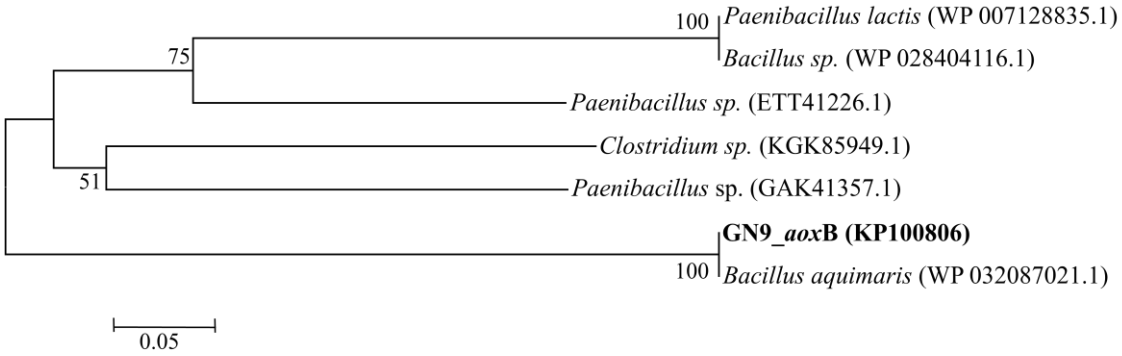


Figure 8.7 Phylogenetic tree built using Mega4 for *aox*B gene in isolate *Bacillus* sp. encoding *aox*B gene, NADH oxidase (KP100806). Percentages of boot strap support are shown on the branches. These values were calculated from 1000 bootstrap re-samplings.