Studies on microbial biodiversity of acidophilic heterotrophs in Acid Rock Drainage samples of Eastern Himalaya

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The only way of finding the limits of the possible is by going beyond them into the impossible.

-Arthur C. Clarke

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Suparna Bhowal
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General Introduction

Bacteria, the simple living entities, are ubiquitous and more abundant than any other life form in the entire biosphere. It is because of their diversity, rapid reproducibility, ratio of surface area to weight which is high and adaptability in any environmental niche. In contrast to the eukaryotic system, in general, the bacterial cells are small, anucleate and rely solely on diffusion for their livelihood (Beveridge, 1989). They are completely exposed to the components of the biosphere. With the simpler physiology they efficiently respond to the changes of any of these components through genetically programmed processes. This is necessary because they cannot selectively reach to their essential nutrient component nor can they fling away a toxic component, or cannot outswim their local aqueous environment to a totally distinct environment (Beveridge, 1988). The extreme heterogeneity of nutrient components present in a given environment supports the microbial world, for the sustenance of diverse populations which enrich and colonize in their favourable sites and further modify and develop specialized ecological niches. Hence, the meaning of the word "diversity" is not really unknown. Biodiversity is an attribute of an area and specifically refers to the varieties within and among living organisms, assemblage of the living organisms, biotic communities and biotic processes, whether naturally occurring or modified by humans. The vast majority of microbial diversity (>95%) remains to be discerned. Very little is known about microbial species and functional diversity, and decisions about the role of microorganisms and their influence on sustainable ecosystems are being made on the basis of very incomplete information.

Based on rRNA trees, it may be said that the main extents of Earth's biodiversity is microbial (Hugenholtz *et al.*, 1998). Our perspective on microbial diversity has improved enormously over the past few decades. The microbial diversity is somewhat different from the macro-organisms. Establishing the diversity of microorganisms present by relying on morphological features alone is not possible; instead, specific physiological capabilities also need to be deciphered in order to establish the different types of microbes present. The specific biochemical and physiological activities of microbial cells are of profound significance for the habitat. The enormous success of enrichment culture and related isolation approaches and considerations (van Neil, 1995), along with applications of new analytical techniques (Amann *et al.*, 1995;

Hugenholtz *et al.*, 1998) has enabled us to see that we understand very little about so many of the presumptive microbes present in nearly every habitat we explore.

Much effort has been expended in the isolation and description of individual micro-organisms. Investigations on pure cultures are important to judge the potential abilities and functions of individual species in nature. Ecology and diversity study deals with interactions between organisms and relations between organisms and environments. Microbial ecology deals only with a segment of the total ecological system.

I. Mineral rich acidic habitats

Extreme environments such as acidic, thermophilic, hypersaline are important 'hot spots' of microbial 'megadiversity'. These are habitats of microorganisms which have the genetic and physiological capacity to survive and grow under these harsh or extreme conditions through which they have evolved while shaping the environment as we know it today. Extremophiles, organisms capable of thriving under extreme conditions, have become the interest from both an academic and biotechnology perspective because of their interesting ecology and physiology and thus have recently attracted considerable attention.

Acidic environments are especially fascinating because, in general, the low pH of the habitat is the consequence of microbial metabolism and not a stipulation imposed by the system as is the case in many other extreme environments like temperature, ionic strength, high pH, radiation, pressure, etc. (Lo´pez-Archilla *et al.*, 2001; Gonzalez-Toril *et al.*, 2001). Acidophiles are organisms that can withstand and even thrive in acidic environments where the pH values range from 1 to 5. Acidophiles or acid-tolerants include certain types of bacteria and archaea that are found in a variety of acidic environments, including ic pools, areas polluted by acid mine drainage, and even our own stomachs. Acidophiles have potential importance in evolution because metabolic processes might have originated on the surface of sulfide minerals (Wächtershäuser, 2006) and structuring of the genetic code could have taken place at acidic pH (Di Giulio, 2005). These organisms are also used to recover metallic minerals lost during the mining of coal and to reduce levels in coal (Marcus, 1997). Probably

first bacteria were isolated from acidic (pH <4.5) environments (Powell and Parr, 1919). Colmer and Hinkle, 1947 found that killing the bacteria stopped production of acids and dissolution of metals in acid mine drainage (AMD), thereby establishing the positive relationship between micro-organisms and environmental acidification. Bacteria contribute in many different ways to the acidic environments in which they cohabit (Robbins, 2000, Bond *et al.*, 2000a).

Acid drainage is a low pH, iron and sulfate bearing water usually formed when rocks containing sulfide minerals (eg. Pyrite, pyrrhotite etc.) are exposed to the atmosphere or an oxidizing environment, and subsequently leached by water. Environments containing high levels of dissolved metals include active and disused mines, where the production of acid mine drainage (AMD) and acid rock drainage (ARD) is catalyzed by the action of microorganisms. The microorganisms that are capable of oxidizing iron usually produce acid resulting into the formation of Acid Rock Drainage (ARD) or Acid Mine Drainage (AMD) systems. ARD is the result of spontaneous oxidation of surface rock outcrops of sulfide masses whereas AMD is the result of the appearance of effluents produced by mining operations (Grande *et al.*, 2005). They are formed when the sulfide ore of a mineral comes in contact with oxygen and atmospheric humidity which leads to a complex set of reactions resulting in the production of acid. The reaction is greatly accelerated in presence of ferric iron and by the action of bacteria that oxidizes ferrous iron to ferric iron (Sand *et al.*, 2001; Rohwerder *et al.*, 2003).

Acid drainage is a major issue affecting the metal mining and coal industry throughout the world. Many old mining sites have a legacy of acid drainage long after the completion of mining (Helms and Heinrich, 1997). As water percolates through the exposed sulfide mineral waste rock, chemical and then microbiological oxidation occur, causing acid production known as acid mine drainage (AMD or ARD). Acid leaching solutions are characterized by high metal concentrations that are toxic to most life. It is well known that AMD solutions are far from "sterile" and those acidophilic microorganisms not only tolerate, but also thrive in these acidic metal rich solutions (Hallberg and Johnson, 2001). However, the biogeochemical mechanisms associated with the production of acid rock drainage are complex and more research will be

necessary to determine if the induced beneficial microbial interactions would dominate in the natural ARD environment (Marchand and Silverstein, 2000).

II. Geological description of the sampling site: the original habitat of the acidophilic autotrophic and heterotrophic strains used in the present study

The geological formations of Darjeeling District comprised of unaltered sedimentary rocks, confined to the hills on the south. A characteristic feature of this area is that the older formation rest on the younger, showing a complete reversal of the original order of superposition. The great range of Himalaya was elevated during the Tertiary period, and the area has accumulated sediments of different geological ages (Dash, 1947).

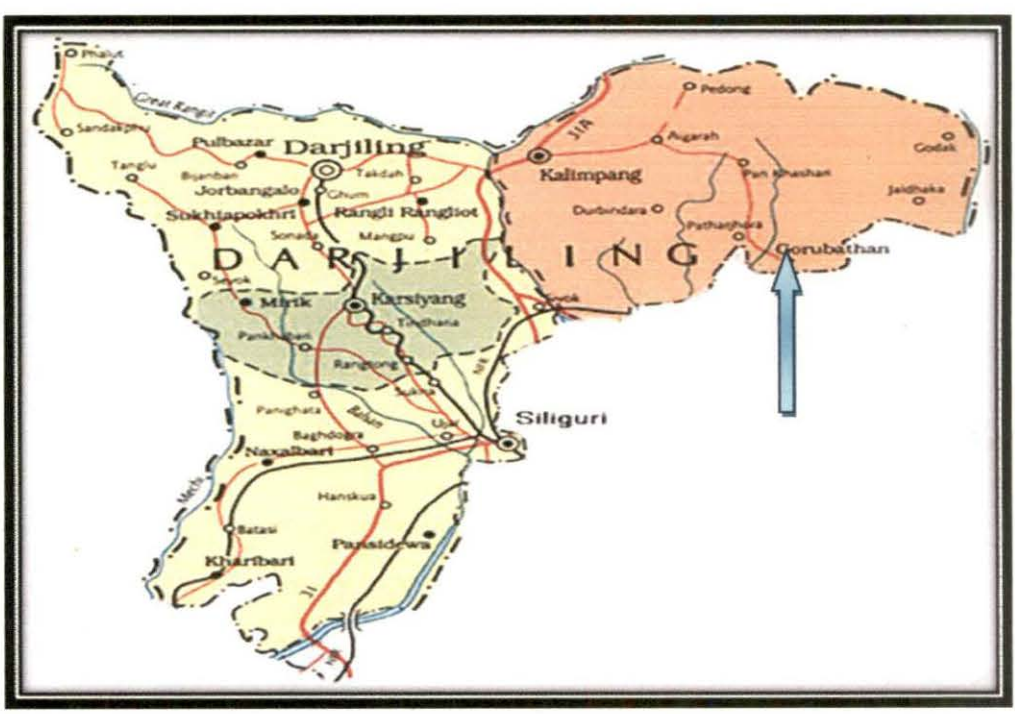

Fig. A: Location of Garubathan (marked with blue arrow) in Darjeeling Map.

Records of the GSI, vol. 109 part I 1982, showed the occurrence of magnetite in Khanikhola, south of Siyokbir (27° 02'00" N Lat. and 88° 30'45" E Long.). Lead-zinc

ore has been found to occur in the area around Garubathan Kalimpong sub-division (Shah *et al.*, 1974-75). An area of 0.247 sq.km on scale 1:2000 was covered by plane table mapping in the Kharkhola Block and Mal Khola block in the East and West of Garubathan. A total of 780.80 m in 4 boreholes were drilled in Mal Khola block which showed an average of 1.49% Pb, 0.01% Zn and 0.33% Cu. A total of 131 samples, comprising of surface channel samples, float-ore samples, borehole samples, composite samples, selective grab samples etc. were collected which showed the probable reserves estimated with an average of 3.54% Pb and 2.7% Zn and that of float ore is 2,910 tons, with an average of 7.147% Pb and 2.87% Zn. The average Cu and Ag in the ore, on the basis of 150 channel sample amount 1.14% and 42 g/ton, respectively (records of GSI, vol. 109, part I, 1982). A systematic geological mapping in parts of Kalimpong sub-division of Darjeeling district, covering an area of 180 sq.km on 1: 25000 scale in parts of toposheet No. 78A/8 and 12 was carried out by Roy, Chowdhury and Ghosh (1974-75). An occurrence of wolframite associated with base metal mineralization was observed at a place (27° 09'55" N Lat. and 88° 34'40" E Long.) near Mansong during the course of mapping. mineralization has been located near Pedong (27° 12'55" N Lat. and 88° 37'00" E Long.), Rishi (27° 11'30" N Lat. and 88° 38'00" E Long.), and Rorathang (27° 12'00" N Lat. and 88° 37'00" E Long.).

III. Microbial ecology of AMD/ARD

Acidophiles grow in environments of low pH (<3) and include bacteria, archaea and eukaryotes that are capable of growing chemolithoautotrophically, chemomixotrophically, and chemoheterotrophically (Hallberg and Jhonson, 2001). Bacterial populations indigenous to acidic drainage streams are heterotrophs such as the fungi *Aspergillus* sp. and *Penicillium* sp.; the bacteria *Acidiphilium* sp., *Flavobacterium acidurans*, and *Bacillus* sp. (Hallberg and Jhonson, 2001).

Traditionally, through culture-dependent methods, *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* were recognized as the major chemolithotrophic bacteria responsible for acid production in AMD (Johnson, 1998; Hallberg and Johnson, 2001). Nowadays, however cultivation-based analysis is not considered a suitable method rather 16S rRNA sequences analysis is considered as more reliable method for

characterizing microbial diversity (De Wulf-Durand *et al.*, 1997; Baker and Banfield, 2003). In the study of Tinto River the most representative bacterial species were found to be *A. ferrooxidans* (23%) and *L. ferrooxidans* (22%) and *Acidiphilium*. Other prokaryotes that were found to be the dominant microflora of Tinto River are *Ferrimicrobium acidiphilum* and closely related archaea *Ferroplasma acidiphilum* (Gonzalez-Torril *et al.*, 2003). 16S rRNA-based analysis has revealed that there are other microorganisms also that occur as dominant microflora of the AMD. *Thermopasmoles, Sulfobacillus, Acidimicrobium* etc.can remain as dominant flora of the AMD (Bond *et al.*, 2002b). The presence of archaebacteria including a group of and/or iron-oxidizers, such as *Sulfolobus, Acidianus, Metalosphaera,* has been reported from acidic environments (Edwards *et al.*, 2000; Fuchs *et al.*, 1995). Beside these, the presence of *Verrucomicrobia* and *Chlorobi* in ARD has also been confirmed by the 16S rRNA analysis of the ARD system (Okabayashi *et al.*, 2005).

A variety of chemolithotrophic and heterotrophic microorganisms are responsible for the solubilization of metals from sulfide minerals in acidic environments (Goebel and Stackebrandt, 1994a; Espejo and Romero, 1997). Although *Acidithiobacillus ferrooxidans* and *A. thiooxidans*, in the presence of heterotrophic *Acidiphilium* bacteria, were commonly regarded as the principle biological catalysts, recent analysis showed the important role played by other acidophilic heterotrophy also (Bhattacharya *et al.*, 1991; Hallberg and Lindstrom, 1994; van Niel, 1995). There is a lack of completed investigations into acidophile metal resistance mechanism (Hallberg and Johnson, 2001; Dopson *et al.*, 2003). Studying acidophiles one can learn about general biological resistance mechanisms too. Inducing biological competition for oxygen by adding a degradable organic carbon substrate has the potential to change the microbial ecology of ARD sites such that bacteria like *A. ferrooxidans* no longer dominate the environment. Furthermore, dominance of heterotrophs may alter the ARD environment sufficiently to allow in situ remediation take place (Marchand and Silverstein, 2000). The singular role of acidophilic heterotrophic bacteria in coupling iron reduction and glucose oxidation is demonstrated in anoxic batch reactor. As addition of organic substrates promote the dominance of heterotrophic bacteria in biofilm that coat rock surfaces, the solubilization of iron resulting from bacterial iron oxidation might cease altogether. On the other hand, investigators have found that several species of

heterotrophic or mixotrophic acidophile are also capable of leaching and oxidizing iron from pyrite (Edwards *et al.*, 1999; Marchand and Silverstein, 2000). Industrial leaching processes use several acidophilic bacteria, including *A. thiooxidans*, *A. ferrooxidans* etc. (Bruins *et al.*, 2000). It is somewhat analogous that commercial bioprocessing of sulfidic minerals has developed into an important and successful area of biotechnology with (in most cases) very limited data on microbial populations that are present in bioleaching systems. One reason for this has been the lack of accurate and appropriate methods for analyzing populations of the diverse acidophiles that are active in the metal enriched, acidic environments that constitute commercial leach liquors. Molecular techniques (gene libraries constructed from extracted DNA, amplification of 16S rRNA gene, restriction enzyme analysis, fluorescence in situ hybridization etc.) have been used to some effect in recent years (Goebel and Stackebrandt, 1994b). To study the ecological relationships, of these microorganisms and the population dynamics during the bioleaching processes, specific methods for their identification and enumerization are required. Molecular methods based on the detection of genomic diversity, such as GC content, DNA-DNA hybridization, and rRNA analysis, have been used to obtain a phylogenetic survey of the iron-oxidizing bacteria (De Wulf-Durand *et al.*, 1997).

Since the acidophilic microorganisms growing in these systems have important biotechnological applications many conventional microbial ecological studies of such acid laden metal rich environments have been performed (Norris, 1990; Hallberg and Johnson, 2001; Lo´pez-Archilla *et al.*, 2001). Recently molecular approaches to examine the microbial diversity of these habitats have also been performed (Goebel and Stackebrandt, 1994a,b; De Wulf-Durand *et al.*, 1997; Bond *et al.*, 2000a). Because of the limited types of substrates available in such environments, the microbial diversity was initially expected to be extremely poor. Cultivation-based studies have however revealed a great diversity of the microbial community in AMD (Johnson, 1998; Hallberg and Johnson, 2001).

Even though AMD environments are diverse and variable microbiologically, relatively little work has been done on their microbial ecology (Leduc *et al.*, 2002). Moreover, very few investigations have examined seasonal variations of bacterial numbers in an AMD environment. On the other hand many studies have been done on one of the key

organism *A. ferrooxidans* (Leduc *et al.*, 2002). The purpose of this study is to explore the acidophilic heterotrophic diversity indigenous to ARD/AMD samples. It has been postulated that their have been large scale swapping of genes amongst organism that share the same ecological niche. The tolerance towards high inorganic acidity and metal concentration has been a common phenotype of acidophilic autotrophs and heterotrophs. The useful genes of the heterotrophs can be manipulated to bring out better leaching microorganisms. The understanding of the genetic basis of tolerance to heavy metals in heterotrophs would therefore be an important area of investigation. The characterization of plasmids from acidophilic heterotrophs might serve as repositories of uncharacterized ORFs for future application in the field of mineral biotechnology.

IV. Physiology of Acidophilic Chemolithoheterotrophs

Acidophilic microorganisms are mainly of two types: Heterotrophs and Chemoautotrophs. Acidophilic chemoautotrophs have in common the ability to use CO_2 as their sole source of carbon and to derive energy from the oxidation of inorganic chemicals. *Acidithiobacillus thiooxidans* grows between the pH values 1.0 and 4.0 with an optimum at pH 2.5 (Rohwerder *et al.*, 2003) and uses elemental or reduced compounds as substrates (Ingledew, 1982). *A. thiooxidans* is an obligate chemoautotroph. *A. ferrooxidans* is probably the most widely studied acidophile. Further, there is a report that *A. ferrooxidans* grows heterotrophically at pH 7 with glucose as carbon and energy source (Harrison, 1984). This finding has been disputed and it has been suggested that *A. ferrooxidans* cultures are contaminated with the facultatively heterotrophic *Thiobacillus acidophilus*, which has since been isolated in axenic culture (Jhonson and Kelso, 1983). *T. acidophilus* is a member of the group of acidophilic thiobacilli capable of both chemoautotrophic and heterotrophic growth. This species, recently isolated from a culture of *A. ferrooxidans*, grows optimally at pH 3.0 to 3.5. The chemoautotrophic ability of this organism is limited to the oxidation of elemental; sugars, amino acids, and carboxylic acids support heterotrophic growth. Other organisms, such as species of *Plantomyces, Pseudomonas, Leptothrix, Aquabacterium, Caulobacter, Ralstonia, Achromobacter,* and *Microcyclus* which have been associated with acid environments, are probably only acid-tolerant (Yang *et al.*, 2008). The bacterium *Caulobacter crescentus* is known for the distinctive ability to

live in low-nutrient environments, a characteristic of most heavily metal-contaminated sites.

The use of acidophilic, chemolithotrophic microorganisms capable of oxidizing iron and in industrial processes to recover metals from minerals containing copper, gold and uranium is a well established biotechnology (Torma, 1983; Brierley, 1982, 1997; Acevedo, 2000; Rawlings, 2002; Olson *et al.*, 2003; Suzuki, 2001). The insoluble metal sulfides are oxidized to soluble metal sulfates by the chemical action of ferric iron, the main role of the microorganisms being the re-oxidation of the generated ferrous iron to obtain additional ferric iron (Rohwerder *et al.*, 2003; Rawlings, 2002; Olson *et al.*, 2003). Currently, there are operations using mesophilic and thermophilic microorganisms (Torma, 1983, 1988; Brierley, 1982, 1997; Lindstrom *et al.*, 1992; Acevedo, 2000; Rawlings, 2002, 2005a, b; Olson *et al.*, 2003). Biomining has distinctive advantages over the traditional mining procedures. For example, it does not require the high amounts of energy used during roasting and smelting and does not generate harmful gaseous emissions such as dioxide (Rawlings, 2002). Nevertheless, acid mine drainage can be generated, which if not properly controlled, pollutes the environment with acid and metals (Rohwerder *et al.*, 2003; Rawlings, 2002, 2005a,b; Olson *et al.*, 2003). Biomining is also of great advantage since discarded low-grade ores from standard mining procedures can be leached in an economically feasible way. There are complete previous reviews regarding methods of bioleaching and their implementation in several countries (Torma, 1983; Brierley, 1982, 1997; Acevedo, 2000; Lindstro¨m *et al.*, 1992; Rohwerder *et al.*, 2003; Rawlings, 2002, 2005a, b; Olson *et al.*, 2003).

Recently, thiosulfate has been postulated as a key compound in the oxidation of the moiety of pyrite (Schippers and Sand, 1999). Iron (III) ions are exclusively the oxidizing agents for the dissolution. Thiosulfate would be consequently degraded in a cyclic process to sulfate, with elemental being a side product. Lithotrophic oxidation is an ancient metabolic process. Ecologically and taxonomically diverged prokaryotes have differential abilities to utilize different reduced compounds as lithotrophic substrates (Ghosh and Dam, 2009). While the mechanisms of oxidation in obligately chemolithotrophic bacteria, predominantly belonging to *Beta-* (e.g. *Thiobacillus*) and

Gamma-proteobacteria (e.g. *Thiomicrospira*), are not well established, the Sox system is the central pathway in the facultative bacteria from *Alpha-proteobacteria* (e.g. *Paracoccus*) (Rother *et al.*, 2001).

V. Sulfur Oxidizing Microorganisms

The sulfur-oxidizing microorganisms are primarily the gram negative bacteria currently classified as species of *Thiobacillus, Thiomicrospira* and *Thiosphaera*, but heterotrophs, such as some species of *Paracoccus, Xanthobacter, Alcaligens* and *Pseudomonas* can also exhibit chemolithotrophic growth on inorganic sulfur compounds (Buonfiglio *et al.*, 1999).

Reduced inorganic sulfur compounds are exclusively oxidized by prokaryotes and sulfate is the major oxidation product. In the domain *Bacteria* sulfur is oxidized by aerobic lithotrophs or by anaerobic phototrophs. Prokaryotes oxidize hydrogen sulfide, sulfite, thiosulfate and various polythionates under alkaline (Friedrich *et al.*, 2005) neutral or acidic conditions (Friedrich *et al.*, 2001). Aerobic oxidizing prokaryotes belong to genera like *Acidianus*, *Acidithiobacillus*, *Aquaspirillum*, *Aquifer*, *Bacillus*, *Beggiatoa*, *Methylobacterium*, *Paracoccus, Pseudomonas*, *Starkeya*, *Sulfolobus*, *Thermithiobacillus*, *Thiobacillus* and *Xanthobacter* and are mainly mesophilic ((Lu and Kelly, 1983; Ghosh and Roy, 2007). Phototrophic anaerobic sulfur oxidizing bacteria are mainly neutrophilic and mesophilic and belong to genera like *Allochromatium, Chlorobium, Rhodobacter, Rhodopseudomonas, Rhodovulum* and *Thiocapsa* (Mittenhuber *et al.*, 1991; Meulenberg *et al.*, 1993; Meyer *et al.*, 2007).

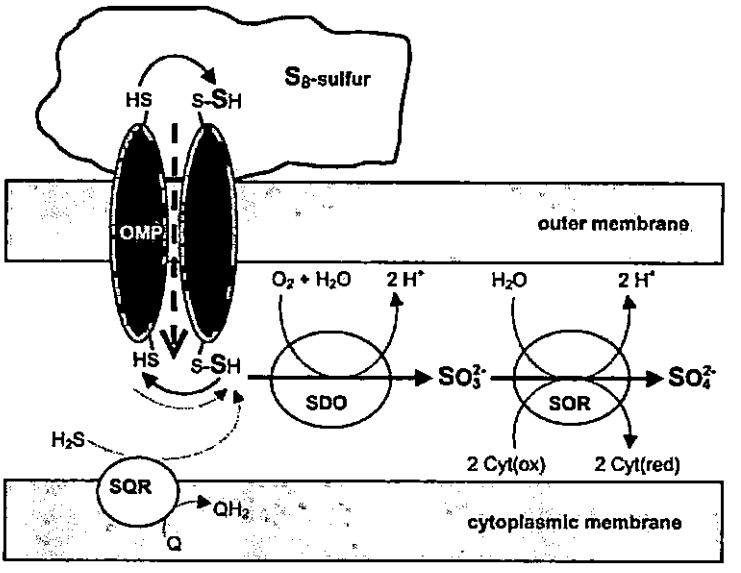

Fig. B. Proposal of a biochemical model for oxidation in *Acidithiobacillus* and *Acidiphilium* spp. In this scheme extracellular elemental (S_8) is mobilized as persulfide sulfane by special outer-membrane proteins (OMP) and oxidized by periplasmic dioxygenase (SDO). The resulting sulfite is oxidized to sulfate by sulfite: acceptor oxidoreductase (SOR), which probably uses cytochromes as electron acceptors (Cyt). Free sulfide is oxidized by a separate dehydrogenase (SQR), which uses quinones (Q) as electron acceptors (Rohwerder and Sand, 2003).

VI. Metal resistance/ tolerance property of acidophilic heterotrophs

Several reports had described the adverse effects of toxic metals on microbial biomass and metabolic activities in soil (Duxbury and Bicknell, 1983; Chander and Brookes, 1992). The interactions between toxic heavy metals and indigenous bacterial flora has become much significant when it was reported that pollution due to organic compounds was also associated with heavy metals like arsenic, mercury, lead, and zinc (Roane and Kellogg, 1996). Metal resistance was thought to be emerged during the early period of prokaryotic evolution in the metal contaminated environment (Ji and Silver, 1995). Environments containing high levels of dissolved metals include active and disused mines (Ledin and Pedersen, 1996). Metals may accumulate above normal physiological

concentrations by the action of unspecific, constitutively expressed transport systems, whereby they become toxic. Intracellular metals can exert a toxic effect by forming co-ordinate bonds with anions blocking functional groups of enzymes, inhibiting transport systems, displacing essential metals from their native binding sites and disrupting cellular membrane integrity (Nies, 1999). The toxicity of As (III) has been documented in *Acidiphilium multivorum*, *A. ferrooxidans*, *Ferroplasma acidarmanus* and *Metallosphaera sedula* (Harvey and Crundwell, 1996). The presence of the *arsB* gene (efflux pump) has been identified by Southern hybridization in various acidophilic micro-organisms; these include *A. caldus*, *A. thiooxidans*, *A. ferrooxidans*, *Acidiphilium acidophilum*, *Thiomonas cuprina* and *Acidocella facilis* (Dopson *et al.*, 2001). *A. ferrooxidans* strains adapted to increased levels of Cu (II) have been found to be tolerant to 800 mM (Dew *et al.*, 1999). Other acidophiles shown to be resistant to copper include *Leptospirillum ferrooxidans*, which shows growth in 5 mM Cu (II) (Johnson *et al.*, 1992). '*Acidiphilium symbioticum*' KM2 has been shown to harbour three plasmids which, when a mini-plasmid library was created and transformed into *E. coli*, conferred resistance to Zn (II) and Cd (II) (Mahapatra *et al.*, 2002). Apart from *A. ferrooxidans* and *Sulfolobus* other acidophiles resistant to Ni (II) include *Acidiphilium multivorum*, *Acidocella aminolytica* and *Acidocella* strain GS19 (Sampson and Phillips, 2001). Many other metals are found in acidic environments, including uranium, molybdenum and chromium. A number of different acidophiles have been isolated from locations containing these metals, and the following species have been isolated from environments containing uranium: *A. ferrooxidans*, *L. ferrooxidans*, *A. thiooxidans*, *Thiomicrospira cuprina*, cells resembling *Sulfolobus*/ *Acidianus* spp., *Acidiphilium* spp. and other heterotrophic iron-oxidizing acidophiles (Tuovinen and Bhatti, 1999).

Acidic waters from AMD cause the dissolution of other resident minerals increasing the concentration of heavy metals in these environments. Therefore, bacteria isolated from AMD exhibit high levels of tolerance to heavy metals. The objective of this work was to isolate and characterize acidophilic, aerobic, chemolithoheterotrophic bacteria from AMD that are tolerant to high concentrations of heavy metal divalent cations. Enrichments and isolated bacteria were characterized by tolerance to heavy metals, presence of extrachromosomal DNA, and presence of known metal tolerance-related

genes. Chemolithoheterotrophic, heavy metal tolerant bacteria were successfully isolated from Garubathan. The majority of the strains were closely related to the genera *Serratia, Burkholderia, Bacillus megaterium, B. cereus, Psychrobacter, Comamonas testosteroni, and Acidiphilium cryptum* respectively. The heavy metal tolerance profiles of all the isolated strains revealed varied tolerance of heavy metals. An attempt was made to correlate tolerance towards heavy metals to the presence of a plasmid. A large portion of the microbial diversity in these acidic metal-rich environments is yet to be cultured and a further challenge will then be to discover the secrets of metal resistance mechanisms in them.

VII. Objectives of the study

1. To study the diversity of acid-tolerant heterotrophic bacteria in ARD samples from mineral occurrences sites of Eastern Himalaya
2. To apply molecular systematics in ascertaining taxonomic status of the isolates
3. To understand the physiology of the acidophilic metal-tolerant chemolithoheterotrophs with special reference to sulfur oxidation phenotype and *sox*B genotype
4. To explore the presence of indigenous plasmids in the isolates and attempt to ascribe their function in relation to metal tolerance

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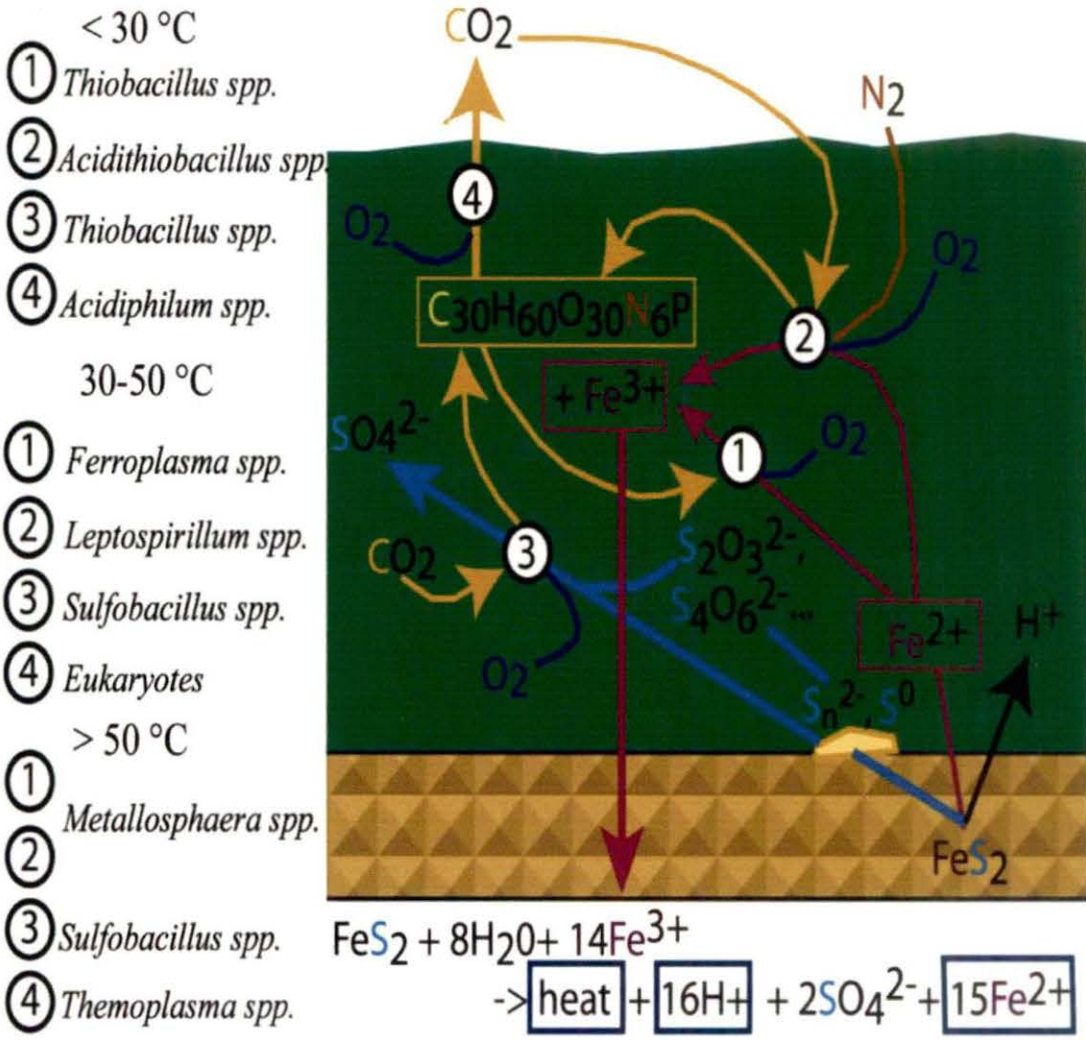
CHAPTER 1

Enrichment and isolation of acid-tolerant Chemolithoheterotrophic bacteria from AMD samples of Garubathan



Potential iron, sulfur, and carbon cycling based on known metabolic capabilities (1, 2, 3, and 4) associated with AMD members. Crystalline pyrite (Fe_2S) is in yellow at the bottom and green is representing AMD solution. Elemental sulfur is shown at the pyrite-water interface as a possible inhibitor of surface dissolution. The overall oxidation of pyrite is shown at the bottom, with Fe^{3+} indicated as the primary oxidant. Intermediate sulfur compounds are indicated as follows: $S_2O_3^{2-}$ being thiosulfate and $S_4O_6^{2-}$ is tetrathionate. $C_{30}H_{60}O_{30}N_6P$ indicates organic carbon compounds (Baker and Banfield, 2003).

1.1 Introduction

Acidophiles have been the spotlight of considerable research in recent years, particularly regarding their role in acid mine drainage. Some of these acidophiles are chemolithotrophs, which means that they are capable of using CO_2 or carbonates as the sole source of carbon for cell biosynthesis, and derive energy from the oxidation of reduced inorganic or organic compounds (Baker and Banfield, 2003). Chemolithotrophic oxidation of sulfur in aerobic habitats produces sulfuric acid ($SO_4^{2-} + 2\ H^+ = H_2SO_4$) during the process of sulfur cycling by chemolithotrophs. Acidophiles are tolerant (and thus considered as acid-tolerant) of the resulting acidic conditions, although the production of sulfuric acid is unfavourable to other microorganisms, plants, and animals.

The huge success of enrichment culture and related isolation approaches and conditions together with application of new analytical techniques, has enabled us to see that we understand very little about so many of the presumptive prokaryotes present in nearly every habitat we explore. The microorganisms deriving energy from dissimilatory oxidation of reduced inorganic sulfur compounds can be enriched in a mineral salts medium having suitable sulfur substrates, which may be supplemented with a mixture of trace metal solution and vitamins or yeast extract as a source of growth factors. Often a desired or known bacterial metabolic type guides the procedure to be adopted for enrichment. Thus, the typical or special environments could be identified by the abundance of inorganic compounds of sulfur and iron, e.g., hydrogen sulfide containing sediments, rivers, canals, estuarine and tidal flats, acid sulfate soils near sulfur stock piles, acid (hot) springs, mine drainage effluents, waste dispose effluents etc. Samples from such specialized ecological niches enriched in purely chemolithotrophic growth condition would enable isolation of obligately chemolithoautotrophic sulfur oxidizers. It is likely that specific chemolithotrophic media containing a reduced sulfur species, when supplemented with an organic compound, should allow enriching growth of facultative and obligate chemolithoautotrophs and also chemolithoheterotrophs. Actually *in situ* succession of these distinct physiological types in the artificial or natural environments is not known and difficult to evaluate. There is no specific

enrichment procedure for facultative chemolithotrophs. The organic matter, growth factor/vitamins supplements to the medium will allow the growth of all physiological types. In such cases limiting factors could be the type of carbon and energy source. pH and temperature selected for enrichments. The enriched culture may be purified by repeated streaking on inorganic thiosulfate agar or mixotrophic agar medium containing both thiosulfate and organic carbon with a specific pH indicator depending on the desired pH range for growth. An indicator present in the sulfur compound containing medium helps in detecting colonies, which produce sulfuric acid as the final product of sulfur metabolism that changes the color of the indicator. After colony purification on thiosulfate agar, the heterotrophic growth potential could be determined by streaking on agar medium containing suitable organic substrate or rich agar or liquid medium of heterotrophic composition. Thiosulfate, due to relatively stable chemical properties compared to other reduced sulfur compounds, is the preferred substrate for enrichment, isolation, purification and further physiological studies as well. Much effort has been expended in the isolation and description of individual micro-organisms. Investigations on purè cultures are important to judge the potential abilities and functions of individual species in nature.

Habitat is the region where a race, species, or individually naturally lives or is found (Odum, 1971). Starting from the origin of life in a historical and evolutionary point of view, it is amazing that versatile and specialized life processes have evolved in different places of inorganic environments on this planet. Habitat gives the first conception of a living system which may represent a group or a type, mainly with an idea of chemical, physical, and organic or biotic embodiment of a particular region on this earth surface. Acid mine drainage (AMD) is a low pH rarely alkaline, high metal concentrated, iron and sulfate bearing water usually formed when rocks containing sulfide minerals are exposed to the atmosphere or an oxidizing environment, and subsequently leached by water (Gonza´lez-Toril *et al.*, 2003; Okabayashi *et al.*, 2005). Acid mine drainage differ significantly from one location to other, and covers a wide range of pH, temperature and mineral content. Undoubtedly, such variations indicate that a variety of acidophilic microorganisms with diverse physiological properties can be found in AMD (Hallberg, 2010). Besides, the detection of acidophiles with varying physiological capabilities allows for the continued exploitation of minerals. AMD microorganisms

and their ecology have recently attracted substantial attention due to their use in biohydrometallurgy. It is known that bacteria play an important role in the formation of AMD. A wide range of heterotrophic and chemolithotrophic bacteria can be detected in the acid-leaching environments (Dopson *et al.*, 2003).

The study of acidophilic heterotroph of AMD has become very important as this is a regular inhabitant of mineral sulphide environments (Berthelot *et al.*, 1997). Several characteristic features of these heterotrophs have been suggested to have beneficial effect on the bioleaching as well as for the sustenance of the iron-oxidising organisms in the environments. Earlier studies have shown that both sulfur- and iron-oxidizing bacteria such as *Acidithiobacillus ferrooxidans*, *A. thiooxidans* and *Leptospirillum ferrooxidans* were present in rather high numbers (Baker and Banfield, 2003). *A. ferrooxidans* and *Ferroplasma* has been considered principally responsible for the extreme conditions of AMD systems (Bond *et al.*, 2000; Okabayashi *et al.*, 2005). The presence of heterotrophs associated with chemolithotrophs like *A. ferrooxidans* has been observed (Wichlacz and Unz, 1981; Bond *et al.*, 2000), but studies of such heterotrophs are largely restricted to obligately acidophilic bacteria like *Acidiphilium* sp. (Johnson and McGinness, 1991; Bruneel *et al.*, 2003; Okabayashi *et al.*, 2005). It was observed that a significant symbiosis exists between heterotrophic and certain autotrophic species. Acidophilic autotrophs produces organic materials as their by product. Heterotrophs makes a way by utilizing/removing organic compounds toxic for the autotrophs (Pronk *et al.*, 1990; Das and Mishra, 1996). Mixotrophic growth was evidenced by certain facultative chemolithotrophs where limited amount of both inorganic and organic substrates are present in AMD (Clark and Norris, 1996; Peccia *et al.*, 2000).

There are only few reports on detection and culture of acidtolerant heterotrophs like *Flavobacterium acidurans*, *Bacillus* sp., and *Sporosarcina* from AMD or coal mine drainage environment (Leduc *et al.*, 2002; Marchand and Silverstein, 2002). Very recently, a culture- independent method was attempted to analyse the microbial communities inhabiting three sites of acid drainage in the Shen-bu, copper mine of China (Yang *et al.*, 2008). 16S rDNA clones, have revealed the tentative presence of bacteria representing diverse genus like *Alicyclobacillus*, *Pseudomonas*, *Caulobacter*,

Brevundimonas, *Sphingomonas*, *Shigella*, *Candidatus*, *Azospirillum*, and few uncultured ones (Yang *et al.*, 2008).

It was known from the survey of Geological Survey of India that an area of 0.247sq. km., had probable reserve of metal sulfide ores of zinc and copper (Shah *et al.*, 1974-75). Assuming larger degree of metabolic plasticity of facultative acidophilic heterotrophs over obligately acidophilic heterotrophs, in the present study we have attempted to cultivate and characterize acidtolerant heterotrophs, from AMD samples from Garubathan ore reserves of West Bengal, India, to study their physiology, to understand their roles and to tap this large biotechnological resource..

1.2 Materials and methods

1.2.1 Sampling site(s) and sampling

Roy, Choudhury and Ghosh (1974-75) carried out a systematic geological mapping in parts of Kalimpong sub-division of Darjeeling district, covering an area of 180 sq. km on 1:25000 scale in parts of Toposheet No. 78 A/8 and 12. [Records of the GSI, vol. 109, Part I, 1982]. A detailed investigation for lead- zinc ore in the area around Garubathan (26° 59' N: 88° 42' E) in Kalimpong sub-division was attained by Saha, Chakraborty and Bandopadhyaya. Records showed the occurrence of magnetite in Khani Khola, south of Siyokbir (27° 02'00" N: 88° 30'45" E). An area of 0.247 sq km on scale 1:2000 was covered by plane table maping in the Khar Khola Block and Mal Khola Block in the east and west of Garubathan. A total of 780.80 m boreholes were drilled in Mal Khola Block. A total of 131 samples, comprising of surface channel samples, float ore samples (2,910 tonnes, with an average of 7.14% Pb and 2.87% Zn), borehole samples, composite samples, selective grab samples etc. were collected which showed the probable reserves, estimated at 0.602 million tonnes with an average of 3.54% Pb, and 2.70% Zn.(records of GSI, vol. 109, Part I, 1982). Sulfide mineralization has been located near Pedong (27° 12'55" N: 88° 37'00" E), Rishi (27° 11'30" N: 88° 38'00" E), and Rorathang (27° 12'00" N: 88° 37'00" E).

AMD site in Dalimkhola, Garubathan was selected as the sampling site. Rock drained, rusty red water samples were collected in the sterilized screw cap polypropylene

bottles. pH of the samples were checked at the sites using pH paper (HiMedia, India). The samples were kept in ice box till arrival at the laboratory.

1.2.2 Enrichment of AMD samples

1.2.2.1 Media

Following autotrophic medium were used for enrichment of acid-tolerant microorganisms

1.2.2.1.1 Modified 9K medium (Yates and Holmes, 1987) *containing ferrous sulfate.*

The modified 9K medium contained a mixture of two solutions. Solution A containing $(NH_4)_2SO_4$, 1.0 g; $MgSO_4.7H_2O$, 0.5 g; K_2HPO_4, 0.5 g; KCl, 0.1 g; in 800 ml distilled water, sterilized at 15 psi for 15 minutes, was mixed with filter sterilized (pore size 0.25 μ) solution B containing $FeSO_4.7H_2O$ 33.0 g and 4 ml 10(N) H_2SO_4.

1.2.2.1.2 Elemental sulfur medium

It was prepared by adding 10.0 g elemental sulfur to a basal salt solution containing $(NH_4)_2SO_4$ 3.0 g; K_2HPO_4 3.0 g; $MgSO_4.7H_2O$ 0.5 g; $CaCl_2$ 0.25 g; $FeSO_4$, 0.01 g; pH was adjusted to 4.0-4.5 with 1 (N) H_2SO_4, in 1000 ml distilled water. Basal salt solution containing elemental sulfur was sterilized at 100 °C for 30 minutes.

1.2.2.1.3 Thiosulfate medium

The medium was prepared by mixing the two solutions. 800 ml solution A contained the same components as in case of basal salt solution of elemental sulfur medium, and 200 ml solution B contained 10.0 g of $Na_2S_2O_3$. pH of solution A was adjusted to 4.0-4.5 with 1 (N) H_2SO_4. Solution A was sterilized at 15 psi for 15 minutes while solution B was filter sterilized by passing it through the bacterial membrane (pore size 0.25 μ).

Following heterotrophic medium were used for the enrichment of the acid-tolerant heterotrophs

1.2.2.1.4 Modified DSMZ 269 medium

Medium contained $(NH_4)_2SO_4$ 2.0 g; K_2HPO_4 0.5 g; $MgSO_4.7H_2O$ 0.5 g; KCl 0.25 g; glucose 1.0 g, yeast extract 0.1 g in 1000 ml distilled water. pH of the medium was adjusted to 3.0 with 1(N) H_2SO_4. Basal salt without glucose and yeast extract was sterilized at 15 psi for 15 minutes. Glucose and yeast extract were separately sterilized as 5 % solution, and the required volume was added to the basal salt solution.

1.2.2.1.5 Modified DSMZ 269 medium containing thiosulfate

All the components of the medium were same as modified DSMZ 269 medium, except $Na_2S_2O_3.5H_2O$ 10.0 g (10 % solution) which was added separately after filter sterilization. pH of the basal salt was adjusted to 4.0-4.5.

1.2.2.1.6 Mixotrophic medium (MSTSY) (Mukhopadhyaya, *et al.*, 2000)

Medium contained $(NH_4)_2SO_4$ 2.0 g; K_2HPO_4 0.5 g; $MgSO_4.7H_2O$ 0.5 g; KCl 0.25 g; $Na_2S_2O_3.5H_2O$ 10.0 g; sodium succinate 5.0 g; yeast extract 5.0 g; trace metal solution 5.0 ml in 1000 ml distilled water. pH of the basal salt solution was adjusted to 4.0-4.5 with 1(N) H_2SO_4. $Na_2S_2O_3.5H_2O$, was filter sterilized as 10 % solution by passing it through the bacterial membrane (pore size 0.25 μ). Sodium succinate and yeast extract were separately sterilized as 5 % solution and the required volume was added to the separately sterilized basal salt solution at 15 psi for 15 minutes.

1.2.3 Preparation of solid media

Solid medium for the heterotrophs was prepared by mixing two solutions. 478 ml solution A contained DSMZ 269 basal salt solution and 500 ml solution B contained 12 g agar. pH of solution A was adjusted to 3.0 with 1(N) H_2SO_4. Both the solutions were then sterilized at 15 psi for 15 minutes. Sterilized solutions were allowed to cool down to about 50 °C and 20 ml of the sterile 5 % glucose and 2 ml of sterile 5 % yeast extract solutions were added to the basal salt solution. The resultant solution was then mixed

with the solution B and poured into the sterile Petri plates. Slants were prepared as described above.

Solid medium for the mixotrophs was also prepared by mixing two solutions. 255 ml solution A contained basal salt composition of MSTSY and 500 ml solution B contained 15 g agar. pH of solution A was adjusted to 5.0. Both the solutions were then sterilized at 15 psi for 15 minutes. $Na_2S_2O_3.5H_2O$, was filter sterilized as 10 % solution by passing it through the bacterial membrane (pore size 0.25 μ). Sterilized solutions were allowed to cool down to about 50 °C. 200 ml of the sterile $Na_2S_2O_3.5H_2O$, and 20 ml each of 5 % sodium succinate; 5 % yeast extract solutions and 5 ml sterile trace metal solutions were added to the basal salt solution of MSTSY. The resultant solution was then mixed with the solution B and poured into the sterile Petri plates.

1.2.4 Isolation and purification of acid-tolerant heterotrophic bacteria

Acid-tolerant heterotrophs were enriched and isolated from the enrichment broth cultures of autotrophic and heterotrophic media. 1.5 ml of the enrichment broth cultures were centrifuged at 10,000 rpm for 10 minutes. The cell pellets so obtained were washed with and re-dissolved in sterile 0.01 (N) H_2SO_4. The cell solution was then seeded in the modified DSMZ 269 broth and MSTSY broth that acted as the enrichment cultures for the heterotrophs. The cultures were then incubated at 28 °C ± 2 °C for 96 hours.

Heterotrophic strains were isolated by serial dilution plating method. A series of 10-fold serial dilutions in test tube was prepared and 0.1 ml of the highest dilution (determined by Haemocytometer count) was used for plating in the modified DSMZ 269 and mixotrophic medium (MSTSY) plates.

In another way of isolating the acidophilic heterotrophs, the peripheral region of the *A. ferrooxidans* colonies, manifested on the ferrous agar plate was scraped by the sterile inoculating loop and then the dilution streaking was performed on the modified DSMZ 269 plates. The streaked plates were then incubated at 28 °C ± 2 °C for 96 hours.

Discrete colonies obtained on the DSMZ 269 plates were further purified by the streaking method.

1.2.5 Screening of the acid-tolerant isolates for further studies

1.2.5.1 Protein profiling

1.2.5.1.1 Reagents used:

- 4X stacking gel buffer (0.5M Tris-HCl at pH 6.8)
- 4X resolving gel buffer (1.5M Tris-HCl at pH 8.8)
- 10X running buffer (30 g of Tris base, 144 g of glycine, and 10 g of SDS in 1000 ml of H_2O, pH should be 8.3)
- 2X SDS-PAGE sample buffer (2 ml of 4X stacking gel buffer, 1.6 ml of glycerol, 3.2 ml of 10 % SDS, 0.8 ml of 2-mercaptoethanol, 0.4 ml of 1 % bromophenol blue)
- Methanol
- Ammonium persulfate solution (10 %, freshly prepared each time)
- TEMED
- SDS (10 %) stock solution
- 30 % T (2.6 % C) Acrylamide stock solution (29.22 g of acrylamide and 0.78 g of bisacrylamide to 100 ml of H_2O). Stock solution was filter sterilized through Whatman filter paper and stored at 4 °C.

1.2.5.1.2 Preparing 12 % resolving gels (10 ml):

- H_2O, 3.3 ml; 30 % acrylamide mix, 4.0 ml; 1.5 M Tris (pH 8.8), 2.5 ml; 10 % SDS, 0.1 ml; 10 % ammonium persulfate, 0.1 ml; and TEMED, 0.005 ml.

1.2.5.1.3 Preparing 5 % stacking gels (3 ml):

- H_2O, 2.1 ml; 30 % acrylamide mix, 0.5 ml; 1.0 M Tris (pH 6.8), 0.38 ml; 10 % SDS, 0.03 ml; 10 % ammonium persulfate, 0.03 ml; and TEMED, 0.003 ml.

1.2.5.1.4 Pouring the slab gel:

- Glass plates were cleaned and the gel-casting unit was assembled.
- The glass plate was marked at a level ~1.0 – 1.5 cm below the bottom of the comb teeth.

- Appropriate resolving gel mixture was prepared and the solution was mixed nicely before the addition of the TEMED.
- The mixture was poured into the glass plate and sandwiched upto the marked level.
- The gel was carefully overlayed with an ~2 mm deep layer of H_2O.
- After completion of the polymerisation (~30 min), overlaying water was poured off and any remaining liquid was carefully removed using filter paper.
- The stacking gel was poured over the resolving gel until the height of the stacking gel was ~2.0 – 3.0 cm.
- A Teflon comb was inserted into this solution, leaving 1.0 – 1.5 cm between the top of the resolving gel and the bottom of the comb. Care was taken so that no air bubble could be trapped.
- The stacking gel mixture was allowed to polymerise for ~2 hrs.
- The sample comb was removed carefully from the stacking gel and the cassette was assembled in the electrophoresis apparatus.
- At first the top reservoir was filled with running buffer and then the bottom tank.

1.2.5.1.5 Preparation of samples:

- To prepare total cellular protein samples, 2X SDS-PAGE sample buffer was directly added to the pellets of purified bacterial strains and vortexed. 100 µl of 2X sample buffer was used per 1×10^5 cells.
- The cellular protein lysates were centrifuged at 10,000 rpm for 15 min and the supernatants were collected.
- The samples were heated in a water-bath for 20 min, at 95 °C to denature the proteins.
- The samples were cooled at room temperature. Insoluble materials were removed by centrifugation.

1.2.5.1.6 Estimation of protein in whole cells: An aliquot of the cell suspension was taken and the volume was made up to 0.25 ml with distilled water. To this, 0.25 ml of 1(N) NaOH solution was added and the mixture was placed on a boiling water bath for 5 min. The mixture was then allowed to cool and 5.0 ml of freshly prepared protein

reagent (a mixture of 2 % Na_2CO_3, 10 ml of 1(N) NaOH, 1.0 ml of 1 % Na-K-tartarate and 0.1 ml of 0.5 % $CuSO_4.5H_2O$, final volume made to100 ml with distilled water) was added. Then 5.0 ml of Folin-Ciocalteu's reagent was added and mixed immediately. The mixture was incubated at 37 ℃ for 30 min and the absorbance was measured at 660 nm against the reagent blank. The amount of protein was calculated from a standard curve using bovine serum albumin fraction V as standard.

1.2.5.1.7 Running the gel:

- 15 µl of each sample was loaded into the bottom of the well.
- A constant voltage of 100 V was passed at room temperature for 4 – 8 hrs.
- Proteins were visualised using sensitive staining method.

1.2.5.2 Coomassie Blue Staining

1.2.5.2.1 Reagents used:

- Coomassie Brilliant Blue (CBR-250) stain- 500 ml of methanol, 100 ml of acetic acid and 1 g of CBR-250 was added to 400 ml of H_2O.
- Destain- 120 ml of methanol and 70 ml of acetic acid was added to 810 ml of H_2O.

1.2.5.2.2 Methods:

- After electrophoresis was complete, the gel was placed in the plastic container containing enough CBR-250 stain to cover the gel.
- The gel was agitated for 1 hr. at room temperature.
- The used CBR-250 stain was discarded.
- Destain was added to absorb excess dye until a suitable background was achieved.
- The gel was stored in 5 % acetic acid and photographed.

1.2.5.3 Gram reaction

Gram reaction was performed following protocols stated by Cappuccino and Sherman, 1996. A thin smear of the culture was made on a glass slide, air-dried and was heat-fixed. The smear was stained with crystal violet for one minute, washed with tap water

and stained with gram's iodine for one minute followed by washing and decolorization with 95% ethanol. It was washed and counter stained with safranin for 45 seconds, washed with tap water, air dried and finally examined under compound light microscope with oil immersion objective lens. Cell size and shape were determined micrometrically. Gram reactions were also confirmed by 3% KOH (Wallace and Gates, 1986).

1.2.5.4 pH tolerance

DSMZ 269 medium was adjusted to different levels of pH range (pH 1-12). Small aliquot of the culture was inoculated and incubated at 28 °C ± 2 °C for 96 hrs. Visible growth at different pH was observed by comparing the O.D. of the inoculated and un-inoculated blank culture.

1.2.5.5 Temperature range

Pure and fresh cultures were inoculated on DSMZ 269 and MSTSY medium for 72 hrs. Visible growth at different temperature range was observed by comparing the O.D. of the inoculated and un-inoculated blank culture.

1.2.6 Maintenance of cultures

Modified DSMZ 269 medium and mixotrophic medium (MSTSY) were used for the maintenance of the acid-tolerant heterotrophs obtained from autotrophic medium and heterotrophic medium. Cultures were transferred monthly and maintained on respective slants and plates.

1.3 Results

1.3.1 Sample collection

A total of four rusty red sludge samples (Fig 1.1) as well as liquid collected from the sample sites showed a pH 1-2 and 4-5 when tested with the pH paper. Homogenized mixture of solid samples as well as liquid samples turned modified 9K medium into rum-red in coloration after about a week of incubation. The modified DSMZ 269

medium, modified DSMZ 269 medium containing thiosulfate and mixotrophic medium acted as the enrichment culture.

Fig. 1.1 : Rusty red sludge sample coming out from the rock in Garubathan mineral rich site or Acid mine drainage site.

1.3.2 Isolation of acid-tolerant heterotrophs from *A. ferrooxidans* culture and enrichment culture media

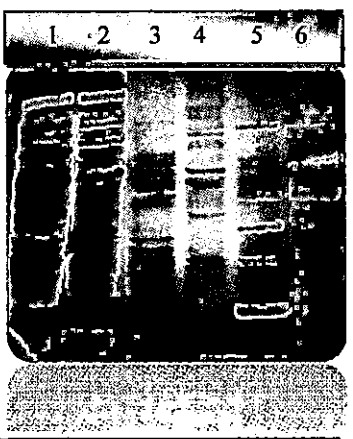
Heterotrophic colonies were obtained from the enrichment culture medium as well as from the colonies of *A. ferrooxidans*. On an average twenty five bacterial colonies were obtained at 10^{-3} dilution from modified DSMZ 269 media and mixotrophic media (MSTSY), viz., GMX1, GMX2, GMX3, GMX4, GMX5, GMX6, GMX7, GMX8, GMX9, GMX10, GMX11, GMX12, GMX13, GMX14, GMX15, GMX16, GMX17, GMX18, GMX19, GMX20, GMX21, GMX22, GMX23, GMX24, and GMX25. Forty six colonies were obtained and purified from modified DSMZ 269 media, viz., GAH1- 46.

Enriched culture of *A. ferrooxidans* from ferrous iron enrichment medium was purified on the 9K agar plates. Ten colonies were obtained from the peripheral region of the colonies of *A. ferrooxidans*. The selected strains were named as DK1AH1, DK2AH1, DK2AH2, DK3AH1, DK3AH2, and CM0AH1, CM1AH1, CM2AH1, CM3AH1, and

CM4AH1. Some colonies were actinomycetes-like with filamentous periphery, greenish at the centre that turned black with age, were excluded from the study.

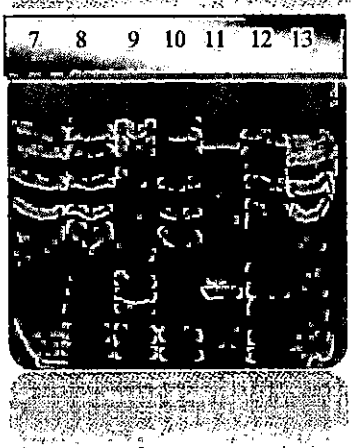
1.3.3 Preliminary selection of acid-tolerant heterotrophic isolates based on total protein profile

Differential banding pattern of the electrophoresed total protein isolated from different isolates was the guiding principle for selection of isolates for further studies. Protein profiles obtained by SDS-PAGE separation were compared; similar banding patterns were grouped; out of several such groups (each having unique banding pattern) representative isolates were chosen (Figl2a,l.2b,l.2c). A total of eighteen acid-tolerant heterotrophic strains were thus selected and maintained for further studies viz., GMX1, GMX2, GMX4, GMX5, GMX6, GMX7, GMX8, GAH1, GAH2, GAH3, GAH4, GAH5, GAH8, GAH9, GAH10, GAH44, DK1AH1, and DK2AH2.



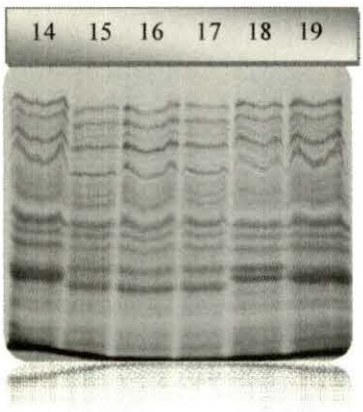
(Fig. 1.2a)

Lane 1, GMX5
Lane 2, GMX6
Lane 3, GAH3
Lane 4, GAH9
Lane 5, GAH10
Lane 6, GMX8



(Fig. 1.2b)

Lane 7, GMX1
Lane 8, GMX2
Lane 9, GAH1
Lane 10, GMX4
Lane 11, GMX7
Lane 12, DK1AH1
Lane 13, DK2AH2



Lane 14 & 19, GAH8

Lane 15, GAH2

Lane 16, GAH4

Lane 17, GAH5

Lane 18, GAH44

(Fig. 1.2c)

Fig. 1 (.2a, .2b, .2c): SDS-PAGE of total protein obtained from AMD isolates.

1.3.4 Grouping of the selected isolates according to pH tolerance, Gram nature, temperature range for growth, and nutritional versatility

Finally, out of eighteen selected acidtolerant isolates twelve were recognized as Gram negative in nature. All the strains have shown heterotrophic growth utilizing glucose as the carbon source. Nine of them could grow mixotrophically with thiosulfate (Table 1.1). Isolates sustained growth at diverse range of pH (1-6; 1-9; 3-6; 3-7; 4-7; 4-12) and temperature (4-37; 16-30; 16-32; 16-37; 16-42; 28-32; 28-37) °C. All the isolates grew best at 30 °C. Gram positive mixotrophs were found to tolerate pH near neutrality. All other Gram negative mixotrophs and heterotrophs were found to tolerate pH till 6 or 7 except one mixotroph, GMX7. The pH range as well as the temperature range for growth of GMX7 was found exceptionally wide i.e. pH 4-12 and temperature 4°C-37 °C.

Table 1.1 : Physiological characteristics of AMD isolates

	Grams nature	Carbon metabolism	Growth at pH range	Growth at temperature range
GMX1	-	mixotrophic	3-7	4°C -37°C
GMX2	-	mixotrophic	3-6	16°C -37°C
GMX4	-	mixotrophic	3-6	28°C -32°C
GMX5	+	mixotrophic	4-7	16°C -42°C
GMX6	+	mixotrophic	4-7	16°C -42°C
GMX7	-	mixotrophic	4-12	4°C -37°C
GMX8	+	mixotrophic	4-7	16°C -42°C
GAH1	-	heterotrophic	1-9	4°C -37°C
GAH2	-	mixotrophic	3-6	16°C -37°C
GAH3	+	heterotrophic	3-6	28°C -37°C
GAH4	-	heterotrophic	1-6	16°C -32°C
GAH5	-	mixotrophic	3-6	16°C -32°C
GAH8	-	heterotrophic	3-6	28°C -37°C
GAH9	+	heterotrophic	3-6	28°C -37°C
GAH10	+	heterotrophic	3-6	28°C -37°C
GAH44	-	heterotrophic	3-7	16°C -37°C
DK1AH1	-	heterotrophic	3-5.9	16°C -30°C
DK2AH2	-	heterotrophic	3-5.9	16°C -30°C

1.4 Discussion

The abundance of any particular inorganic compound and accumulation of the transformed compound of the same inorganic species in nature sets a clue to investigate the involvement of biological species and thus had led to the discovery of many specialist microorganisms. These specialists enjoy the freedom and avoid the competition by deriving energy from the reactions not obtainable by the heterotrophs with which it shares the habitat. But facultative chemolithotrophs with dual mode of nutrition may also inhabit the same community. On the other hand, many other heterotrophs could thrive in that inorganic environment at the expense of the excreted organic compound from the specialists. Often this type of association breeds commensalism, where both specialist and non-specialist live in close association. The scavenging of organic acids, released from the specialists' cell, by the heterotrophs is

sometimes helpful or essential to the specialists. In the complex inorganic environment such association of diverse specialists and non-obligates constitutes a unique ecological niche, studying which, alienation of the specialists from the other nutritional types becomes very complicated. The enrichment, isolation, purification and preliminary screening of acid-tolerant heterotrophic isolates from AMD sites of Garubathan have been described in this chapter. In general, acidophilic heterotrophs from acidulated inorganic environment has received lesser attention in terms of their acidophily, nutritional flexibility, metal tolerance, physiological diversity and above all their contribution to microbial ecology of acid-rich environment (Wichlacz and Unz, 1981). One of the chief hindrances for such eco-physiological studies was due to the failure in culturing these heterotrophs under laboratory condition. Earlier observations have indicated a predominance of gram negative bacteria among the isolates recovered on acid culture media (Tuttle *et al.*, 1968). Using exclusively mineral salts medium (pH 3.0) and agar as solidifying agents, there were recovery of several gram negative heterotrophic bacterial strains from acid mine drainage, of which *Pseudomonas acidophila* strains were the best characterized ones (Manning, 1975). For recovery of acid-tolerant heterotrophs like flavobacteria, tryptone-yeast extract (pH 6.0) was often used (Millar, 1973). It was noted by the earlier authors that proper formulation of selective media has an important bearing on the result (Bruneel *et al.*, 2003). There were also reports on isolation of obligately acidophilic heterotrophs from *A. ferrooxidans* culture (Harrison, 1985). Though cultivation-based studies have been capable of reflecting to some extent the microbial diversity in acid mine drainage (Johnson, 1998; Hallberg and Johnson, 2003), methods using culture-independent 16S rDNA sequences to study bacterial communities of acidic environments have revealed the phenomenon to a great extent (Okabayashi *et al.*, 2005; Yang *et al.*, 2008). The authors are also of opinion that since the putative presence of the bacteria can only be stated from these culture-independent studies, further investigation should be concentrated on isolating these bacteria to understand their physiological role in ecology (Bruneel *et al.*, 2006; Yang *et al.*, 2008). In the present study, we have been able to enrich and isolate acid-tolerant heterotrophs from the AMD samples. We could overcome the problem of colony generation on solid medium by restricting acid

hydrolysis of agar and thiosulfate (details has been described in the materials and methods) during preparation of plates.

Primarily, eighty one acid-tolerant heterotrophic isolates were obtained from the AMD samples. Out of which ten heterotrophic isolates were purified from the autotrophic culture of *Acidithiobacillus ferrooxidans* colonies. Earlier authors (Johnson and Kelso, 1983; Mishra *et al.*, 1983) have also detected acidophilic heterotrophs in *A. ferrooxidans* cultures. It has been found that some organic substrates such as pyruvate, glutamate, aspartate, serine, glycine, and other amino acids excreted by *A. ferrooxidans* can be utilized by the heterotrophs for their growth (Schnaltman and Lundgren, 1965; Arkesteyn *et al.*, 1980; Ingledew, 1982). Several types of acidophilic heterotrophs (*Acidiphilium cryptum, A. acidophilum, A. organovorum* etc.) that are gram negative in nature were recovered from *A. ferrooxidans* enrichment cultures (Guay and Silver, 1975; Harrisson *et al.*, 1980; Lobos *et al.*, 1986).

An important molecular technique that has proved to be useful in typing bacterial strains is Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of whole cell bacterial proteins, wherein differences seen in protein bands have been successfully used to group bacteria (Krech *et al.* 1988; Huey and Hall, 1989; Maiti *et al.*, 2009). In our study, we too used SDS-PAGE to screenout the similar banding patterns generated by the acid-tolerant heterotrophic isolates and finally selected eighteen of them showing differential banding patterns of protein.

Twelve acid-tolerant isolates were found to be Gram negative. All the isolates showed growth at diverse range of pH, (from 1 to 12) and temperature (from 4 °C to 42 °C). The isolates showed best growth at pH 3 and temperature (28-30) °C except GMX1, GMX7, and GAH1which showed psychro-tolerant nature growing best at 16 °C. Further the present study showed that nine out of eighteen acid-tolerant heterotrophic isolates, were tentatively found to be mixotrophic (facultative sulfur-oxidizing) strains (Table1.1). Reduced sulfur species of sulfidic mineral origin in Garubathan AMD sites of Darjeeling Himalayas favour abundant microbial populations of sulfur-oxidizers (both mesophilic and psychro-tolerant), including autotrophic *Acidithiobacillus* species as well as heterotrophic sulfur-oxidizing strains. Similar diversity of thiosulfate-

oxidizing bacteria has been reported from marine sediments and hydrothermal vents (Teske *et al.*, 2000). The association of microorganisms with acidic mine drainage occurs in several ways: (i) increased production of acid via the metabolic activity of the acidophilic sulfur-oxidizing bacteria; (ii) the inhibitory influence of sulfuric acid on the organisms normally present in receiving streams; (iii) growth of acid-tolerant microbes which will aid in recovery of acid contaminated streams;(iv) the ability of sulfate-reducing bacteria to convert sulfate (e.g. H_2SO_4) back to sulfide which can be precipitated as iron sulfide (FeS).

An attempt was made in this study to understand the presence of diverse bacteria which could withstand the relative influence of the prominent chemical parameters that constitute acid mine drainage as they effect the aerobic heterotrophic microflora of receiving streams. The approach was based on the premise that physiological activity of microorganisms is fundamental in triggering the ecological succession which ultimately results in re-establishment of higher forms of life (e.g. insects, fish, etc.) in the streams. Although iron, sulfate and hydrogen ions are all characteristic of acidic mine effluents, they appear to vary independently with respect to concentration in drainage which originates at different locations. Isolation and identification of acid-tolerant heterotrophs from AMD samples from Garubathan, India, has partially revealed diversity of the strains in terms of pH tolerance, growth at a wide range of temperature, and chemolithotrophy. These strains may provide genetic resources for the development of novel biotechnological processes. Hence, in the following chapter the detailed study of facultative sulfur lithoautotrophy, organotrophy and biochemical characterization of these isolates were done.

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CHAPTER 2

Facultative sulfur lithoautotrophy, organotrophy and general biochemical characterization of the acid-tolerant isolates

2.1 Introduction

2.1.1 Facultative sulfur lithoautotrophy

The term 'chemolithotrophy' is used to describe the energy metabolism of bacteria; it is a bioenergetic process operative only in a few groups of microorganisms which can derive energy stored in reduced inorganic compounds (sulfur, iron etc.) and carbon from the atmospheric CO_2 (Kelly and Wood, 2006). The bacteria growing by metabolizing different forms of reduced sulphur compounds can be called as "thiotrophs"-and the property is termed as "thiotrophy" (Kelly, 1989). From the physiological point of view the "thioptrophs" are defined as the 'sulfur chemolithotrophic bacteria'. Until the reorganization of the genus *Thiobacillus* the members of this genus constituted the most widely studied among the 'unicellular sulfur bacteria' and even in 'thiotrophs' in general. The properties of sulfur lithotrophic mode of nutrition by the different life form proposed by Winogradsky were as follows: (1) Development in wholly inorganic medium containing an oxidizable inorganic compound providing the sole source of energy, (2) its vital activity was crucially dependent on the availability of thio inorganic compound, (3) lack of demand for organic nutrition as a source of material and energy, (5) inability to degrade organic compounds, (6) the only source of carbon is assimilation of carbon dioxide in the process of chemosynthesis.

Among the different chemolithotrophic processes operative in nature, the lithotrophic sulfur oxidation (SOX) is most widespread and is encountered in almost all the major prokaryotic groups. Phylogenetically diverse sulfur-lithotrophs are however assorted in terms of their ability to utilize different reduced sulfur compounds as substrates, as well as the biochemical mechanisms by which they do so (Lu *et al.*, 1985; Mason *et al.*, 1987; Kelly, 1989; Brune, 1995; Kelly *et al.*, 1997; Brüser *et al.*, 2000; Kletzin *et al.*, 2004). The inequality of efficiency of energy conservation from the same substrate by different organisms (at their optimum pH and temperature) has strengthened the probability of the existence of different substrate oxidation pathways, electron transport mechanisms, and modes of energy conservation. Although an array of sulfur

compounds are used by different groups of bacteria, thiosulfate is the common substrate oxidized by most of the photo-, or chemo-, lithotrophic sulfur-oxidizer, and atleast three pathways for the oxidation of this compound are known: (I) the **SOX** pathway (II) the **Branched Thiosulfate Oxidation** pathway and (III) the **Tetrathionate Intermediate [S_4I]** pathway.

Thiosulfate, one of the most abundant reduced inorganic sulfur species in the environment, plays a central role in the biogeochemical sulfur cycle as it happens to be a common substrate oxidized by almost all sulfur lithotrophs. Since thiosulfate can chemically decompose to sulfur and sulfite at pH values below 4.0 (Johnston and McAmish, 1973) and the rate of the process is strongly dependent on thiosulfate concentration. Thiosulfate-oxidizing enzymes have been studied from phylogenetically and metabolically diverse bacteria and different biochemical systems appear to be involved in the dissimilatory oxidation of thiosulfate. At least three pathways for the complete oxidation of thiosulfate are known to be operating in different sulfur-oxidizing chemo-, or photo-, lithotrophic bacteria (Kelly *et al.*, 1997; Brüser *et al.*, 2000; Friedrich *et al.*, 2001; 2005).

2.1.2 Thiosulfate oxidation

Thiosulfate is the common oxidizable substrate that is most suitable for the investigations of sulfur lithotrophic processes. Thiosulfate oxidation via formation of tetrathionate as an intermediate is widespread among sulfur- chemolithotrophic beta-, and gamma-, proteobacteria and though archetypal of obligately chemolithotrophic species of *Acidithiobacillus, Thermithiobacillus* and *Halothiobacillus* that live in extreme habitats, (Trudinger, 1961a, b, 1964, 1965; Pronk *et al.*, 1990; Kelly *et al.*, 1997), it is also known to be followed by facultatively sulfur-chemolithotrophic alphaproteobacteria like *Acidiphilium acidophilum* (formerly *Thiobacillus acidophilus*), betaproteobacteria like *Thiobacillus aquaesulis* and gammaproteobacteria like *Acidithiobacillus caldus* (Meulenberg *et al.*, 1992, 1993a, b; Hallberg *et al.*, 1996). An exhaustive series of physiological studies have documented the fact that the initial step of thiosulfate oxidation leads to the formation of tetrathionate in the above mentioned bacteria. Complete oxidation of thiosulfate to sulphate via polythionates by acidophilic bacteria like species of *Acidithiobacillus* and *Acidiphilium* have been explained by

conceding that thiosulfate is chemically unstable under acidic conditions and converting it to acid-stable intermediates like tetrathionate would be preferred (Johnston and McAmish, 1973). Again, the anoxygenic photolithotrophic gammaproteobacterium *Allochromatium vinosum* preferably forms tetrathionate from thiosulfate at pH values below 7.0 while sulfate is the main product under alkaline conditions (Smith, 1966).

Interestingly, while a periplasmic localization of tetrathionate-oxidizing enzymes have been indicated in species like *Acidiphilium acidophilum*, *Acidithiobacillus ferrooxidans*, and *Acidithiobacillus thiooxidans* (De Jong *et al.*, 1997a, b; Meulenberg *et al.*, 1993a; Tano *et al.*, 1996), studies with *Thermithiobacillus tepidarius* and *Acidithiobacillus caldus* have shown thiosulfate to be oxidized to tetrathionate in the periplasmic space while tetrathionate hydrolysis yielding sulfite as one of the intermediates took place in the cytoplasm or in close vicinity of the inside of the cell membrane, followed by oxidation of sulfite to sulfate in the same cellular compartment (Kelly, 1989; Hallberg *et al.*, 1996; Kelly *et al.*, 1997). There seems to be atleast one consensus among all these models in that they all essentially envisage thiosulfate to be oxidized to tetrathionate in the periplasmic space by the enzymic action of thiosulfate dehydrogenase, which either use *c*-type cytochromes or artificial electron acceptors (ac) as co-substrates and/or themselves contain *c*-type hemes. Though several thiosulfate-oxidizing and tetrathionate forming thiosulfate dehydrogenases have been identified or characterized from the periplasmic or soluble fractions of thiosulfate-grown cells of both neutrophilic and acidophilic sulfur-chemolithotrophs (Lu and Kelly, 1988b; Meulenberg *et al.*, 1993b; Visser *et al.*, 1996; Nakamura *et al.*, 2001), no molecular genetic basis of this obscure enzyme having variable structural features in diverse organism is available. Again of late, a periplasmic thiosulfate dehydrogenase having a pH optimum of 4.25 responsible for the oxidation of thiosulfate up to tetrathionate has been reported for *Allochromatium vinosum* (Hensen *et al.*, 2006) that is independent of the *sox* gene-encoded multienzyme complex essential for the oxidation of thiosulfate to sulfate.

2.1.3 Sulfite oxidation

Earlier biochemical studies had indicated that tetrathionate hydrolysis in the S4I pathway yielded sulfite as an intermediate and the oxidation of the latter in the cytoplasm was catalysed by sulfite oxidases (Hallberg *et al.*, 1996). However, two distinct pathways for the oxidation of sulfite have been identified, one involving APS reductase and ATP sulfurylase and looked upon as the reversion of the initial steps of the dissimilatory sulphate reduction pathway (Dhal, 1996; Sanchez *et al.*, 2001), and the other concerning the direct oxidation of sulfite to sulphate by a type of mononuclear molybdenum enzyme known as sulfite oxidoreductase not found in any sulphate reducer (Kappler and Dhal, 2001). Mononuclear molybdenum enzymes, in their turn, fall into three distinct groups viz., the xanthine oxidase, sulfite oxidase (SO) and DMSO reductase families (Kisker, 2001). The SO family, in particular, comprises of both plant assimilatory nitrate reductases and sulfite-oxidizing enzymes found in all the three domains of life, out of which the latter can directly catalyse the two electron oxidation of the highly reactive, and hence toxic, sulfite to sulfate (Aguey-zinsou *et al.*, 2003) [$SO_3^{2-} + H_2O = SO_4^{2-} + 2H + 2e^-$] with oxygen and/or heme-coordinated iron ions as the final electron acceptor (Hille, 1996; Kappler and Dhal, 2001).

Depending on the ability to transfer electrons to molecular oxygen, the sulfite-oxidizing enzymes are further divided into two categories: the eukaryotic sulfite oxidases and the prokaryotic sulfite dehydrogenases, even though both types are together preferred to as sulfite: acceptor oxidoreductases. As such, mainly three types of sulfite-oxidizing enzymes have been characterized so far, *viz.*, the homodimetric, heme b and molybdenum-containing enzymes from humans, rats and birds (Kisker *et al.*, 1997; Garrett and Rajagopalan, 1994); the homodimetric, molybdenum-containing enzymes from plants (Eilers *et al.*, 2001); and a third heterodimetric, heme c and molybdenum-containing bacterial enzyme (Kappler *et al.*, 2000) that can not transfer electrons to molecular oxygen and is therefore classified as a sulfite dehydrogenase. The typical bacterial sulfite dehydrogenase of the chemolithotrophic alphaproteobacterium *Starkeya novella*, is a heterodimetric complex of a catalytic molybdopterin and a *c*-type cytochrome subunit (Kappler and Dhal, 2001). It is now proven that *Starkeya novella* can oxidize sulfite by such a sulfite dehydrogenase encoded by its *sor*AB genes (Kappler *et al.*, 2001) located distantly from the *sox* operon.

2.1.4 Organotrophy

Microorganisms inhabiting AMD play important roles in the cycling of several elements, including carbon, iron, and sulfur. These include heterotrophs (organotrophs) that are sustained by organic carbon originating from primary production, as well as chemolithotrophs who get their energy from the inorganic products of heterotrophic metabolism. Availability of organic carbon is important to the microbial biochemistry at AMD-producing sites. Organic carbon inhibits the growth of many extreme acidophiles, a result of their reversed membrane potential and accumulation of organic acids that results in acidification of the cytoplasm (Alexander *et al.*, 1987). However, *Ferroplasma* spp. grow chemo-organotrophically by aerobic and anaerobic respiration at pH 1·0 (Baumler *et al.*, 2005; Dopson *et al.*, 2004). Heterotrophic microorganisms represent a numerically significant proportion of the AMD community (Sand *et al.*, 1992; Walton and Johnson, 1992; Johnson *et al.*, 2001) despite the fact that these environments typically contain low concentrations of dissolved organic carbon (<20 ppm) (Johnson, 1998). Members of the genus *Acidiphilium* represent the most frequently isolated heterotroph, although *Acidobacterium capsulatum*, and *Acidocella* spp. have also been retrieved (Johnson 1998). Sulfur (eg. *Acidophilium acidophilium*) and iron oxidizers (eg. *Ferromicrobium acidophilium*) that require organic carbon represent a distinct type of acidophilic heterotroph. *F. acidophilium* is able to increase pyrite oxidation rates in the presence of organic carbon or even in the absence of exogenously supplied organic carbon when a sulfur oxidizer such as *T. thiooxidans* is present (Bacelar- Nicolau and Johnson, 1999). Heterotrophy may also affect sulfide mineral oxidation by enhancing the growth rate of autotrophic, iron oxidizing bacteria (Johnson 1998). Acidophilic heterotrophs and lithoautotrophs are thought to form a mutualisic relationship as the heterotrophs obtain organic carbon from exudates and lysis products of the latter and, in turn, consume low molecular weight organic acids toxic to many iron-oxidizing bacteria (Johnson, 1998). In fact, early reports of *A. ferrooxidans* exhibiting mixotrophic growth on glucose and ferrous iron are now attributed to contamination by heterotrophic bacteria (Harrison, 1984).

2.2 Materials and methods

2.2.1 Growth of acid-tolerant isolates in autotrophic thiosulfate (MST) medium

Primarily, purified colonies of acid-tolerant strains isolated on modified DSMZ 269 medium (pH 4.0) were replicated on modified DSMZ 269 agar medium (pH 5.0) devoid of glucose but supplemented with $Na_2S_2O_3.5H_2O$ (10.0 g l^{-1}), and phenol red as indicator. The orange-red replicated plates were incubated for 4 days at 30 °C and the colonies surrounded by yellow zones (due to acidification) were identified.

Cells of the acid-tolerant isolates were grown in MST for 72 h and standardized inocula (diluted in sterile MS [(NH_4Cl, 1.0; K_2HPO_4, 4; KH_2PO_4, 1.5; $MgSO_4.7H_2O$, 0.5) g l^{-1} and trace metal solution 5 ml] were transferred to MS (control; without thiosulfate) or MST (thiosulfate 10.0 g l^{-1}) media so that initial cell densities in the growth flasks were $\leq 10^4$ cells ml^{-1}. Cultures were incubated at 30 °C and viable cell counts at different time intervals were done by plating serially diluted culture suspension on modified DSMZ 269 agar plates.

2.2.2 Growth of acid-tolerant isolates in autotrophic elemental sulfur medium

Preparation of elemental sulfur medium was described in chapter 1 under the section 1.2.2.1.2.Acid-tolerant isolates were grown in modified DSMZ 269 medium (pH 4.0), overnight at 30 °C. The freshly grown cultures were centrifuged at 7,000 rpm and washed with sterile MS devoid of glucose. This step was done thrice to remove the organic particles from the cell surface. Finally the washed pellet was re suspended in sterile MS which served as the inocula for elemental sulfur medium.

2.2.3 Growth of acid-tolerant isolates in mixotrophic (MSTSY) medium

Preparation of mixotrophic (MSTSY) medium was described in chapter 1 under the section 1.2.2.1.6.The standardized inocula was prepared as described in the section 2.2.2. The cultures were incubated at 30 °C for 4days. The MSTSY medium without inoculum served as the control or blank. Growth was measured after every 24 h interval

by taking O.D. at 550 nm in a Digital Spectrophotometer model 302 (Electronics, India).

2.2.4 Consumption of thiosulfate by acid-tolerant chemolithotrophic isolates

Thiosulfate was estimated following the spectrophotometric and iodometric determination (Kelly and Wood, 1994). Sulfur free control and standards were prepared with thiosulfate ranging from 0.01-0.2 µmole. The cultures were grown in MST medium and the concentrations of thiosulfate in the spent MST medium were determined. For this, the cultures at different period of incubation were centrifuged and the supernatant was collected for determining the concentration of the left-over thiosulfate. Finally, the percentage of thiosulfate consumption was calculated using the formula: (X-Y/X) ×100

Where, X= concentration of thiosulfate in the medium at zero hour and Y= concentration of thiosulfate in the spent medium after a defined period of incubation.

2.2.5 Thiosulfate and sulfite dehydrogenase assay of acid-tolerant isolates

2.2.5.1 Preparation of cell-free extract

Cells of the strains GAH1, GAH4, and GMX2 were harvested from culture broth of MST medium (Mukhopadhyaya *et al.*, 2000) and GAH2, and GAH5 in modified DSMZ 269 medium supplemented with thiosulfate, by centrifugation at 11,000 × g for 20 min. The harvested cells were washed and resuspended in 0.03 M potassium phosphate buffer (pH 7.0). The cell suspension was disrupted by sonication at 20 kHz for 10 min at 0 °C and the homogenate was centrifuged at 15000 × g for 45 min at 4 °C. The clear supernatant was used as the cell-free extract for enzyme assay. Protein concentrations were determined by the standard method (Bradford, 1976).

2.2.5.2 Thiosulfate dehydrogenase (EC 1.8.2.2) assay

The thiosulfate dehydrogenase enzyme assay was based on that of Trudinger (Trudinger, 1961a) in which ferricyanide reduction was measured

spectrophotometrically at 420 nm. The reaction mixture was in a total volume of 3 ml in a 1 cm cuvette containing phosphate buffer pH 7.0 (300 µmol), $Na_2S_2O_3$ (30 µmol), $K_3Fe(CN)_6$ (3 µmol). The reaction was started by addition of cell-free extract (2 mg) and substrate. Decrease in absorbance at 420 nm was recorded and ferricyanide reduction was measured using an extinction coefficient of 1.0 mM^{-1} cm^{-1} Enzyme activity was expressed as nmol ferricyanide reduced/min/mg protein.

2.2.5.3 Sulfite dehydrogenase (EC 1.8.2.1) assay

Sulfite-dependent reduction of ferricyanide was measured in a reaction mixture (3 ml) containing 1 ml of Tris buffer (300 µmol) and 25 mM EDTA, 0.1ml of potassium ferricyanide (3 µmol), 0.05 ml of Na_2SO_3 (9 µmol) dissolved in 25 mM EDTA, and cell-free extract (2 mg). The reaction was started by adding sulfite, and decrease in absorbance at 420 nm was recorded, using buffer plus water as a blank. Ferricyanide reduction was measured using an extinction coefficient of 1.0 mM^{-1} cm^{-1}. The rates of sulfite autooxidation (before the addition of cell-free extract) and any endogenous reduction of ferricyanide by the protein preparation (in the absence of sulfite) were assayed as controls.

2.2.6 Demonstration of organotrophic growth by the acid-tolerant isolates

2.2.6.1 Growth of acid-tolerant isolates in heterotrophic (MSSY) medium

Freshly grown cultures of acid-tolerant isolates were inoculated in heterotrophic MSSY medium maintaining the initial cell densities in the growth flasks $\leq 10^4$ cells ml^{-1}. The cultures were incubated at 30 °C and growth measurement at different time intervals were done by taking O.D. at 600 nm in a Digital Spectrophotometer model 302 (Electronics, India). The MSSY medium without inoculum served as control or blank.

2.2.6.2 Growth of acid-tolerant isolates in R2A medium

Growth of acid-tolerant isolates in R2A medium [composition (g/l): yeast extract, 0.5; proteose peptone, 0.5; casein hydrolysate, 0.5; dextrose, 0.5; starch, 0.5; dipotassium phosphate, 0.3; magnesium sulphate, 0.024; sodium pyruvate, 0.3 (pH of the medium was adjusted to 6.0)] was done as described above (under section 2.2.6.1) where MSSY medium was replaced by R2A medium. The cultures were incubated at 30 °C and

growth measurement was taken after 24 hr. of incubation by taking O.D. at 600 nm in a Digital Spectrophotometer model 302 (Electronics, India). The R2A medium without inoculum served as control or blank.

Acid-tolerant isolates were also streaked on R2A agar plates to obtain single colonies.

2.2.7 Biochemical tests

The cultures were characterized following biochemical tests described in the Bergey's Manual of Systemic Bacteriology. The acid-tolerant strains were differentiated on the basis of catalase, oxidase, caseinase, gelatinase, urease, amylase, indole, citrate, MR, VP, nitrate reduction, lipase, and carbohydrate fermentations. Modified DSMZ 269 medium was used for carbohydrate fermentations where glucose was replaced by other sugars. Mixotrophic traits of the strains were determined by growing them on MSTSY medium (Mukhopadhyaya *et al.*, 2000).

Information about the properties of organisms was converted into a form of suitable numerical analysis. The resulting evaluation of the isolates were based on general similarity as judged by comparison of many characteristics, each given equal weight. The process began with a determination of the presence or absence of selected characters in the group of organism under study. A character usually is defined as an attribute about which a single statement can be made. After character analysis, an association coefficient, a function that measures the agreement between characters possessed by two organisms, was calculated for each pair of organisms in the group. The simple matching coefficient (S_{SM}) is the proportion of characters that match regardless of whether the attribute is present or absent. Jaccard coefficient (S_j) is calculated by ignoring any characters that both organisms lack. Both coefficients increase linearly in value from 0.0 (no matches) to 1.0 (100% matches).

The calculation of association coefficients for two organisms

The simple matching coefficient (S_{SM}) = a+d/a+b+c+d

The Jaccard coefficient (S_j) = a/a+b+c

a = number of characters coded as present (1) for both organisms

b and c =numbers of characters differing (1,0 or 0,1) between the two organisms

d = number of characters absent (0) in both organisms

Total number of characters compared = a+b+c+d

The following biochemical tests were conducted for the characterization of the acid-tolerant heterotrophic isolates.

2.2.7.1 Indole test

Cells were grown in 5 ml tryptophan broth (g/l; NaCl, 5.0; $MgSO_4.7H_2O$, 0.2; $NH_4H_2PO_4$, 1.0; K_2HPO_4; L-tryptophan, 10.0, pH 7) and were incubated at 35 ^{0}C for 24 h. 0.2 ml of the test reagent (p-dimethylaminobenzaldehyde, 0.5 gm; Isoamyl alcohol, 0.5 ml; Conc. HCl, 2.5 ml) was then carefully added into the full-grown culture and the tubes were allowed to stand for 10 min after a gentle shaking. A dark red colored ring on the surface was regarded as positive test.

2.2.7.2 Methyl Red test

The organisms were grown in 10 ml of glucose peptone broth for 2-3 days at 30 ^{0}C. The medium contained (g/l) - peptone, 5.0; KH_2PO_4, 5.0; glucose 10.0 and the pH of the medium were adjusted to 7.4. Few drops of methyl red reagent (5 mg dissolved in 30 ml of 95 % ethanol and diluted to 50 ml with water) were added to the growing culture. Methyl red positive reaction was indicated by bright red coloration and negative reaction was indicated by yellow color of the culture medium.

2.2.7.3 Voges-Proskauer test

One loop full of overnight grown test culture was inoculated in 5ml of the sterilized buffered glucose broth (g/l, Protease peptone, 7.0; Glucose, 5.0; K_2HPO_4, 5.0; pH 7.0) and was incubated at 35 ^{0}C for 48 h. One ml aliquot of the full-grown culture were taken in a separate test tube and 0.6ml of α-napthol solution (α- napthol, 5.0 gm dissolved in 100 ml ethanol) and 0.2 ml 7(N) KOH were sequentially added and mixed

well by shaking. Development of pink to crimson color at the surface within 5 min considered as positive test.

2.2.7.4 Catalase test

The organisms were grown on Tryptic Soy Agar (TSA) plates for 24-28 h at 35 ^{0}C to get isolated colonies. A drop of H_2O_2 was put over a colony. As a negative control H_2O_2 was always dropped on any part of the plate devoid of any bacterial growth. Catalase positive character was indicated by effervescence of gas bubbles from the colony surface, immediately after the addition of H_2O_2.

2.2.7.5 Oxidase test

The test was performed by adding bacterial culture on a strip of filter paper which was initially impregnated with 1% (w/v) aqueous solution of N-N-dimethyl-p-phenylenediamine. Development of pink color within 30 seconds indicated the oxidase positive tests.

2.2.7.6 Citrate utilization test

The Simon's Citrate medium was used for this test. The composition of the Simon's Citrate medium is as follows (g/l); NaCl, 5.0; $MgSO_4.7H_2O$, 0.2; $NH_4H_2PO_4$, 1.0; K_2HPO_4, 1.0; Na-Citrate, 5.0; bromothymol blue, 0.02; Yeast Extract, 0.05 agar powder 20; pH 6.8. Blue coloration of the media after 24- 48 h of inoculation was recorded as positive reaction.

2.2.7.7 Urease test

Urease activity was tested with a medium having following composition (g/l): part A: peptone, 1.0; glucose, 1.0; NaCl, 5.0; KH_2PO_4, 2.0; phelol red, 0.12; agar, 20.0; pH 8.0; and part B (g/l): urea 40.0. After filter sterilization part A and part B were mixed together aseptically and were cooled to approximately 40-50 ^{0}C, and petri-plates were poured with the medium. In each plate, a loopful of overnight-grown culture was

streaked and incubated at 30 ^{0}C for 1-2 days. Development of deep red color surrounding the bacterial growth was indicative of positive test.

2.2.7.8 Nitrate reduction test

The enzyme nitrate reductase is a molybdenum-containing membrane bound enzyme, which reduces nitrate to nitrite in presence of an electron donor. Nitrate reduction and denitrification (as a function of dissimilatory reduction of nitrate denoting the presence of the enzyme nitrate reductase) was examined in the medium having following composition (g/l); KNO_3, 1.0; peptone, 5.0; pH 7.5. 5 ml volume of medium were dispensed in each tube and sterilized by autoclaving at 15 psi for 15 min. Each culture tube was inoculated with a single isolate and incubated at 30 ^{0}C for 24 h. Formation of nitrite in the culture medium was tested by adding 1.0 ml test reagent (0.8 g of sulfanilic acid dissolved in 100 ml of 5 (N) acetic acid and 0.5 g of α–napthalamine dissolved in 100 ml acetic acid, mixed immediately before use). Development of red color indicates a nitrate reductase positive test. To monitor the production of gas by nitrate respiration, inverted Durham tubes were placed in culture medium.

2.2.7.9 Gelatin hydrolysis

Gelatin hydrolysis was examined in a medium with components (g/l); gelatin, 20.0; nutrient broth, 1.0. Melted medium of 6 ml aliquots were dispensed into different culture tubes, plugged with cotton and sterilized by autoclaving at 15 psi for 15 min. each tube was inoculated with freshly grown culture by stabbing, and incubated for 2-3 days at 35 ^{0}C. The culture tubes, after growth of the organisms, were kept at 4 ^{0}C along with an un-inoculated (control) tube. The tubes that did not solidify even at 4 ^{0}C indicated the liquefaction of gelatin and the isolates were recorded as gelatinase positive.

2.2.7.10 Starch hydrolysis

For this test organisms were streaked on Starch agar plates [(g/l); NA, 23; soluble starch, 5.0; pH 7.2] and incubated at 37 ^{0}C for 1-2 days. After the growth of the culture the petri-plates were flooded with Lugol's iodine solution. Appearance of colorless

zone surrounding the bacterial growth indicated the hydrolysis of starch leaving the other part dark blue in color due to the reaction of starch with iodine.

2.2.7.11 Casein hydrolysis

Milk Agar plates were streaked with overnight grown loop-full culture(s) and caseinase producing bacteria showed a clear zone along the growth.

2.2.7.12 Lipase test

One loop full of overnight grown culture was streaked on Tributyrin agar plate (1.0 ml Tributyrin added in 100 ml sterile Tributyrin agar base) and was incubated at 37 ^{0}C. Isolates showed opaque zone around the growth area was considered as lipase positive.

2.2.7.13 Hydrogen Sulfide production test

The isolates were evaluated for Hydrogen Sulfide production using Sulfide Indole Motility (SIM) agar medium [(g/l); Peptone, 30.0; Beef extract, 3.0; Ferrous ammonium sulfate, 0.2; Sodium thiosulfate,0.025; and Agar, 3.0] autoclaved at 121^{0}C for 15 min. The isolates were inoculated by stabbing and incubated at 28^{0}C for 48-72 hours. The presence of black coloration along the line of stab inoculation was recorded as positive for H_2S production (Aneja,1996)

2.2.7.14 Fermentation of sugars

Fermentation of sugars was tested using 'Phenol Red Broth base' medium [(g/l); protease peptone, 10.0; beef extract, 1.0; Sodium chloride, 5.0; phenol red 0.018; pH 7.4] containing different sugars at a concentration of 1 % (w/v). An aliquot of 50 µl overnight grown culture was inoculated in 5 ml of test media and incubated at 30 ^{0}C for 24-48 h. Change in the color of the media from red to yellow was recorded as positive test.

2.2.7.15 Utilization of organic acid salts

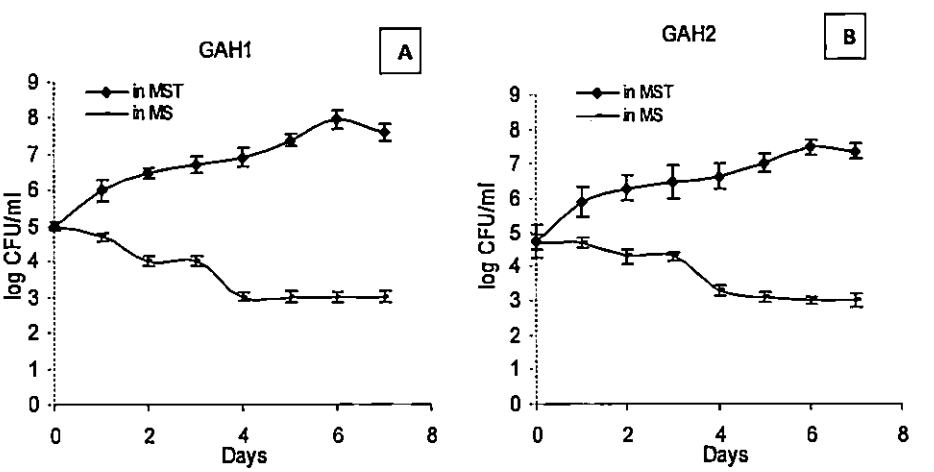
The test for the utilization of organic acid salts was carried out using HiBio-ID/HiCarbo system according to manufacturer's instruction (HiMedia, Mumbai, India). 50 µl of growing cultures were added to the media and growth was observed after 2-3days after incubation at 30 ^{0}C.

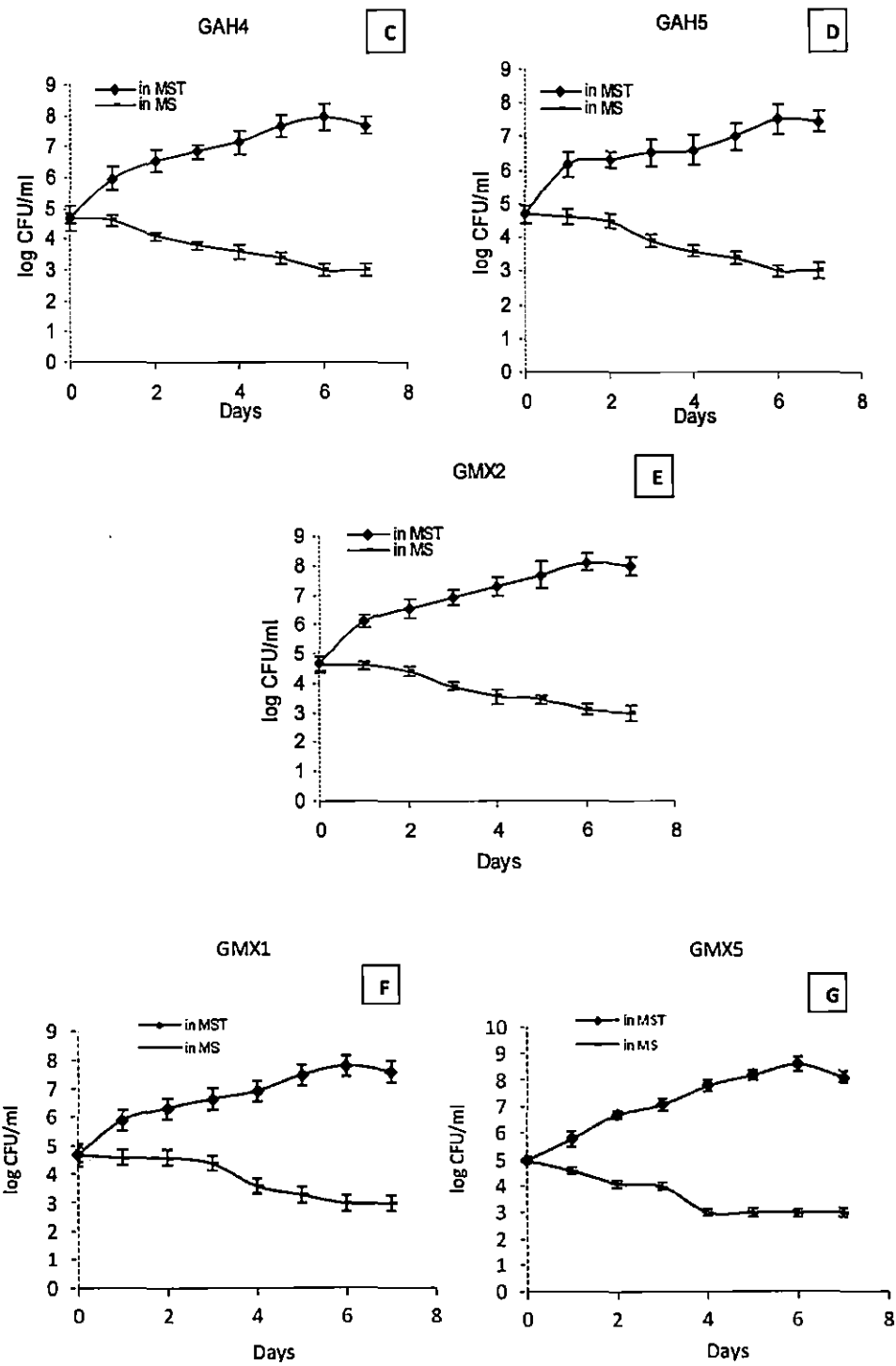
2.3 Results

2.3.1 Demonstration of chemolithotrophic growth by the acid-tolerant isolates

Details of chemolithotrophic growth of the acid-tolerant isolates in MST medium [(MS medium supplemented with thiosulfate ($Na_2S_2O_3$. $5H_2O$, 10 g l^{-1})] were studied over a total incubation period of 7 days (Fig.2.1). Maximum increase in viable cell numbers by a factor > 900 has been noted in case of GAH1, GAH4, GMX2, GMX5, and GMX7 while the other four strains, GAH2, GAH5, GMX1, and GMX6 grew relatively poorer with increment by a factor approximately ≤ 700 in MST (Fig. 2.1J). No growth by the acid-tolerant isolates was observed in MS medium. pH of the medium gradually lowered during the growth of the isolates except GMX7 where the pH value increased from 5 to 9.

Chemolithotrophic growth of the acid-tolerant isolates in elemental sulfur medium were studied for a total incubation period of 30 days. But none of the acid-tolerant isolates were found to grow in this medium.




GAH4
C
in MST
in MS
log CFU/ml
Days
GAH5
D
GMX2
E
GMX1
F
GMX5
G

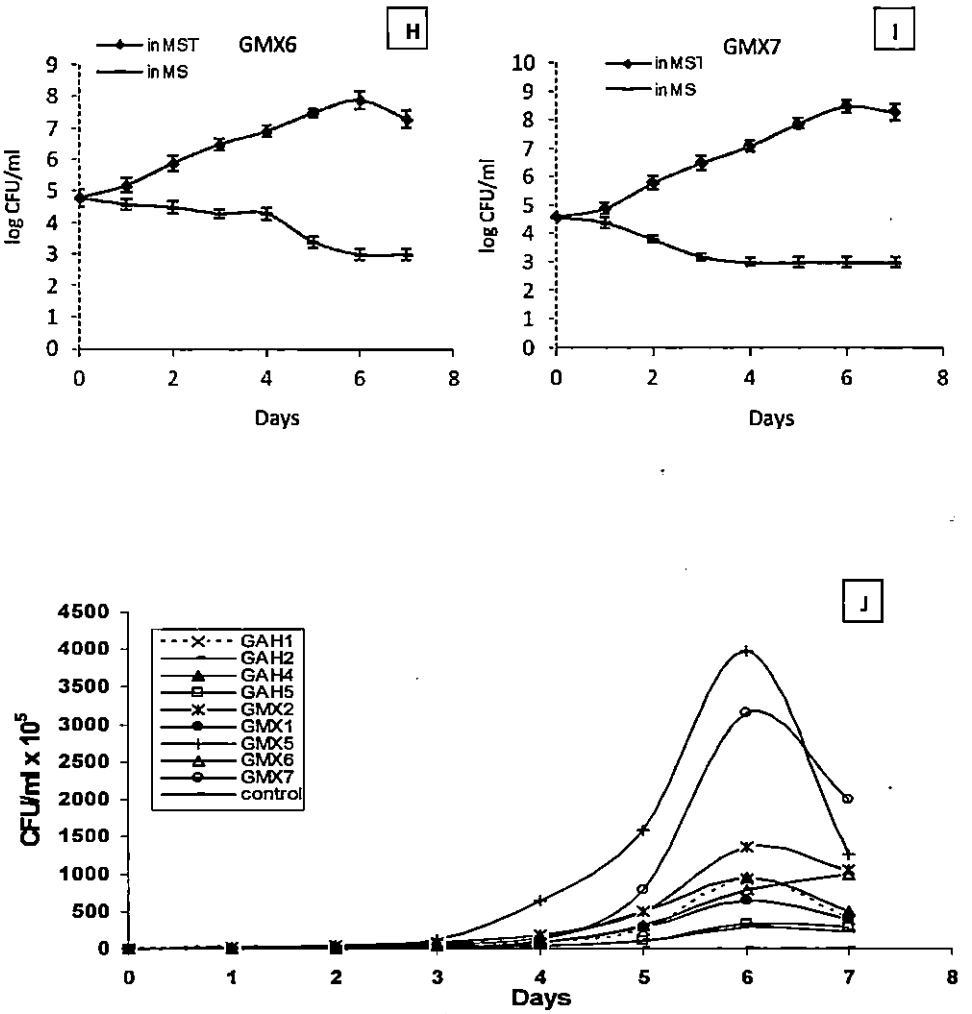


Fig. 2.1: Chemolithotrophic growth of acid-tolerant isolates on thiosulfate (10 gl^{-1}) in MST medium. (A) GAH1, (B) GAH2, (C) GAH4, (D) GAH5, (E) GMX2, (F) GMX1, (G) GMX5, (H) GMX6, (I) GMX7, and (J) results of all test isolates represented together. MS medium was used as control in all the experiments. Error bar represent the standard error for 5 replicates.

2.3.2 Mixotrophic growth by the acid-tolerant isolates

Mixotrophic growths by the acid-tolerant isolates were studied over a total incubation period of 4 days (Fig. 2.2). Among all the acid-tolerant isolates tested, ten were able to grow in mixotrophic (MSTSY) medium. GAH4 could not grow further after two days. GAH1, GMX1, and GMX6 showed exponential growth till 4th day. GMX6 showed maximum growth among all the isolates.

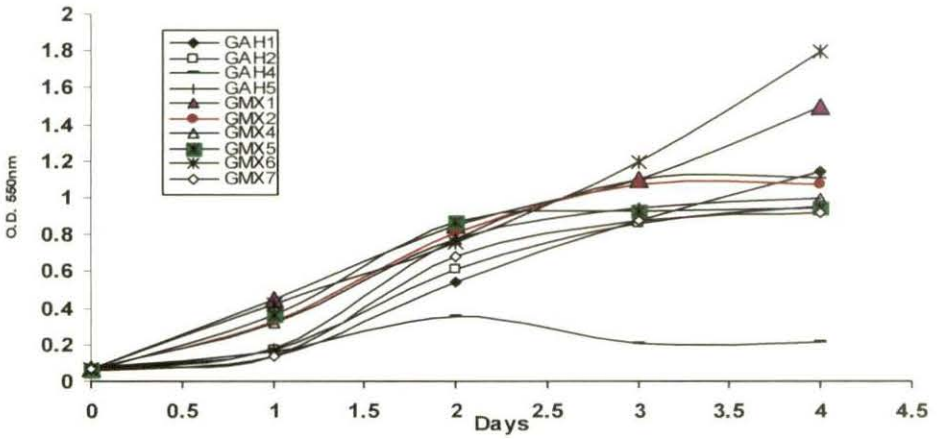


Fig. 2.2: Mixotrophic growth of acid-tolerant isolates on thiosulfate (10 gl^{-1}), and succinate (5 gl^{-1}) in MSTSY medium at $O.D._{550nm}$. Results of all test isolates have been represented together. MSTSY medium devoid of inoculum was used as negative control.

2.3.3 Consumption of thiosulfate by acid-tolerant mixotrophic isolates

Nine of the acid-tolerant heterotrophic isolates were found to be facultatively heterotrophic and have demonstrated growth by utilizing thiosulfate as the energy source (Fig. 2.3). Rate of depletion of thiosulfate in the MST medium with GMX7 was highest (70.2%) and least consumptions were recorded for GAH2 (4.6%), GAH5 (6.0%). Chemolithotrophic growth demonstrated by the acid-tolerant isolates in thiosulfate containing MST medium (Fig. 2.1) corroborated well with the data on consumption of thiosulfate by these isolates (Fig. 2.3).

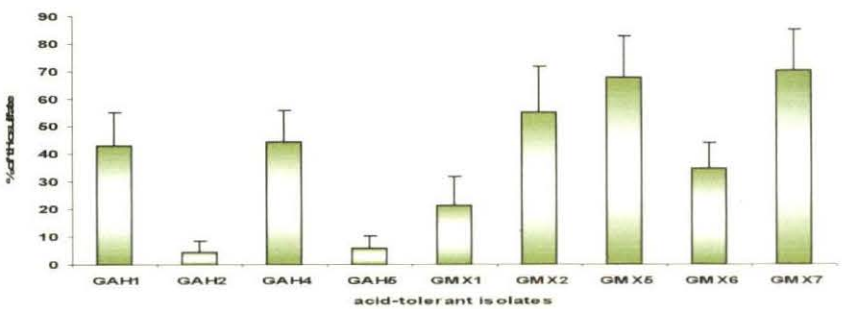


Fig 2.3: Thiosulfate consumption (percent consumption) by acid-tolerant heterotrophic isolates in 72 h. Error bar represent the standard error for 3 replicates.

2.3.4 Thiosulfate and sulfite dehydrogenase assay of acid-tolerant isolates

The activities of the enzymes associated with thiosulfate metabolism, particularly, thiosulfate dehydrogenase and sulfite dehydrogenase were observed in cell free extracts of the thiosulfate oxidising strains tested. The strains, GAH2, GMX1, GMX5 and GMX6 failed to demonstrate thiosulfate dehydrogenase activity (Table 2.1). The highest activities of thiosulfate dehydrogenase (200 nmol ferricyanide reduced/min/mg protein) and sulfite dehydrogenase (152 nmol ferricyanide reduced/min/mg protein) were found in GMX7, and GMX6 respectively (Table 2.1).

Table. 2.1: Thiosulfate dehydrogenase and Sulfite dehydrogenase activities of the acid-tolerant thiosulfate metabolizing strains isolated from AMD

Strains	***Thiosulfate dehydrogenase (ferricyanide reduced in nmole/mg cell free extract/min)***	***Sulfite dehydrogenase (ferricyanide reduced in nmole/mg cell free extract/min)***
GAH1	25±2	36±12
GAH2	3.5±2	16±15
GAH4	11±3	36±12
GAH5	10±2	24±10
GMX1	-	34±13
GMX2	57±5	25±11
GMX5	-	62±12
GMX6	-	152±15
GMX7	200±3	70±12

2.3.5 Organotrophic growth by the acid-tolerant isolates

Organotrophic growth of the acid-tolerant isolates in MSSY was studied over a period of 72 h while in R2A medium it was studied over a period of 24 h (Fig. 2.4 and Table 2.2). All the isolates were indeed found to grow well in both MSSY and R2A medium. Distinct single colonies were also obtained on R2A agar plates.

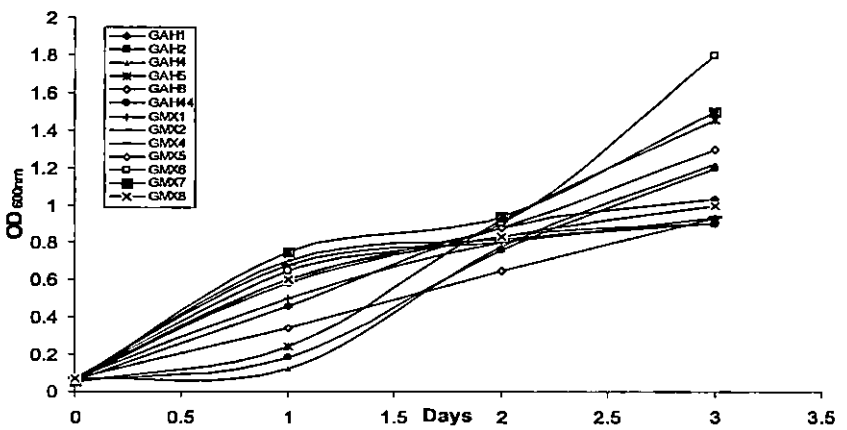


Fig. 2.4: Organotrophic growth of acid-tolerant isolates on succinate (5 gl^{-1}) in MSSY medium at $O.D._{600nm}$. Results of all test isolates represented together. MSSY medium devoid of inoculum was used as control.

Table. 2.2: Growth of acid-tolerant isolates in R2A medium. Final reading at OD_{600} was taken after 24 h of incubation.

Acid-tolerant isolates	Initial OD_{600} reading	Final OD_{600} reading
GAH1	0.05	2.5
GAH2	0.05	1.8
GAH4	0.06	2.2
GAH5	0.05	2.0
GAH8	0.06	2.8
GAH44	0.06	1.5
GMX1	0.05	2.6
GMX2	0.06	2.4
GMX4	0.05	2.0
GMX5	0.06	2.2
GMX6	0.05	1.8
GMX7	0.05	2.8
GMX8	0.05	1.5

2.3.6 Biochemical characterization of acid-tolerant isolates and determination of similarity matching coefficient (S_{SM}) amongst the isolates

None of the acid-tolerant isolates could produce indole from tryptophan. Variations with respect to different biochemical tests conducted with these isolates have been displayed in Table 2.3. All the isolates were lipolytic. With respect to the C- source utilization, all the isolates failed to utilize dulcitol, α-CH_3 D-mannoside, xylitol and sorbose. All the isolates failed to utilize Na-gluconate and α-CH_3 D-glucoside except GMX1 and GAH5 respectively. D-arabinose supported the growth of the only strain GAH4 and GAH10. Variations in utilization of other C- sources have been shown in Table 2.3. The isolate DK1AH1 and DK2AH2 were not included in this study as they could not grow at pH more than 5.5.

Numerical analyses of all the available comparative phenotypic characteristics were performed using the simple matching coefficient (S_{SM}) (Sneath and Sokal, 1973). Higher S_{SM} value (93.1%) was obtained in all pairwise combination with GAH8, GAH9, and GAH10. A high S_{SM} value of 91.4% was obtained with GAH3 in relation to GAH8 or GAH9 or GAH10. Similar S_{SM} value of 91.4% was obtained with the matching pair GAH2 and GAH5. Lower S_{SM} value was obtained with pairs like GAH4 and GAH8 (29.3%), GMX7 and GAH8 (39.7%), GMX2 and GAH4 (43.1%), GMX6 and GMX8 (75.9%). The obtained S_{SM} values were considered for selecting the acid-tolerant isolates for doing the 16S rRNA phylogeny.

Table. 2.3: Comparison of biochemical characteristics and carbon source utilization of acidtolerant heterotrophic isolates.

+, positive; -, negative

	GMX1	GMX2	GMX4	GMX5	GMX6	GMX7	GMX8
Indole production	-	-	-	-	-	-	-
Methyl red	+	-	+	-	+	-	-
Voges-Proskauer	-	-	-	+	+	+	-
Citrate,Simmons'	+	+	+	+	+	+	+
Urease,Christensens'	-	+	+	-	-	-	-
Gelatine liquefaction at 22°C	+	+	+	-	-	-	-

	GMX1	GMX2	GMX4	GMX5	GMX6	GMX7	GMX8
Malonate utilization	+	+	+	+	+	+	+
Lactose	-	+	-	-	-	-	-
Sucrose	+	-	-	+	+	+	+
D-Mannitol	+	-	-	+	-	-	-
Dulcitol	-	-	-	-	-	-	-
Salicin	+	+	-	-	-	-	+
D-Adonitol	-	-	-	-	-	-	-
myo-Inositol	+	-	-	-	-	-	-
D-Sorbitol	+	-	-	-	-	-	-
L-Arabinose	-	-	-	-	-	-	-
Raffinose	-	-	-	+	-	-	-
L-Rhamnose	-	-	-	-	-	-	-
Maltose	+	+	+	+	+	-	-
D-Xylose	+	-	-	+	-	-	-
Trehalose	+	-	-	-	+	-	-
Cellobiose	-	-	-	-	-	-	-
α-Methyl-D-glucoside	-	-	-	-	-	-	-
Esculin hydrolysis	+	-	-	-	-	+	+
Melibiose	+	-	-	-	-	-	-
Lipase, corn oil	+	+	+	+	+	+	+
Nitrate reduction	+	+	+	-	+	+	-
Oxidase, Kovacs'	-	-	-	-	+	+	-
ONPG(ß-galactosidase)	+	+	+	+	-	-	-
D-Mannose	+	+	-	-	+	-	+
Caseinase	-	+	-	+	+	-	+
Catalase	+	-	-	+	+	+	+
Amylase	-	-	-	+	+	-	+
Fructose	+	-	-	+	+	-	-
Dextrose	+	+	+	+	+	+	+
Galactose	+	+	+	-	-	-	-
Inulin	-	-	-	+	-	-	+
Na-gluconate	+	-	-	-	-	-	-
Glycerol	+	+	-	+	-	-	-
Glucosamine	-	+	-	-	-	-	-
Ribose	+	+	-	+	-	-	+
Melezitose	-	-	-	-	-	-	-

	GMX1	GMX2	GMX4	GMX5	GMX6	GMX7	GMX8
Mannoside	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-
D-Arabinose	-	-	-	-	-	-	-
Sorbose	-	-	-	-	-	-	-

	GAH1	GAH2	GAH3	GAH4	GAH5	GAH8	GAH9	GAH10
Indole production	-	-	-	-	-	-	-	-
Methyl red	+	+	+	-	+	+	+	+
Voges-Proskauer	+	-	+	+	-	-	+	+
Citrate,Simmons'	-	-	-	+	-	-	-	-
Urease,Christensens'	-	+	+	-	+	+	+	+
Gelatine liquefaction at 22°C	-	-	+	-	-	+	+	+
Malonate utilization	-	-	-	+	-	-	-	-
Lactose	+	+	+	-	+	+	+	+
Sucrose	+	-	+	+	-	+	+	+
D-Mannitol	-	-	-	+	-	+	-	+
Dulcitol	-	-	-	-	-	-	-	-
Salicin	-	-	-	-	-	-	-	-
D-Adonitol	+	-	-	+	-	-	-	-
myo-Inositol	-	-	-	+	-	-	-	-
D-Sorbitol	-	-	-	+	-	-	-	-
L-Arabinose	-	-	-	+	-	-	-	+
Raffinose	+	-	+	-	-	+	+	+
L-Rhamnose	+	+	-	+	+	-	-	-
Maltose	+	-	+	-	-	+	+	+
D-Xylose	+	+	+	+	-	+	+	+
Trehalose	+	-	+	-	-	+	+	+
Cellobiose	+	-	-	+	-	-	-	-
α-Methyl-D-glucoside	-	-	-	-	+	-	-	-
Esculin hydrolysis	+	-	+	-	-	-	-	+
Melibiose	-	-	+	-	-	+	+	+
Lipase, corn oil	+	+	+	+	+	+	+	+
Nitrate reduction	-	-	-	+	-	-	-	-
Oxidase, Kovacs'	-	-	+	-	-	+	+	+
ONPG(ß-galactosidase)	+	-	+	-	-	+	+	+

	GAH1	GAH2	GAH3	GAH4	GAH5	GAH8	GAH9	GAH10
D-Mannose	+	-	+	+	-	+	+	+
Caseinase	-	+	-	-	+	+	+	+
Catalase	+	-	-	+	-	-	-	-
Amylase	-	-	+	-	-	+	-	+
Fructose	+	-	+	+	-	+	+	+
Dextrose	+	+	+	+	+	+	+	+
Galactose	+	-	+	-	-	+	+	+
Inulin	-	-	+	-	+	+	+	+
Na-gluconate	-	-	-	-	-	-	-	-
Glycerol	-	-	+	-	-	+	+	+
Glucosamine	+	+	-	-	+	-	-	-
Ribose	-	+	+	-	+	+	-	-
Melezitose	+	-	+	-	-	+	+	+
Mannoside	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-
D-Arabinose	+	-	+	+	-	+	+	+
Sorbose	-	-	-	-	-	-	-	-

Table. 2.4: S_{SM} value of acid-tolerant heterotrophic isolates obtained by comparing their biochemical characters.

	GMX1	GMX2	GMX4	GMX5	GMX6	GMX7	GMX8	GAH1	GAH2	GAH3	GAH4	GAH5	GAH8	GAH9	GAH10
GMX1	100	62.07	63.79	62.07	63.79	56.90	60.34	53.44	44.82	56.89	56.89	39.65	58.62	55.17	56.89
GMX2		100	74.13	55.17	60.34	60.34	60.34	53.44	72.41	56.89	43.10	67.24	62.06	62.06	55.17
GMX4			100	58.62	65.51	63.79	22.41	51.72	74.13	51.72	55.17	68.96	53.44	56.89	50.00
GMX5				100	74.13	67.24	81.03	46.55	58.62	50.00	67.24	56.89	55.17	51.72	51.72
GMX6					100	82.75	75.86	55.17	53.44	51.72	65.51	51.72	53.44	56.89	53.44
GMX7						100	75.86	51.72	56.89	44.82	67.24	55.17	39.65	46.55	43.10
GMX8							100	41.37	63.79	41.37	65.51	65.51	43.10	39.65	39.65
GAH1								100	56.89	75.86	48.27	51.72	70.68	77.58	74.13
GAH2									100	50.00	53.44	91.37	55.17	55.17	48.27
GAH3										100	27.58	48.27	91.37	91.37	91.37
GAH4											100	48.27	29.31	32.75	32.75
GAH5												100	53.44	53.44	46.55
GAH8													100	93.10	93.10
GAH9														100	93.10
GAH10															100

Table. 2.5. Shortlisted pairs of isolates which produced > 60% similarities

ISOLATE	SIMILARITY %	ISOLATE
GMX1	63.79	GMX4/GMX6
GMX2	74.13	GMX4
GMX4	74.13	GMX2/GAH2
GMX5	81.03	GMX8
GMX6	82.75	GMX7
GMX7	82.75	GMX6
GMX8	81.03	GMX5
GAH1	77.58	GAH9
GAH2	91.37	GAH5
GAH3	91.37	GAH8/GAH9/GAH10
GAH4	67.24	GMX5/GMX7
GAH5	91.37	GAH2
GAH8	93.10	GAH9/GAH10
GAH9	93.10	GAH8/GAH10
GAH10	93.10	GAH8/GAH9

2.4 Discussion

Lithoautotrophic sulfur oxidizers are found in environments rich in H_2S, such as AMD. Particularly most sulfur-oxidizing bacteria are both acidophilic and autotrophic (Pronk *et al.*, 1991). As in the previous chapter we have discussed about the isolation of heterotrophic acid-tolerants from AMD, in this chapter we have studied the facultative sulfur lithoautotrophy, organotrophy, and biochemical characterizations of these isolates. Nine of the acid-tolerant isolates could grow chemolithoautotropically on thiosulfate (Fig. 2.1) however four isolates, GAH2, GAH5, GMX1, GMX6 grew relatively poorer than the others; whereas, elemental sulfur medium could not support the growth of any of the acid-tolerant isolates. Concurrent utilization of organic (succinate) and inorganic (thiosulfate) substrates, termed as mixotrophic nutritional ability (Matin, 1978), were shown by ten of the isolates reported in this study (Fig. 2.4). GMX4 which could not sustain in MST medium was able to grow when yeast extract was added to the medium (MSTSY). These mixotrophic behaviours of the isolates were also evident during the study of *Thiobacillus* A2 which showed mixotrophic metabolism when in the natural environment both inorganic sulfur compounds and organic substrates were available simultaneously (Gottschal and Kuenen, 1980).

Nine of the isolates have demonstrated growth by utilizing thiosulfate as the energy source (Fig. 2.3). Rate of depletion of thiosulfate in the medium with GMX7 and GMX5 was highest among all the nine isolates. Chemolithotrophic growth of acid-tolerant isolates on thiosulfate in MST medium (Fig. 2.1J) and the data regarding consumption of thiosulfate (% consumption) by these isolates (Fig. 2.3) supported each other which means cell growth was coupled with the utilization of thiosulfate. Isolation and classification of bacteria as the strains of *Thiobacillus* solely on the unifying property of sulfur chemolithotrophy resulting in "Physiological lumping" had a great impact on comparative microbial biochemistry and physiology. It made possible to pursue concentrated work on individual species, due to concept of unity in biochemistry that they would have similar functions regarding the sulfur compound metabolism (Kelly, 1989). It is now clear that the genus is heterogeneous assemblage of gram-negative bacteria, which shares many common physiological, biochemical and genetic properties within them and also with species of other genera, e.g. *Paracoccus, Thiomicrospira, Rhodobacter, Aquaspirilum, Acidiphilium* etc. The diversity of physiology was evident from the wide range of physicochemical requirements like temperature, pH tolerance etc. Similarly autotrophic growth of different isolates in this study in batch cultures on thiosulfate produced different growth yields. It was inferred from the growth experiment that external physical parameters do not influence the growth yield on thiosulfate for different isolates. Concurrently it also enabled to set up a working hypothesis that there may be difference in the mode of dissimilar sulfur compounds oxidation pathways, electron transport mechanisms and modes of energy conservation. Reduced sulfur species of sulfidic mineral origin in Garubathan AMD sites of Darjeeling Himalayas must have favoured abundant microbial populations of sulfur-oxidizers (both mesophilic and psychrotolerant), including autotrophic *Acidithiobacillus* species (Gurung and Chakraborty, 2009) as well as heterotrophic sulfur-oxidizing strains. Similar diversity of thiosulfate-oxidizing bacteria has been reported from marine sediments and hydrothermal vents (Teske *et al.*, 2000). Chemolithoautotrophy on thiosulfate is also known in non-thiobacilli of facultative lithotrophs like *Paracoccus pantotrophus*, hydrogen oxidizing heterotrophs, and heterotrophic marine pseudomonads (Ruby *et al.*, 1981; Kelly, 1989). Further investigations of all these isolates would be helpful in the study of widespread distribution of sulfur lithotrophy.

The final product of chemolithotrophic sulfur compounds oxidation is sulfate. The role and production of polythionates as intermediates in oxidations of sulfur, sulfide and thiosulfate to sulfite had been addressed starting from the initial studies (Kelly, 1982, 1989). Peck (1960) first proposed a separate view of thiosulfate oxidation independent of tetrathionate formation, in which $S_2O_3^{2-}$ was cleaved to S^{2-} and SO_3^{2-} by the enzyme thiosulfate reductase. He showed that S^0 was produced from sulfide (S^{2-}) oxidation, while the SO_3^{2-} was oxidized to sulfate forming APS (adenylyl sulfate) as the intermediate product. Kelly (1989) proposed a 'common pathway", a hypothetical scheme proposed on the basis of the discrete available data generated with the various strain, viz., *T. concretivorus* (strain *T. thiooxidans*), *T. thiooxidans*, *T. thioparus*, *T. novellus*, *T. ferrooxidans*, *T. denitrificans*, and *T. neapolitanus*. All these strains oxidize thiosulfate. The differences on the oxidative metabolism of thiosulfate by different members of sulfur lithotrophs were primarily due to the differences in the mechanism of initial cleavage of S-S bond of thiosulfate. Tabita *et al.*, (1969) proposed the involvement of two different enzymes in the metabolism of thiosulfate- (i) Rhodanese and (ii) thiosulfate oxidizing enzyme. Rhodanese cleaves the S-S bond, thereby producing membrane associated (S^0) and sulfite (SO_3^{2-}). The thiosulfate oxidizing enzyme, thiosulfate cytochrome c oxidoreductase catalyzes union of two molecules of thiosulfate to form one molecule of tetrathionate ($S_4O_6^{2-}$), which could be further oxidized to produce thiosulfate ($S_2O_3^{2-}$) and sulfite (SO_3^{2-}). The oxidation of thiosulfate without the formation and accumulation of polythionates has been best studied with facultative species *T. versutus*. Cytochrome-dependent complete oxidation of thiosulfate to sulfate was demonstrated with enzyme preparations (Kelly, 1989). A multi-enzyme system that performs the sequence of oxidation reactions of thiosulfate was identified in some greater detail (Kelly, 1989). Thiosulfate oxidation pathways operating in sulfur-oxidising bacteria have been tentatively grouped in three categories (Meyer *et al.*, 2007): (a) pathway involving breakdown of thiosulfate to polythionate intermediates by thiosulfate dehydrogenase and tetrathionate hydrolase, which is common in extremophilic sulfur oxidisers (*Acidithiobacillus*, *Thermothiobacillus*, and *Halothiobacillus*) (Kelly *et al.*, 1997); (b) pathway for direct conversion of thiosulfate to sulfate without sulfur globule formation by multienzyme complex (Sox) system, active in photo- and chemotrophic alphaproteobacteria (Friedrich *et al.*, 2001; Mukhopadhyaya *et al.*, 2000); (c) branched thiosulfate oxidation pathway involving formation of sulfur globules operating in sulfur-storing bacteria (Hensen *et al.*, 2006).

Sox enzyme system is present in diverse thiosulfate oxidizing bacteria. Activity of thiosulfate dehydrogenase, which occurs in a wide range of chemolithotrophic bacteria, was observed in the cell-free extracts of nine of the thiosulfate oxidizing strains, with GAH2 showing the least activity; GMX1, GMX5 and GMX6 showing no activity. (Table 2.1). Even if cell free extracts produces tetrathionate, doubts prevail about the nature of the intermediate; as tetrathionate may not always be a normal intermediate in thiosulfate oxidation by normal cells (Dam *et al.*, 2007).

Studies in energy conservation in sulfur oxidation constitute electron transport (ETS) and translocation of proton. Electrons generated from sulfur substrates oxidations may couple the redox reaction at the level of cytochrome *c* or electron enters at cytochrome *b* or more higher status in the electron transport chain for energy generation (Kelly, 1989). The terminal reaction, $SO_3^{2-} + H_2O \rightarrow SO_4^{2-} + 2H^+ + 2e^-$, is common in all the sulfur compound oxidation to produce sulfate from sulfite. In almost all the members of thiobacilli the enzyme responsible for the catalysis of this reaction, sulfite cytochrome *c* oxidoreductase, has been found to be actively present. The electron negative redox couple of SO_3^{2-}/ SO_4^{2-} is adequate to couple to the electron transport system at the level of flavin-quinone-cytochrome *b*. In some species it has been shown that cytochrome *c* is the physiological electron acceptor and the start point where the energy is coupled to the electron transport chain. Alternatively, adenosine phosphosulfate (APS) reductase has been shown to be operative in thiosulfate metabolism in several strains of *Thiobacillus*. It calyses the same terminal oxidation reaction of sulfite to sulfate. The reaction steps are:

$$SO_3^{2-} + AMP \rightarrow APS + 2e^-$$

APS will then be desulfurylated to SO_4^{2-} and ADP or ATP catalyzed by either ADP or ATP sulfurylase:

$$APS^{2-} + PO_4^{3-} \rightarrow ADP^{3-} + SO_4^{2-}$$

$$APS^{2-} + P_2O_7 \rightarrow ATP^{4-} + SO_4^{2-}$$

The electrons entering the chain at level of cytochrome *b*, are benefited in two ways- by conserving energy synthesizing ATP at the site of transfer in between cytochrome *b* to cytochrome *c* sector in the ETS and also at the cytochrome *c* / oxidase sector. Whereas this cytochrome *c* / oxidase sector is the only location for the organisms coupling the electron at cytochrome *c*. These differences in major electron transport chain cause the differences in growth yields e.g., *T. denitrificans* and *T. tepidarius*, produce better

yields of growth when compared to the growth of *T. versutus* and *T. neapolitanus* (Kelly, 1989). Sulfite dehydrogenase activity was observed in cell-free extracts of all of the nine test strains (Table 2.1). *soxC* encodes a sulfite dehydrogenase, the requirement for which in thiosulfate-dependent lithotrophic growth, experimentally verified in *Paracoccus denitrificans* (Mukhopadhyaya *et al.*, 2000).

Organotrophic growth for the acid-tolerant isolate was done on MSSY and R2A medium. Thirteen of the acid-tolerant isolates showed good growth on both the media (Fig. 2.4 and Table 2.2). R2A medium was greatly preferred (better growth within 24 h) by them. The isolates also displayed a wide range of substrate utilization as well (Table 2.3). R2A is a low-nutrient containing medium. Oligotrophic bacteria are generally tested for their ability to grow in R2A medium (Bhowal and Chakraborty, 2011). Growth of acid-tolerant isolates in R2A medium has impelled us for the identification of oligotrophic property of these isolates (discussed in chapter 3).

The isolates DK1AH1 and DK2AH2 were excluded from biochemical characterization study as they could not survive in pH more than 5.5. The rest of the acid-tolerant heterotrophic isolates showed varying differential properties from each other biochemically. The results of S_{SM} values scored between the isolates have reflected similar behaviour as obtained from total protein profile (discussed in chapter 1).

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CHAPTER 3

Oligotrophic Growth characteristics, metal and antibiotic tolerance of acid-tolerant heterotrophic isolates

3.1 Introduction

The growth of the micro-organisms are greatly affected by chemical and physical nature of their surroundings, nutrient availability on habitat, pH of the medium, presence or absence of metals, presence or absence of antibiotics etc. An understanding of these factors control the microbial growth on different habitat and it also helps for studying the ecological distribution of microorganisms. Bacterial degradation and transformation of dissolved organic matter is the key factor in the cycling of inorganic and organic matter. Organotrophic bacteria are active at any level in the aquatic food webs, and have more than one possibility to use energy, as it passes through the ecosystem. Organotrophic bacteria in natural waters appear in double role: they are limited by the degree of nutrient supply and are responsible for the actual nutrient concentration. Otherwise, Oligotrophic bacteria inhabit very low concentaration of dissolved organic matter media but as facultative organisms can be found in conditions with higher concentrations of dissolved organic matter.

3.1.1 Oligotrophy

Nutrient concentration is an important intrinsic factor of the medium for microbial growth. The amount of nutrient varies from one habitat to another. Bacteria grow best when optimal amount of nutrients are provided; however, the nutritional needs of bacteria vary tremendously. Some strains require nutritionally rich medium full of amino acids, peptides and sugars. These rich broths sometimes kill other bacteria. Nutrient broth is moderately rich medium that allows good growth of most of the bacteria used for regular use in laboratories. It lacks sugars, which increase the growth rate but also increase the death rate because the metabolism of sugar produces acids that kill the cells. Minimal media which provide only the essentials that will allow many bacteria to make their own amino acids and vitamins, is often used in the laboratory. However bacteria growing in minimal media have a long lag phase and they grow slowly (Watve *et al.*, 2000).

Oligotrophic bacteria are a type of bacteria that survive in oligotrophic environments such as the ocean, blue water and nutrient-deficient soils and other environments, in which organic substances are deficient. Oligotrophic bacteria are defined as a type of bacteria which can grow in 1-15 mg CL^{-1} culture medium when they were cultured first. Some organisms can live in a very low carbon concentration less than one part per million, known as **oligotrophs.** They may be contrasted with copiotrophs, which prefer environments rich in carbon (Valiela, 1995; Koch, 2001). Most oligotrophs are bacteria, though archaean oligotrophs also exist. Oligotrophs are characterized by slow growth, low rates of metabolism, and generally low population density (Lauro *et al.*, 2009). Oligotrophs are ubiquitous in the environment and have been isolated from the soil (Hattori, 1984), rivers (Yanagita *et al.*, 1978; Kumar *et al.*, 2011), lakes (Lango, 1988), oceans (Deming, 1986; Stahl *et al.*, 1992), tap water (Jaeggi and Schmidt-Lorenz, 1990), distilled water (Favaro *et al.*, 1971; Suwa and Hattori, 1984), and even clinical materials (Tada *et al.*, 1995) lacking organic substances. Two different types of oligotrophs can be distinguished. Those oligotrophs that can grow on only a low concentration of carbon are called *obligate oligotrophs.* Those that are able to grow at both high and low concentration of organic substances are called *facultative oligotrophs.* The mechanism by which they grow under extremely poor nutritional conditions is not known. They did not grow or grew very poorly on blood agar, and some hardly grow on nutrient agar, although they grew on purified agar with NB that was diluted 1:100 or less (Tada, *et al.*, 1995). These are a group of microbes living in oligotrophic environments. Their diversity and biomass are dominant in biosphere, and thus, play an important role in biogeochemical cycles. Since 1980s, their oligotrophic mechanisms, responses to starvation, and roles in ecosystems have been one of the most advanced subjects in microbial ecological research. The nutritional flexibility of oligotrophic and copiotrophic bacteria from an Antarctic freshwater lake sediment was investigated (Upton and Nedwell, 1989). Bacteria isolated on plates of oligotrophic and copiotrophic media were replica plated onto media containing different substrates, and their ability to utilise the different substrates was determined. The oligotrophs were shown to be able to utilise a significantly broader range of organic substrates than the copiotrophs, consistent with the idea that nutritional flexibility is adaptive for oligotrophic bacteria.

A large proportion of bacterial diversity in natural habitats is uncultured and therefore unexplored. A large fraction, if not all, of uncultured diversity from a variety of aquatic and terrestrial habitats are oligotrophic. Oligotrophic bacteria form small or microscopic colonies. Slow growth rates and high yields indicate that they are 'K' selected species and the fast growing as 'R' selected bacteria (Watve *et al.*, 2000).

Oligophiles are known to constitute the majority of marine bacterial communities (Akagi *et al.*, 1977). Studies on oligophiles of soil and fresh water are scanty, but confirm their presence. R2A medium is typically used for isolation and purification of oligotrophic bacteria from different samples (Tada *et al.*, 1995). An earlier study from our laboratory had also validated the capability of diluted (10^{-2}-10^{-3}) Luria-Bertani broth in quantifying oligotrophic bacteria (Kumar *et al.*, 2010). Mine-water inhabiting heterotrophic isolates like *Acidiphilium* spp. was shown to demonstrate oligotrophic growth in elemental sulfur spent medium of *Acidithiobacillus ferrooxidans* (Gurung and Chakraborty, 2009).

In the present work oligotrophic growths of the acid-tolerant heterotrophic strains were studied in R2A, diluted modified DSMZ 269, and diluted Luria-Bertani media along with sterile acid mine water (pH 1.5) and elemental sulfur spent medium (pH 2.0) of *Acidithiobacillus ferrooxidans* culture.

3.1.2 Metal tolerance in Acid Mine Drainage

Bacteria resistant to heavy metal(s), characterized so far, were isolated from metal contaminated environments, such as, zinc decantation tank of Belgium (Mergeay *et al.*, 1978), waste water treatment plant of Germany (Timotius and Schlegel, 1987), metal working industries of Germany and Sweden (Mattsby-Baltzer *et al.*, 1989), low grade ore deposits of Belgium and Zaire (Mergeay, 1991), sewage contaminated water (Pickup *et al.*, 1997), etc. Heavy metal resistance is a prevalent trait among microorganisms isolated from mining environments. Bacteria isolated from acid mines viz., *Acidiphilium* and *Acidocella* genera are able to resist to levels as high as 1M Cd, Zn, Ni and Cu. (Mahapatra and Banerjee, 1996; Ghosh *et al.*, 1997). The high incidence of heavy metal resistance in mine microorganism indicates the potential of these microorganisms as bioremediation agents (Castro-Silva *et al.*, 2003). Many

commercially viable metals themselves occur as sulfides, including copper, gold, lead, silver and zinc. Excavation of sulfide minerals during coal and metal mining results in sulfuric acid production mediated either biologically by sulfur oxidizing bacteria attached to the mineral surface. It is now well established for the use of acidophilic, chemolithotrophic iron- and sulfur-oxidizing microbes in processes to recover metals from certain types of copper, uranium, and gold-bearing minerals or mineral concentrates (Rawlings, 2002). Much of the effort has been focused on *A. ferrooxidans*; however, more recent molecular ecological investigations indicate that other bacteria and archaea are numerically dominant in acid-leaching environments (Bond *et al.*, 2000).

It was known from the survey of Geological Survey of India that an area of 0.247sq. km., in Kalimpong subdivision had probable reserve of metal sulfide ores of zinc and copper (Shah *et al.*, 1974-75). Acidic sample from Garubathan AMD cause the dissolution of other resident minerals increasing the concentration of heavy metals in these environments. Therefore, bacteria isolated from AMD could exhibit high levels of tolerance to heavy metals. In the present work we had isolated and characterized acid-tolerant, facultative chemolithotrophic bacteria from AMD that were tolerant to high concentrations of heavy metal divalent cations.

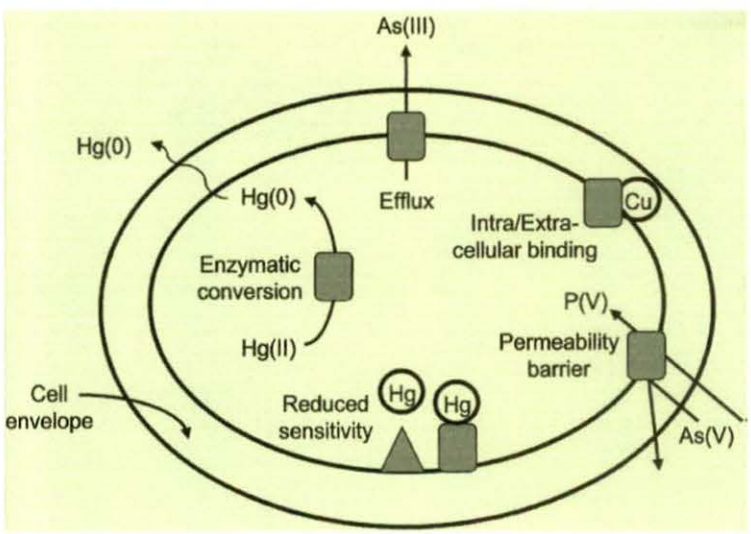

Fig. 3.1 : Schematic diagram of metal resistance mechanisms in acidophilic micro-organisms. The resistance mechanisms (clockwise from the top) include: efflux of the toxic metal out of the cell, e.g. As(III) efflux by the ArsB protein; intra/extracellular binding of the metal reducing

its toxic effect; exclusion of the metal via a permeability barrier, in this instance the expression of the phosphate-specific transport protein that is specific for phosphate but not As(V); alteration of a cellular component to lower its sensitivity to the toxic metal, for example the less sensitive cytochrome c oxidase in some mercury-resistant strains; and finally conversion of the metal to a less toxic form, an example being the reduction of Hg(II) to Hg(0), which then volatilizes out of the cell (Dopson *et al.*, 2003).

3.1.3 Antibiotic tolerance

Presence of antibiotic resistant bacteria in the aquatic environment has been studied by many researchers. A detailed descriptive information about the antibiotic resistances of gram-negative bacteria isolated from four tributaries has been provided which enter Tillamook bay, Oregon and the Bay itself (Kelch and Lee, 1978). Several others have demonstrated the wide spread occurrence of such organisms in many rivers and streams (Jones, 1986; Sokari *et al.*, 1988; Magee and Quinn, 1991; Ogan and Nwiika, 1993; Leff *et al.*, 1993). Boon and Cattanach, 1999 have studied the antibiotic resistance of native and faecal bacteria isolated from rivers, reservoirs and sewage treatment facilities in Victoria, Southeastern Australia. The results of one of the study implied that heavy metal pollution might contribute to increased antibiotic resistance through indirect selection. (McArthur and Tuckfield, 2000).

Micro-organisms resistant to both metals and antibiotics have been isolated repeatedly from different environments and clinical samples (Henriette *et al.*, 1991; Sundin and Blender, 1993). This led to the proposition that the combined expression of antibiotic resistance and metal tolerance is caused by selection resulting from metals present in the particular environment (Nakahara *et al.*, 1977a,b; Calomiris *et al.*, 1984; De Vicente *et al.*, 1990; Sabry *et al.*, 1997). The presence of metal and/or antibiotic-resistant bacteria in natural habitats can pose a public health risk (Brown *et al.*, 1991; Qureshi and Qureshi, 1992).

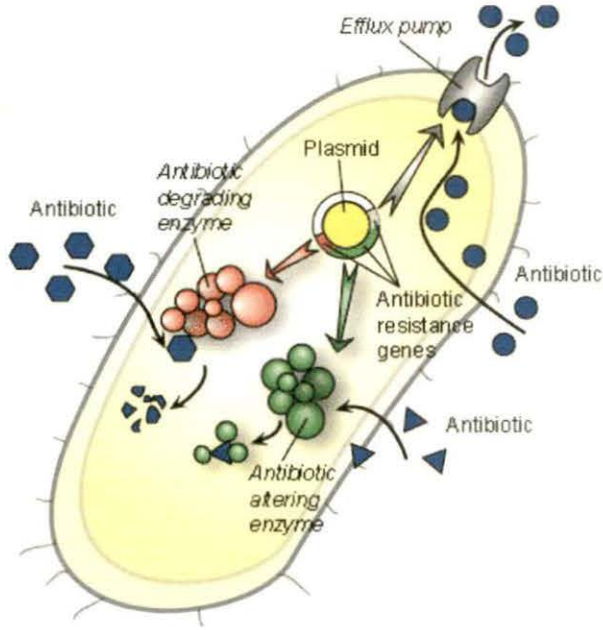

Fig. 3.2: Mechanisms of Antibiotic Resistance (Yim, 2011).

3.2 Materials and methods

3.2.2 Determination of oligotrophic property of acid-tolerant heterotrophic isolates

Growth of the acid-tolerant heterotrophic strains were studied in R2A, diluted modified DSMZ 269, and diluted Luria-Bertani media along with sterile acid mine water (pH 1.5) and elemental sulfur spent medium (pH 2.0) of *Acidithiobacillus ferrooxidans* culture.

3.2.2.1 Growth of acid-tolerant heterotrophic isolates in diluted LB, modified DSMZ 269, and undiluted R2A medium

Purified single colonies of the acid-tolerant heterotrophic isolates from 24 hr-old culture were seeded into the 10 ml sterile modified DSMZ 269 medium (pH 3.0) supplemented with separately sterilized glucose (1.0 g/l) and yeast extract (0.1 g/l) in a 100 ml Erlenmeyer flask for the preparation of the inoculum. *E. coli* K-12 strain

(standard copiotrophic strain) was used as a negative control and its inoculum was prepared in standard 1x Luria- Bertani (LB) medium at pH 7.3. The inoculated media of acid-tolerant heterotrophic isolates were incubated at 30 °C for 12 hr and *E. coli* K-12 at 37 °C. The cultures were harvested by centrifuging at 8,000 rpm for 8 min at 4 °C and afterwards washed twice with sterile saline (0.5% NaCl) water to ensure removal of traces of media. The washed pellets were resuspended in sterile saline water. 0.5 ml of cell suspension(s) were added to 5 ml volume(s) of diluted (0.1x, 0.01x, 0.001x) modified DSMZ 269 (where by glucose and yeast extract were diluted likewise) and LB media; and undiluted modified DSMZ 269, R2A, and LB in 25 ml culture tube(s). All the culture tubes were made in triplicates and the pH of all the media were maintained at 3.0 for acid-tolerant heterotrophic isolates and at pH 7.3 for *E. coli* K-12. The tubes were kept at 30 °C for acid-tolerant heterotrophic isolates and at 37 °C for *E. coli* K-12 during the period of incubation. Growth was measured by taking O.D. at 550 nm in a Digital Spectrophotometer model 302 (Electronics, India).

3.2.2.2 Viability and growth of acid-tolerant heterotrophic isolates in sterile acid mine water

DOC of AMD water of Garubathan was analyzed by the high temperature oxidation method using a Shimadzu TOC-5000 Carbon analyzer. The inoculum of acid-tolerant heterotrophic isolates were prepared as described above (section 3.2.2.1). The washed cultures were then grown in sterile acid mine water (pH 1.5; and pH 3.0-4.0 which isolates have pH range more than 2.0) was adjusted to without any supplementation of additional carbon source or yeast extract. Viable cell count was assessed through dilution-plating at different time intervals on modified DSMZ 269 agar medium.

3.2.2.3 Growth of acid-tolerant heterotrophic isolates in elemental sulfur-spent medium of *Acidithiobacillus ferrooxidans* DK6.1 pure culture

Acid-tolerant heterotrophic cultures were grown in four weeks old elemental sulfur spent medium of autotrophic *Acidithiobacillus ferrooxidans* DK6.1 pure culture. Four weeks old elemental sulfur culture of *A. ferrooxidans* DK6.1 was filtered through Whatman filter paper no.1 to remove the suspended elemental sulfur particles. The filtrate was centrifuged at 10,000 rpm to exclude DK6.1 cells as pellet. The supernatant

was passed through bacterial filter (pore size: 0.25 μm) and sterilized by autoclaving with flowing steam for 20 min. By this process, any traces of sulfide and sulfite that may be present in the spent elemental sulfur medium would be oxidized to sulfate. Presence of any other oxyanions in the spent medium may be ruled out as no other oxyanions are produced during the course of elemental sulfur oxidation by *A. ferrooxidans*. Soluble form of elemental sulfur, if present in the spent medium, would be as low as 5 μg/l (Roy and Trudinger, 1970; Steudal *et al.*, 1987). The cells of acid-tolerant heterotrophic cultures were grown in elemental sulfur spent medium without any supplementation of additional carbon source. Viable cell count was assessed through dilution-plating at different time intervals on modified DSMZ 269 agar medium.

3.2.3 Heavy metal tolerance of acid-tolerant heterotrophic isolates

All pure cultures of acid-tolerant heterotrophic isolates were tested for their tolerance to elevated concentrations of Chromium [Cr(II)], Cobalt [Co(II)], Nickel [Ni(II)], Copper [Cu(II)], Zinc [Zn(II)], Arsenite [As(III)], Cadmium [Cd(II)], and Mercury [Hg(II)]. Maximum tolerable concentration (MTC) was determined on liquid modified DSMZ 269 medium supplemented with a series of different concentrations of heavy metal(s). Deionised double distilled water and analytical grades of metal salts ($K_2Cr_2O_7$, $CoCl_2.6H_2O$, $NiCl_2.6H_2O$, $CuSO_4.5H_2O$, $ZnSO_4.7H_2O$, $Na_2HAsO_4.7H_2O$, $CdCl_2.H_2O$, and $HgCl_2$) were used to prepare 1 M or 0.1 M stock solutions (Bhadra *et al.*, 2007). Growth was determined by measuring O.D. at 550 nm in spectrophotometer after 2 days incubation at 30 °C. The medium without any inoculum was considered as negative control, while the medium containing inoculum but without any heavy metal(s) was considered as positive control.

3.2.4. Preparation of antibiotic stock solutions and determination of antibiotic tolerance of the acid-tolerant isolates

Antibiotic stock solutions were prepared by dissolving measured amounts of respective antibiotics to its suitable diluents. These concentrated stock solutions were made at least once a month and were stored at -20 °C. Tests with known sensitive isolates of *E. coli* indicated adequate storage stability for all antibiotics stored under these

conditions. Antibiotic powders were weighed to 0.1 mg accuracy; liquids were quantified by micropipette. Modified DSMZ 269 medium was used as the basal medium to determine the antibiotic resistances of acid-tolerant bacterial isolates. The pH of the medium was adjusted to 5.0. A panel of 12 antibiotics, representing 7 different classes were used. Antibiotics and their concentrations employed in this investigation were as follows: Aminoglycosides (azithromycin, 10 μgml^{-1}; kanamycin, 10 μgml^{-1}; netilmicin, 1 μgml^{-1}; streptomycin, 5 μgml^{-1}); Antifolates (sulfamethoxazole, 10 μgml^{-1}); Cephalosporins (cefepime, 10 μgml^{-1}; cefotaxime, 10 μgml^{-1}); Penicillin (ampicillin, 50 μgml^{-1}); Quinolones (ciprofloxacin, 5 μgml^{-1}; livofloxacin, 5 μgml^{-1}); Others (chloramphenicol, 150 μgml^{-1}; oxytetracycline, 2 μgml^{-1}). The desired concentrations of the antibiotics (diluted from the stock) were stirred into the modified DSMZ 269 medium and incubated for 2 days at 30 °C. Growth of the isolates were determined by taking O.D. at 550 nm in spectrophotometer. The medium without any inoculum was considered as negative control, while the medium containing inoculum but without any antibiotic was considered as positive control.

3.3 Results

3.3.1 Demonstration of oligotrophy of acidtolerant heterotrophic isolates from AMD

All the isolates were facultative oligotrophs except DK1AH1 and DK2AH2 which were obligate oligotrophs. All have demonstrated growth in 10^{-2} diluted modified DSMZ 269 and LB media. GAH1, GAH2, GAH3, GAH8, GAH44, DK1AH1 and DK2AH2 have shown growth in 10^{-3} diluted modified DSMZ 269 medium. The R2A medium has supported growth of all the tested strains (Table 3.1). The standard copiotrophic strain *E. coli* K-12 failed to show good growth in R2A medium and failed to grow in 10^{-2} diluted modified DSMZ 269 medium and LB.

Table. 3.1: Oligotrophic growth of acid-tolerant heterotrophic isolates in modified DSMZ 269, undiluted R2A and LB, and diluted LB media.

Strain	*DSMZ 269 (modified)*				*R2A*	*LB*			
	1x	**0.1x**	**0.01x**	**0.001x**		**1X**	**0.1X**	**0.01X**	**0.001X**
GAH1	++	++	+	+	++	+	+	+	-
GAH2	++	++	+	+	++	-	+	+	-
GAH3	++	++	+	+	+	+	+	+	-
GAH4	++	++	+	-	++	++	++	+	-
GAH5	+	+	+	-	++	-	+	+	wp
GAH8	++	++	+	+	+	+	+	-	-
GAH44	++	++	+	+	++	++	++	+	wp
GMX1	++	+	+	-	++	++	++	+	-
GMX2	++	+	+	-	++	-	+	+	+
GMX4	++	+	+	-	++	-	+	+	+
GMX5	++	+	+	-	++	+	++	++	+
GMX6	+	+	+	-	++	+	++	++	+
GMX7	+	-	-	-	++	++	++	+	-
GMX8	+	+	+	-	++	+	+	+	-
DK1AH1	++	++	+	+	+	-	-	wp	-
DK2AH2	++	++	+	+	+	-	-	wp	-
E.coli K-12	+	wp	-	-	+	+++	++	wp	-

+++, Δ $O.D._{550}$ reaching >0.7 after 20 hr of incubation; ++, Δ$O.D_{550}$ reaching ≤ 0.35 after 20 hr of incubation; +,Δ $O.D_{550}$ reaching ≤ 0.15 after 20 hr of incubation; wp- weakly positive Δ $O.D_{550}$ reaching ≤ 0.1 after 20 hr of incubation; - growth absent. E. coli K-12 was used as negative control (standard copiotrophic strain)

3.3.2 Viability and growth of acid-tolerant heterotrophic isolates, GAH1 and GAH4 in sterile acid mine water (pH 1.5) and elemental sulfur spent medium of *Acidithiobacillus ferrooxidans* DK6.1 pure culture (pH 2.0)

GAH1, GAH4, GAH8, GMX5, GMX6, DK1AH1, and DK2AH2 strains, were able to grow in sterile acid mine water (pH 1.5) (Fig. 3.3) and elemental sulfur spent medium

of *Acidithiobacillus ferrooxidans* DK6.1 pure culture (pH 2.0) (Fig. 3.4), without supplementation of any other carbon or nitrogen source or growth factors. DOC concentrations of the AMD water samples and elemental sulfur spent medium were 17-22 and 65- 71 mg l^{-1} respectively.

Elemental sulfur spent medium supported much better growth for four strains GAH1, GAH4, GAH8, and GMX5 compared to growth in sterile acid mine water. Increases of approximately 12 times, 8 times, 5 times, and 10 times the initial cell number were noted in a span of two days in GAH1, GAH4, GAH8, and GMX5 respectively. On the other hand, in sterile acid mine water increases of approximately 3.5 times, 1.5 times, 1.4 times, and 0 times the initial cell number were noted in case of GAH1, GAH4, GAH8, and GMX5 respectively.

On the contrary, three strains DK1AH1, DK2AH2, and GMX6 showed better growth and survivability in sterile acid mine water. . Increases of approximately 8 times, for DK1AH1 and DK2AH2; and twice for GMX6 the initial cell number were scored in a span of two days in acid mine water. In elemental sulfur spent medium the increment was restricted to 5 times for DK1AH1 and DK2AH2; and 1 times for GMX6. The ability of the seven strains to survive (without reduction in viable cells since inoculation) and grow in a low nutrient media up to five days of incubation explains the oligotrophic nature of the strains.

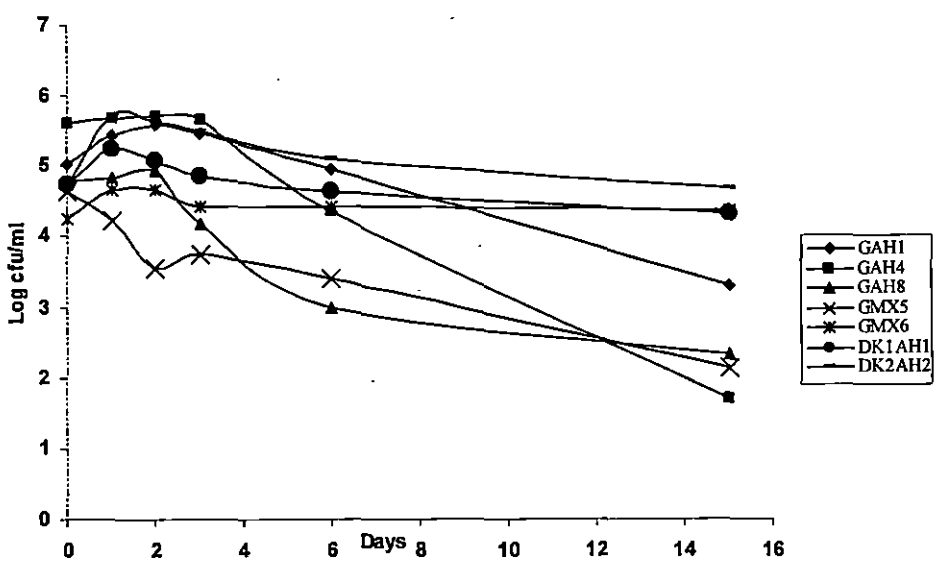

Fig. 3.3: Viability and growth of acid-tolerant heterotrophic isolates, GAH1, GAH4, GAH8, GMX5, GMX6, DK1AH1, and DK2AH2 in sterile acid mine water (pH-1.5).

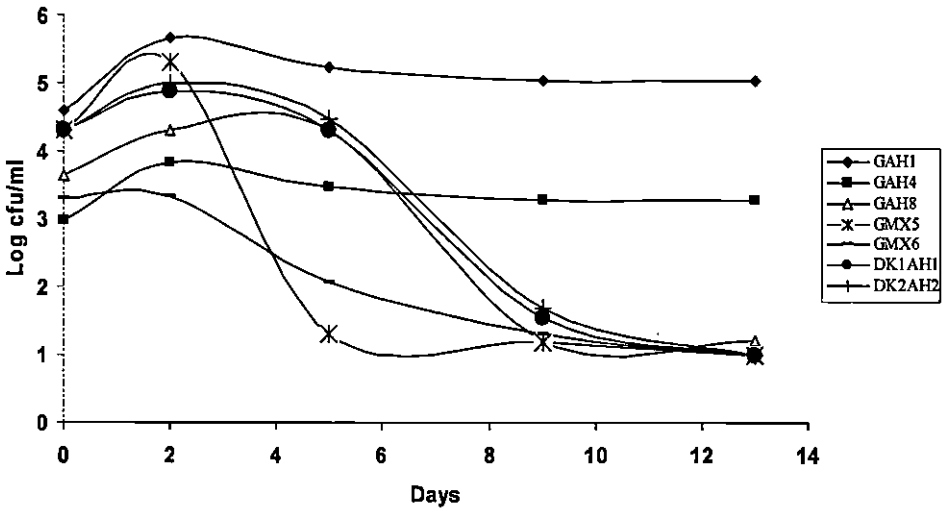

Fig. 3.4: Viability and growth of acid-tolerant heterotrophic isolates, GAH1, GAH4, GAH8, GMX5, GMX6, DK1AH1, and DK2AH2 in elemental sulfur spent medium of *A. ferrooxidans* DK6.1 pure culture (pH- 2.0)

3.3.3 Metal tolerance in acid-tolerant heterotrophic isolates

A wide diversity of the AMD isolates was noted in terms of metal tolerance. At least nine different groups could be made on the basis of maximum concentration of a particular metal that an isolate could withstand metabolically along with the resistance profile against the number of different metal salts used (Table 3.2). GAH2 and GAH5 representing group III could tolerate Co (II), Ni (II), and Zn (II) to the extent of 35, 30, and 40 mM respectively. The maximum tolerance of 100 mM Zn (II) was shown by GAH4. DK1AH1 and DK2AH2 could tolerate Ni (II) to the level of 90 and 450 mM respectively.

Table.3.2: Upper level concentrations of metals,where metabolic activity has been recorded

Metal concentration where by metabolic activity occurs (mM)

Name of the group	Name of the isolates	Metal resistance profile of acid-tolerant heterotrophs							
		Cr(II)	Co(II)	Ni(II)	Cu(II)	Zn(II)	As(III)	Cd(II)	Hg(II)
Group I	GMX2	0.5	2	7	3	7	9	1	0.1
	GMX4	0.5	1	7	3	7	7	1	0.1
Group II	GMX5	1.5	2	3	2	1	8	<0.01	<0.01
	GMX8	1	2	3	0.5	0.1	9	<0.01	<0.01
Group III	GAH2	<0.01	35	30	0.05	40	<0.01	<0.01	<0.01
	GAH5	<0.01	35	30	0.05	40	<0.01	<0.01	<0.01
Group IV	GAH3	<0.01	0.5	0.5	0.5	5	<0.01	<0.01	<0.01
	GAH8	<0.01	0.5	0.5	0.5	5	<0.01	<0.01	<0.01
	GAH9	<0.01	0.5	0.5	0.5	5	<0.01	<0.01	<0.01
	GAH10	<0.01	3	0.5	0.5	5	<0.01	<0.01	<0.01
Group V	GMX1	1.5	2	6	5	10	10	0.5	<0.01
Group VI	GMX6	1.5	2	3	2	1	7	0.5	<0.01
Group VII	GMX7	1.5	2	3	0.5	0.1	1	1	<0.01
Group VIII	GAH4	<0.01	15	10	0.05	100	<0.01	<0.01	<0.01
	GAH44	<0.01	10	10	0.05	90	<0.01	<0.01	<0.01
Group IX	DK1AH1	0.03	5	90	0.3	20	nd	-	<0.01
	DK2AH2	0.05	5	450	0.3	30	1	0.01	<0.01

3.3.4 Determination of the antibiotic tolerance profile of the acid-tolerant heterotrophic isolates

The acid-tolerant heterotrophic isolates displayed different antibiotic resistance profile. The strains GAH1, GAH3, GAH8, GAH9, and GAH10 showed resistance towards all the 12 panel of antibiotics tested. On the other hand four strains GAH4, GAH44, DK1AH1, and DK2AH2 were found to be sensitive towards antibiotics tested. GMX5

and GMX8 showed resistance towards two antibiotic ampicillin and sulfamethoxazole. The remaining isolates resisted more than two antibiotics (Table 3.3).

Table.3.3: Antibiotic resistance profile of acid-tolerant heterotrophic isolates

Antibiotics ued	GMX 1	GMX 2	GMX 4	GMX 5	GMX 6	GMX 7	GMX 8	DK1A H1	DK2A H2
Livofloxacin	-	-	-	-	-	-	-	-	-
Chloramphenicol	+	+	+	-	-	-	-	-	-
Streptomycin	-	+	+	-	-	+	-	-	-
Cefotaxime	+	+	-	-	+	+	-	-	-
Tetracycline	+	+	+	-	-	-	-	-	-
Ampicillin	+	+	+	+	+	-	+	-	-
Netilmicin	-	+	+	-	-	-	-	-	-
Sulfomethoxazole	+	-	-	+	+	+	+	-	-
Ciprofloxacin	-	-	-	-	-	-	-	-	-
Azithromycin	+	-	-	-	-	-	-	-	-
Kanamycin	-	+	-	-	-	-	-	-	-
Cefepime	-	+	-	-	+	+	-	-	-

Antibiotics ued	GAH 1	GAH 2	GAH 3	GAH 4	GAH 5	GAH 8	GAH 9	GAH 10	GAH 44
Livofloxacin	+	-	+	-	-	+	+	+	-
Chloramphenicol	+	+	+	-	+	+	+	+	-
Streptomycin	+	+	+	-	+	+	+	+	-
Cefotaxime	+	-	+	-	-	+	+	+	-
Tetracycline	+	+	+	-	+	+	+	+	-
Ampicillin	+	+	+	-	+	+	+	+	-
Netilmicin	+	+	+	-	-	+	+	+	-
Sulfomethoxazole	+	-	+	-	-	+	+	+	-
Ciprofloxacin	+	-	+	-	+	+	+	+	-
Azithromycin	+	-	+	-	-	+	+	+	-
Kanamycin	+	+	+	-	+	+	+	+	-
Cefepime	+	-	+	-	-	+	+	+	-

3.4 Discussion

Acid mine drainage contains relatively low concentration (10 mg/l) of dissolved organic carbon (Kolmert and Johnson, 2001), and, as such, is called an oligotrophic environment. For the purposes of enumeration, oligotrophic aquatic bacteria have been tentatively defined as bacteria that develop on first cultivation on media with a minimal content of organic matter of either 1 mg or 1 to 15 mg of C per liter, usually supplied as complex mixtures of peptone, Trypticase, and other nutrients. Eutrophic bacteria, on the other hand, have been considered to be organisms able to grow on similar nutrients but at levels supplying 2 or more g of C per liter (Akagi *et al.*, 1977). The two types of media, differing widely in nutrient level, have been used to determine the distribution of oligotrophic and eutrophic bacteria in the sea (Akagi *et al.*, 1977) and in lake water (Ishida *et al.*, 1980). Oligotrophic bacteria were considered to be those organisms which grew at the lower nutrient level but failed to grow when transferred to the higher level. In another study organisms able to grow only at the lower concentration of nutrients were called obligate oligotrophs, whereas those which grew at both low and high concentrations of nutrients were termed facultative oligotrophs (Ishida *et al.*, 1980, 1982). When one detects growth by measuring changes in numbers of viable cells in a liquid medium by using the plate count technique (Van der Kooij *et al.*, 1980; Zoßell and Grant, 1942), the concentrations of nutrients required to produce detectable increases in cell numbers are at least two orders of magnitude smaller than those needed to produce turbidity in a liquid medium or colonies on a solid medium. Thus, using plating techniques, Zoßell and Grant in 1943 were able to show that *Escherichia coli*, *Staphylococcus citreus*, *Bacillus megaterium*, *Proteus vulgaris*, and *Lactobacillus lactis* multiplied in solutions containing 0.1 mg of glucose (supplying 40 µg of C per liter) (Zoßell and Grant, 1942). Lower concentrations were not tested for technical reasons. Even *Pseudomonas aeruginosa* has been shown to grow in tap water at 25 µg of C per liter supplied by any one of a number of compounds (Van der Kooij *et al.*, 1982), and *Aeromonas hydrophila* multiplied when C supplied as glucose was added at 10 µg/liter (Van der Kooij *et al.*, 1980). These organisms are ordinarily not considered to be oligotrophs, yet they more than qualify when the current definition of an oligotroph as an organism which can grow in a medium containing nutrient supplying 1 to 15 mg of C per liter is applied. Oligotrophic bacteria are generally tested

for their ability to grow in R2A medium. Very recently, diluted Luria-Bertani broth has been used to detect oligotrophic bacteria from environmental water samples (Kumar *et al.*, 2010; Oh *et al.*, 2009). Here we have reported that all the sixteen acid-tolerant heterotrophic strains from Garubathan AMD are facultatively oligotrophic capable of growing in R2A and diluted Luria-Bertani as well as in diluted modified DSMZ 269 media (Table 3.1).

There are numerous reports of the isolation of acidophilic heterotrophs from extensibly pure culture of *Acidithiobacillus ferrooxidans* (Guay and Silver 1975, Harrison *et al.*, 1980) and they are the part of the consortium present in the sulfide mineral occurrence sites (Harrison, 1981, Berthelot *et al.*, 1997). Although these environments are very poor in organic materials, the heterotrophs probably survive by scavenging on the low concentration of organic compounds excreted by *A. ferrooxidans* and other autotrophic chemolithotrophs. Heterotrophic acidophiles are able to utilize organic materials produced by acidophilic autotrophs. The culture filtrate from the autotroph *A. ferrooxidans* contained sufficient organic matter to support heterotrophic growth of *Sulfobacillus thermosulfidooxidans* TH1 (Norris and Kelly, 1980) and *Acidiphilium* sp. DKAP1.1 (Gurung and Chakraborty, 2009). Of the seven strains reported in this study, two strains, which tolerated pH to the extreme of 1.0, GAH1 and GAH4, were able to grow in sterile acid mine water (pH 1.0-1.5) and elemental sulfur spent medium of *A. ferrooxidans* (pH 1.5-2.0) while the others (lesser acid-tolerant; Table 1.1 of chapter 1) have shown growth in the said media only when pH was increased to 3.0 - 4.0. DOC measurements of AMD samples from Garubathan (17-22 mg l^{-1}) and elemental sulfur spent medium of *A. ferrooxidans* (65-71 mg l^{-1}) have confirmed very low carbon content which supported the oligotrophic growth of the acid-tolerant strains. This shows that these acid-tolerant heterotrophs have adapted to a low nutrient condition that usually prevails in the mineral rich environments. The ability to grow in low nutrient condition, on the other hand is highly beneficial to the autotrophic partner *A. ferrooxidans.*

Microorganisms surviving in AMD environment meet substantial selective pressure to develop resistance mechanism to metal ions, supporting them with a competitive selective advantage. As a result, in shaping the characteristics of microbial communities in acidic environments in terms of both structure and function, the efficacy of diverse

heavy metal resistance mechanisms would play a significant role (Dopson *et al.*, 2003). Acidophilic heterotrophic bacteria representing *Acidiphilium* and *Acidocella* genera were found to resist high levels of Cd, Zn, Ni, and Cu (Ghosh *et al.*, 1997; Mahapatra and Banerjee, 1996). Distinct patterns of heavy metal resistance in isolates from coal mining environments of Brazil were evidenced, being the Zn and Ni resistance the most widespread (Castro-Silva *et al.*, 2003). The AMD sites selected in this study is restricted to an area of 0.247 sq.km where zinc ore has been found to occur (Shah *et al.*, 1974-75). Not surprisingly, the strains isolated from Garubathan had been able to resist the heavy metal ions. Acid-tolerant heterotrophic isolates from AMD samples could be classified into nine groups on the basis of distinct patterns of metal resistance (Table 3.2). Isolates of Group III, strains GAH2 and GAH5, tolerated Co(II), Ni(II), and Zn(II) as high as 35, 30, and 40 mM respectively. Similarly, isolates of Group IX, strains DK1AH1 and DK2AH2 tolerated Ni(II) and Zn(II) at its most 90, 20; and 450, 30 mM. The maximum tolerance of 100 mM Zn (II) was shown by GAH4. They could tolerate cobalt, nickel, copper, zinc, and arsenite concentration up to the level of 35 mM, 450 mM, 5 mM, 100 mM, and 10 mM respectively. However, maximum tolerable concentration of chromium, cadmium, and mercury was found to be only 1.5 mM, 1 mM, and 0.1 mM respectively. The strains have shown multiple metal tolerance as well as higher metal tolerance ability due to the selective pressure as the site from where these strains were obtained was rich in mineral occurrences.

For further characterization, it was important to test the sensitivity of the acid-tolerant isolates towards several antibiotics. The antibiotics tested were selected to represent 7 different classes: Aminoglycosides, Antifolates, Cephalosporins, Penicillin, Quinolones, and Others. The detailed antibiotic tolerance profile of the acid-tolerant isolates were given in Table 3.3. Four (GAH4, GAH44, DK1AH1, and DK2AH2) out of eighteen strains were found to be sensitive towards antibiotics tested. However five (GAH1, GAH3, GAH8, GAH9, and GAH10) of them were resistant to all the 12 panel of antibiotics tested. The rest of the strains showed resistance towards two or more than two antibiotics. The resistance to a particular heavy metal has been correlated to antibiotics and other heavy metal resistance in a variety of organisms (Austin and Colwell, 1977; Luli *et al.*, 1983; Sabry *et al.*, 1997) and the role of plasmids in confering resistance to both antibiotics and metals has been previously demonstrated

(Foster, 1983; Lyon and Skurray, 1987). Now the plasmid characterization of the acid-tolerant isolates become the next findings.

Isolation and identification of acid-tolerant heterotrophs from AMD samples from Garubathan, India, revealed diversity of the strains in terms of growth characteristics, overall carbohydrate metabolism, thiosulfate chemolithoautotrophy, metal tolerance, and antimicrobial resistance. These strains now require the phylogenetic affiliation which is discussed in the next chapter.

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CHAPTER 4

16S rRNA gene and *sox*B gene phylogeny of the acid-tolerant heterotrophic as well as thiosulfate metabolizing isolates

4.1 Introduction

Both numerical taxonomy and growth characteristics of the acid-tolerant heterotrophic isolates have produced results identifying the potentiality in them to enrich the field of microbial as well as genetic diversity. The remarkable diversity exhibited by them, in terms of oligotrophy, conventional biochemical tests, facultative sulfur chemolithoautotrophy, metal tolerance, and antimicrobial resistance phenotype, compelled an active scientific urge to ascertain their proper systematic positions.

4.1.1 Phylogenetic analyses using 16S rRNA gene sequences

4.1.1.1 Molecular Phylogeny:

Although the morphologies and physiologies of prokaryotes are much simpler than those of eukaryotes, there is a large amount of information in the molecular sequences of their DNA, RNAs and proteins. Thus, it is possible to use molecular similarities to infer the relationships of genes, and, by extension, to learn the relationships of the organisms themselves.

4.1.1.2.DNA-DNA Hybridization:

One important technique for comparing prokaryotes at the molecular level is DNA-DNA hybridization. In this test, the genomic DNA from one species is mixed with the DNA from a second species and the similarity of the DNAs is reflected in the extent to which strands of DNA from one organism anneal with strands of DNA from the other organism. The sensitivity of DNA-DNA hybridization declines rapidly as the organisms become more diverged, limiting the method to characterization of closely related strains, species and genera. In addition, testing the relationships of a new organism can require many hybridizations. If no close relative is found by this test, we have only learned what the new organism is not - whereas we wish to know what it is.

4.1.1.3 Phylogenies Based on Specific Genes:

DNA-DNA hybridization gives a measure of relatedness across the whole genome. As mentioned above, the average similarity falls off rapidly when looking at more diverged species. In principle, the range accessible to molecular analysis could be increased by looking at specific genes with above average conservation. There are several important requirements if we wish to use a gene phylogeny to infer organismal relationships: (i) The gene must be present in all organisms of interest. Thus, to infer relationships that span the diversity of prokaryotes (or life), we must look at the central (universal) cellular functions. Examples include genes whose products function in replication, transcription, or translation - the processes constituting the "Central Dogma" of molecular biology; (ii) The gene cannot be subject to transfer between species (lateral transfer). Since we wish to infer organismal relationships, if a gene is transferred, then the gene history is not the same as the organismal history. If a gene performs a central function, an organism is unlikely to acquire a copy by lateral transfer, since the organism must already have a functional copy to be alive; (iii) The gene must display an appropriate level of sequence conservation for the divergences of interest. If there is too much change, then the sequences become randomized, and there is a limit to the depth of the divergences that can be accurately inferred. If there is too little change (if the gene is too conserved), then there may be little or no change between the evolutionary branchings of interest, and it will not be possible to infer close (genus or species level) relationships; (iv) The gene must be sufficiently large to contain a record of the historical information. Thus, although transfer RNA (tRNA) genes are present in all species, they are too small (about 75 nucleotides) to provide an accurate sample of evolutionary history.

4.1.1.4 Ribosomal RNA Genes and Their Sequences:

To infer relationships that span the diversity of known life, it is necessary to look at genes conserved through the billions of years of evolutionary divergence. An example of genes in this category are those that define the ribosomal RNAs (rRNAs). Most prokaryotes have three rRNAs, called the 5S, 16S and 23S rRNA.

4.1.2 Ribosomal RNAs in Prokaryotes

Name[a]	***Size*** (nucleotides)	***Location***
5S	120	Large subunit of ribosome
16S	1500	Small subunit of ribosome
23S	2900	Large subunit of ribosome

a *The name is based on sedimentation coefficient*

The 5S has been extensively studied, but it is usually too small for reliable phylogenetic inference. The 16S and 23S rRNAs are sufficiently large to be quite useful.l8

The extraordinary conservation of rRNA genes can be seen in these fragments of the small subunit (16S) rRNA gene sequences from organisms spanning the known diversity of life:

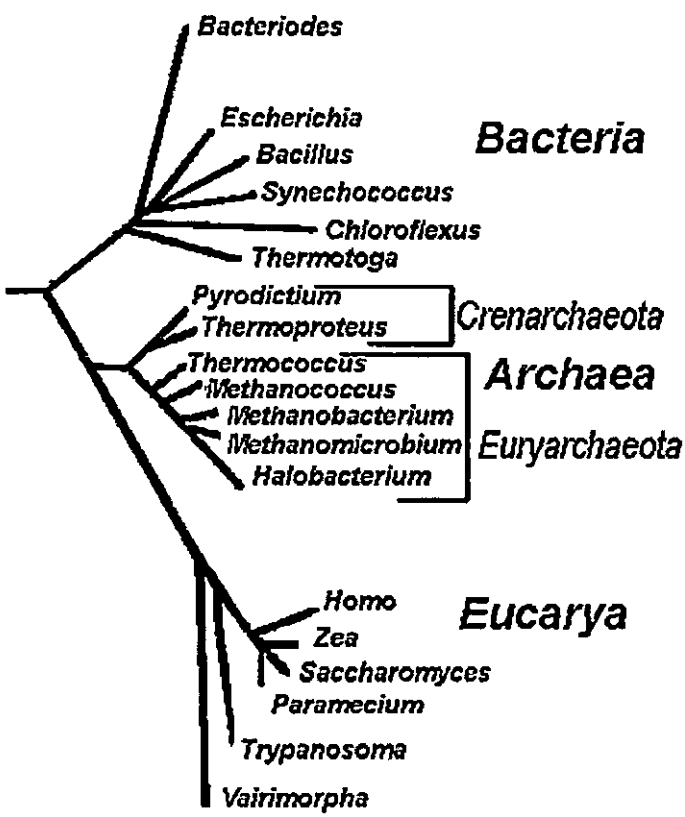
human	...GTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGCTG CAGTTAAAAAG...
yeast	...GTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTG CAGTTAAAAAG...
corn	...GTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTTAAGTTGTTG CAGTTAAAAAG...
Escherichia coli	...GTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTG GGCGTAAAGCG...
Anacystis nidulans	...GTGCCAGCAGCCGCGGTAATACGGGAGAGGCAAGCGTTATCCGGAATTATTG GGCGTAAAGCG...
Thermotoga maratima	...GTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTACCCGGATTTACTG GGCGTAAAGGG...
Methanococcus vannielii	...GTGCCAGCAGCCGCGGTAATACCGACGGCCCGAGTGGTAGCCACTCTTATTG GGCCTAAAGCG...
Thermococcus celer	...GTGGCAGCCGCCGCGGTAATACCGGCGGCCCGAGTGGTGGCCGCTATTATTG GGCCTAAAGCG...
Sulfolobus sulfotaricus	...GTGTCAGCCGCCGCGGTAATACCAGCTCCGCGAGTGGTCGGGGTGATTACTG GGCCTAAAGCG...

Carl Woese recognized the full potential of rRNA sequences as a measure of phylogenetic relatedness. He initially used an RNA sequencing method that determined about 1/4 of the nucleotides in the 16S rRNA (the best technology available at the

time). This amount of data greatly exceeded anything else then available. Using newer methods, it is now routine to determine the sequence of the entire 16S rRNA molecule. Today, the accumulated 16S rRNA sequences (about 10,000) constitute the largest body of data available for inferring relationships among organisms.

4.1.3 Molecular Phylogenies can Reflect Genealogy and Amount of Change

By comparing the inferred rRNA sequences (or those of any other appropriate molecule) it is possible to estimate the historical branching order of the species, and also the total amount of sequence change. An example of a 16S rRNA-based phylogenetic tree showing the three (identified) Domains of life : Bacteria, Archaea and Eucarya is given below. In this tree, lineages diverge from a common ancestral lineage on the far left. The lengths of the individual lines reflect the amount of sequence change (note that some lineages have modified the gene sequence substantially more than others, and thus have accumulated longer total branch lengths).



Besides functional constancy, ubiquitous distribution and size (1.5 kb), genes encoding for 16S rRNA exhibited both evolutionary conserved locus as well as highly variable regions. For these reasons, comparison of 16S rRNA gene sequences of the organisms could be used to calculate evolutionary distances between organisms (Woese *et al.*, 1990). The 16S rRNA gene of bacteria can be amplified and sequenced by using specific primers, designed from conserved regions. To identify the systematic position of an isolate, the 16S rRNA gene was sequenced and the sequences were used to generate phylogenetic trees with nearest relatives.

4.1.4 *sox*B gene phylogeny of sulfur chemolithotrophs

Sulfur compounds are metabolized by an operon called *sox* operon. It codes for a number of proteins. A considerable progress in the genetics of sulfur lithotrophy is noted but the structural details of the proteins are not available. Corresponding to the first mechanism of thiosulfate oxidation a sulfur oxidation (*sox*) operon, *soxTRS-VW-XYZABCDEFGH*, has been characterized from photo- as well as chemo-, lithotrophic alphaproteobacteria (Friedrich *et al.*, 2000; Mukhopadhayaya *et al.*, 2000; Appia-Ayme *et al.*, 2001; Kappler *et al.*, 2001) and shown to encode all the essential components of the Sox multienzyme system, viz., the thiosulfate-induced periplasmic proteins SoxXA, SoxYZ, SoxB and $Sox(CD)_2$ that are all catalytically inactive by themselves, but together govern the oxidation of thiosulfate, sulfite, sulfide and elemental sulfur directly to sulfate. In quite a few alphaproteobacteria this operon has been shown to govern the oxidation of tetrathionate, in addition to thiosulfate, as a chemolithotrophic substrate (Mukhopadhayaya *et al.*, 2000; Lahiri *et al.*, 2006).

So far as the oxidation of thiosulfate is concerned, in a nutshell, the Sox complex covalently binds thiosulfate via cysteinyl residue and oxidises both the sulfur atoms of thiosulfate to sulfate by transferring electrons to c-type cytochromes without the formation of any free intermediate. To start with, the SoxXA heterodimer oxidatively couples the sulfane sulfur of thiosulfate to a SoxY-cysteine-sulfhydryl group of the SoxYZ complex from which the terminal sulfone group is released by the activity of the SoxB component. Subsequently, the sulfane sulfur of the residual SoxY-cysteine persulfide is further oxidized to cysteine-S-sulfate by $Sox(CD)_2$ from which the

sulfonate moiety is again hydrolysed by SoxB regenerating SoxYZ in the process (Wodara *et al.*, 1994; Kelly *et al.*, 1997; Friedrich *et al.*, 2001: Bamford *et al.*, 2002).

A truncated Sox system (SoxXAYZB) deficient in SoxCD has lately been found responsible for the oxidation of the sulfone sulfur of thiosulfate in sulfur-storing photo-, or chemo-, lithotrophs that follow the Branched Thiosulfate Oxidation Pathway (Hensen *et al.*, 2006). All or some of the genes essential to assemble a fully functional Sox complex having lately been reported from the genomes of quite a few obligate, as well as facultative, sulfur-chemolithotrophs that generate polythionate intermediates during the oxidation of thiosulfate or sulfur; e.g., *sox* genes dispersed in the genome as discrete *soxXYZA*, *soxB*, and *soxCD* clusters have been identified in the gammaproteobacterium *Thiomicrospira crunogena* (Scott *et al.*, 2006), which oxidizes thiosulfate by depositing sulfur globules outside the cell under low pH (Javor *et al.*, 1990); *soxB* homologues have been detected by PCR amplification from species like *Thiobacillus thioparus, T. aquaesulis, T. plumbophilus* and *T. prosperus* (Meyer and Kuever, 2007), which derive energy from the oxidation of sulfides, sulfur, thiosulfate, polythionates or thiocyanate with sulfate as the general end product and occasional transient accumulation of sulfur, sulfite or polythionates. These findings apparently point towards the involvement of the Sox complex, or its components, in the seemingly distinct S_4I process.

Earlier authors have developed a PCR protocol for the detection of sulfur oxidation bacteria based on *soxB* gene that is essential for thiosulfate oxidation by S-oxidising bacteria of various phylogenetic groups which use the '*Paracoccus* S-oxidation pathway (Kelly *et al.*, 1997; Petri *et al.*, 2001). The primary structure of the SoxB is about 30% identical to zinc-containing 5'-nucleotidases; however, besides its essential enzymatic activity as sulfate thioesterase component in the Sox enzyme system, no other *in vivo* function has been reported for this protein (Epel *et al.*, 2005). A variety of S-oxidising bacteria have shown amplification with degenerate soxB primers (Anandham *et al.*, 2008; Dam *et al.*, 2007; Meyer *et al.*, 2007; Pandey et.al., 2009; Petri *et al.*, 2001).

4.2 Materials and methods

4.2.1 Extraction of total genomic DNA

Total cellular DNA preparation was made following modification of Marmur's procedure (Marmur, 1961) as described by Yates and Holmes, 1987. Cells were grown in modified DSMZ 269 medium at 30 °C up to late log phase. Cells were harvested from 200 ml of the culture by centrifuging at 10,000 rpm for 10 min at 4 °C. The cell pellet was washed with distilled water and re-suspended in 2 ml of 0.15 M NaCl-0.1 M EDTA (pH 8.0) and the concentrated solution was frozen at -20 °C for at least 4 hrs. The frozen cells were rapidly thawed at 55 °C and lysozyme (1.5 mg/ml) was added. The mixture was then incubated at 37 °C for 15 min followed by the addition of SDS to the final concentration of 1% (w/v). The mixture was incubated at 55 °C for 1 hr. Proteinase K was then added (5 mg/ml) and the incubation was continued at 55 °C until the solution became clear. The cell lysate was extracted with phenol, phenol:chloroform and then with chloroform at 4 °C. Finally the aqueous phase was collected in a beaker placed in ice. 1/10 volume of 3 M Na-acetate and double volume of chilled ethanol was then added. The DNA was spooled with a sterile bent glass rod. The DNA obtained was air dried and suspended in 0.1X TE buffer (10:1). For quantification of DNA a UV-vis spectrophotometer (Thermospectronic) was used, and absorbance at 260 and 280 nm was measured.

4.2.2 PCR amplification of DNA

PCR amplification was performed using 'PCR Amplification Kit' (GENEI, India), in 50 µl reaction volume, following instructions provided by the supplier. Each 50 µl PCR mix contained; 3 µl of 10mM dNTP mix, 5 µl of 10X buffer containing 15 mM $MgCl_2$, 12.5 pmol of each forward and reverse primer, 10 ng of template DNA and 1U *Taq* DNA Polymerase. Taq DNA polymerase was added after adding all the ingredients of the mixture. The PCR was done in a GenAmp PCR system (Applied Biosystems).

Purity of DNA was tested with the help of *UV* spectrophotometer by determining the O.D. values at 260 and 280 nm (ratio of the two readings for pure DNA was considered

to be 2.0), while concentration was determined by using the standard that the concentration of DNA will be 10 times the O.D. reading at 260 nm.

4.2.2.1 Primers used

A. for 16S rRNA gene

To amplify the 16S rDNA a set of eubacterial universal primers 27f and 1492r (Brosius *et al.*, 1978) were used. Sequence of the primers used were:

27f: 5′-AGAGTTTGATCCTGGCTCAG3′
1492r: 5′-TACGGTTACCTTGTTACGACTT3′

The program was set to amplify 16S rDNA was: 5 min of initial denaturation at 94 °C, 30 sec of denaturation at 94 °C, 30 sec for annealing at 58 °C, and 1.5 min at 72 °C for extension, for a total of 25 cycles.

B. For *soxB* gene

To amplify the *soxB* gene four sets of degenerate primers soxB 432F, soxB 693F, soxB 1446B, and soxB 1164B (Petri *et al.*, 2001) were used. Sequence of the primers used were:

soxB432F: 5′-GAYGGNGGNGAYACNTGG-3′
soxB693F: 5′-ATCGGNCARGCNTTYCCNTA-3′
soxB1446B: 5′-CATGTCNCCNCCRTGYTG-3′
soxB1164B: 5′-AARTTNCCNCGNCGRTA-3′

PCR amplifications were performed as a two-step PCR program: After an initial denaturation step of 2 min at 94 °C , 10 cycles with an annealing temperature of 55 °C consisting of 30 sec elongation at 72 °C, 40 sec annealing and 30 sec denaturing at 94 °C were performed. Then, additional 25 cycles were performed with an annealing temperature of 47 °C.

4.2.3 Agarose gel electrophoresis

0.8-1 % agarose in 1X TAE buffer supplemented with 1 μgml^{-1} ethidium bromide was melted in a microwave or boiling water bath and then cooled to 50-60 °C before pouring in a casting tray fitted with a Teflon comb forming wells. DNA loading buffer (1X) was mixed to the sample DNA prior to loading in the wells. Electrophoresis was performed in horizontal electrophoresis tank using 1X TAE buffer containing 1 μgml^{-1} ethidium bromide for 1-2 hours. DNA could be directly viewed in a UV-transilluminator.

4.2.4 Cloning of PCR products

4.2.4.1 Chemicals and reagents

All the chemicals used in the experiment were purchased from SRL Fine Chemicals (SRL India Ltd.). De-ionized double distilled water was used for the preparation of reagents. The reagents were either filter-sterilized or autoclaved, wherever required, before use and preserved in glass containers.

4.2.4.2 Ligation of PCR product to the vector

The purified PCR products were cloned in pGEM®T-easy vector system II following company's protocol (Promega, USA). The vector (25 ng) and the insert DNA were taken in the molar ratio of 1:2 and were suspended in 4 µl sterile double distilled water. 5 µl of 2X rapid ligation buffer and 1 µl of T4 DNA ligase were added and thoroughly mixed by vortexing and centrifugation. The mixture was kept at 4 °C for at least 16 hr, heated at 60 °C for 10 min and 5-7 µl was used to transform competent *E. coli* cells.

4.2.5 Transformation of recombinant plasmids

4.2.5.1 Preparation of fresh competent *E. coli* cells using Calcium Chloride

A single colony of *E. coli* XL1 blue from an agar plate freshly grown for 16-20 hours at 37 °C was taken and transferred into 100 ml of LB broth in a 1 L flask. The culture was incubated for approximately 3 hours or more at 37 °C with vigorous shaking to attain the viable cell number of 10^8 cells/ml. The cells were transferred aseptically to a sterile, ice-cold 50 ml polypropylene tube. The culture was cooled to 0 °C by storing the tube

on ice for 10 min. The cells were then recovered by centrifugation at 5000 rpm for 10 min at 4 °C. The medium was decanted from the cell pellet. The pellet was re-suspended in 10 ml of ice cold 0.1 M $CaCl_2$ and stored on ice for 30 min. The cells were recovered by centrifugation at 4000 rpm for 10 min at 4 °C. The fluid was decanted from the cell pellet, and the tube was placed in an inverted position for 1 min to drain all the traces of fluid. The pellet was re-suspended in 2 ml of ice cold 0.1 M $CaCl_2$ for each 50 ml of original culture.

4.2.5.2 Transformation

Using a chilled, sterile pipette tip, 200 µl of each suspension of the competent cells were transferred to a sterile microfuge tube. DNA (not more than 50 ng in a volume of 10 µl) was added to each tube. Gentle swirling of the microfuge tubes was done to mix the contents. The tubes were stored on ice for 30 min. The tubes were then transferred to a rack placed on a circulating water bath (42 °C) for exactly 90 sec followed by immediate transferring to an ice bath for chilling for 1-2 min. 800 µl of Luria-Bertani (LB) medium was added to each tube and incubated for 45 min in a water bath set at 37°C to allow the bacteria to recover and to express antibiotic resistance marker encoded for the plasmid. Appropriate volume (upto 200 µl per 9 cm petriplates containing 15 ml medium each) of competent cells were transferred onto LB agar plate containing appropriate antibiotic. A sterile bent glass rod was used to spread the transformed cells over the surface of the agar plate. The plates were left at room temperature until the liquid had been absorbed. Finally plates were inverted and incubated at 37 °C for 12-16 hours for the appearance of colonies.

4.2.5.3 Identification of bacterial colonies that contain recombinant plasmids by α-complementation method

To sterile Luria–Bartani agar plate containing 50 $\mu g ml^{-1}$ ampicillin, 0.5 mM isopropylthiogalactoside (IPTG) and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) (80 $\mu g \mu l^{-1}$) transformed competent cells were plated as described earlier. It was possible to recognize colonies that carried putative recombinant plasmids by blue-white screening of the colonies. Insertion of foreign DNA into the polycloning site of plasmid vectors leads to the incapability of complementation. Bacteria carrying recombinant colonies therefore produce white colonies. Re-circularized vectors

containing bacteria could utilize chromogenic substrate X-Gal and thus form blue colonies. Recombinant-plasmids were isolated and screened by alkaline lysis method (Brinboim and Doly, 1979). Purified clones were checked by *Eco*RI restriction digestion followed by agarose gel electrophoresis.

4.2.6 DNA sequencing

The recombinant plasmids were directly used for sequencing of the inserts using primers for T7 and SP6 promoters. Nucleotide sequencing was performed with the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kits (Perkin-Elmer) using specific primer and the reaction was analyzed in an 'AB1 PRISM 377 DNA Sequencer'.

4.2.7 Computer analysis of the sequences

4.2.7.1 Similarity search

For similarity search studies of nucleic acid and amino acid sequences the BLAST N and BLAST P programs were used (Altschul *et al.*, 1997) from the website http://www.ncbi.nlm.nih.

4.2.7.2 Restriction analysis

For the determination of restriction map of the sequence, the software package NEB CUTTER v. 2.0 of New England Biolabs Inc. was used from the website http://tools.neb.com/NEBcutter2.

4.2.7.3 Determination of open reading frame

The possible open reading frame was obtained from the sequence by using the NCBI tool for ORF finder (http://www.ncbi.nlm.nih.gov/projects/gorf/).

4.2.7.4 Conserve domain search (CDD)

The Domain analysis of the amino acid sequence of the inserts was done using the program of Marchler-Bauer and Bryant, 2004 from the website http://www.ncbi.nlm.nih.

4.2.8 Phylogenetic analysis of the acid-tolerant heterotrophic strains

The 16S rDNA sequences showing high similarities were retrieved from the GenBank database and were aligned with 16S rRNA gene sequences of the isolates by using CLUSTAL W software (Thompson *et al.*, 1997) of the Europeon Bioinformatics Institute website (http://www.ebi.ac.uk/clustalw). PHYLIP v 3.6 software package (Felsenstein, 2002) was used for the generation of phylogenetic trees. Distances were calculated using Jukes and Cantor one-parameter and Kimura two-parameter methods. Phylogenetic trees were inferred using the neighbour-joining (Saitou and Nei, 1987), and parsimony (Felsenstein, 1983) analysis methods. Bootstrap analysis was based on 100 resamplings. For neighbour-joining and parsimony analysis PHYLIP 3.6c software packages were used. Reference sequences were retrieved from GenBank under the accession numbers indicated in the trees.

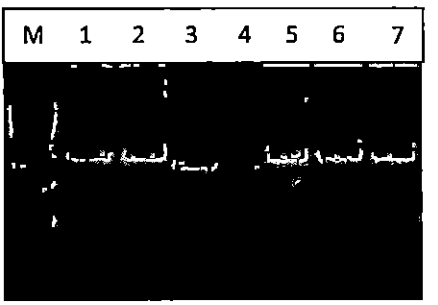
The *soxB* nucleotide sequences were translated into amino acid sequences, and the deduced amino acid sequence data were compared to the available databases by using the BLAST search programs (Atschul *et al.*, 1997). A phylogenetic tree was generated using the neighbour joining and maximum parsimony with an NJ plot and ProPars based on amino acid alignment from CLUSTAL X. Bootstrap analyses with 1000 re-samplings were performed to obtain confidence estimates for the phylogenetic tree topologies on the basis of 20 SoxB sequences.

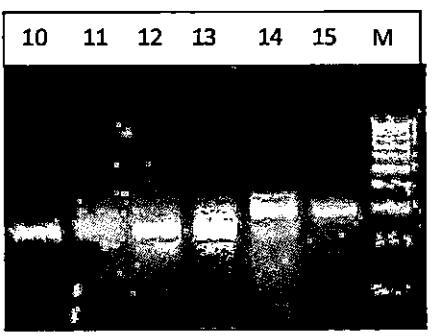
4.3 Results

4.3.1 16S rRNA gene amplification and cloning

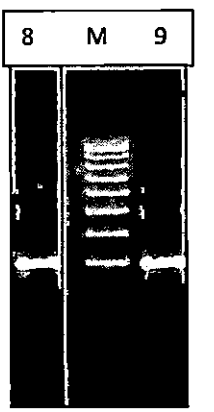
About 1.5 kb 16S rDNA amplicons of the isolates GAH1, GAH2, GAH4, GAH5, GAH8, GAH44, GMX1, GMX2, GMX4, GMX5, GMX6, GMX7, GMX8, DK1AH1, and DK2AH2 were checked in an agarose gel comparing with a molecular size marker (Fig. 4.1). The PCR product was purified and cloned in pGEM T-easy vector (Fig. 4.2) prior to sequencing. The recombinant plasmid containing 1.5 kb PCR product of the acid-tolerant heterotrophic isolates GAH1, GAH2, GAH4, GAH5, GAH8, GAH44, GMX1, GMX2, GMX4, GMX5, GMX6, GMX7, GMX8, DK1AH1, and DK2AH2

were named as pSH1, pSH2, pSH4, pSH5, pSH8, pSH44, pSX1, pSX2, pSX4, pSX5, pSX6, pSX7, pSX8, pSDK1, and pSDK2 respectively.





Lane M: 500bp DNA ladder
Lane 1: 16S of GMX1
Lane 2: 16S of GMX2
Lane 3: 16S of GMX4
Lane 4: 16S of GMX5
Lane 5: 16S of GMX6
Lane 6: 16S of GMX7
Lane 7: 16S of GMX8
Lane 8: 16S of DK1AH1



Lane 9: 16S of DK2AH2
Lane 10: 16S of GAH1
Lane 11: 16S of GAH2
Lane 12: 16S of GAH4
Lane 13: 16S of GAH5
Lane 14: 16S of GAH8
Lane 15: 16S of GAH44

Fig. 4.1: Amplified 16S rDNA of AMD strains

The recombinant plasmids were transformed into the competent cells of *E. coli* XL1 Blue and the clones containing recombinant plasmids were detected using blue-white screening method in X-gal containing plates. White cells were taken and minipreparation of plasmid (methodology is described in chapter 5) was carried out to check the presence of insert in the plasmid. Once the presence of desired insert in the plasmid was confirmed by restriction digestion, maxipreparation of plasmid

(methodology is described in chapter 5) was carried out. And the sequencing of the inserts of the recombinant plasmids was done using SP6 and T7 primers.

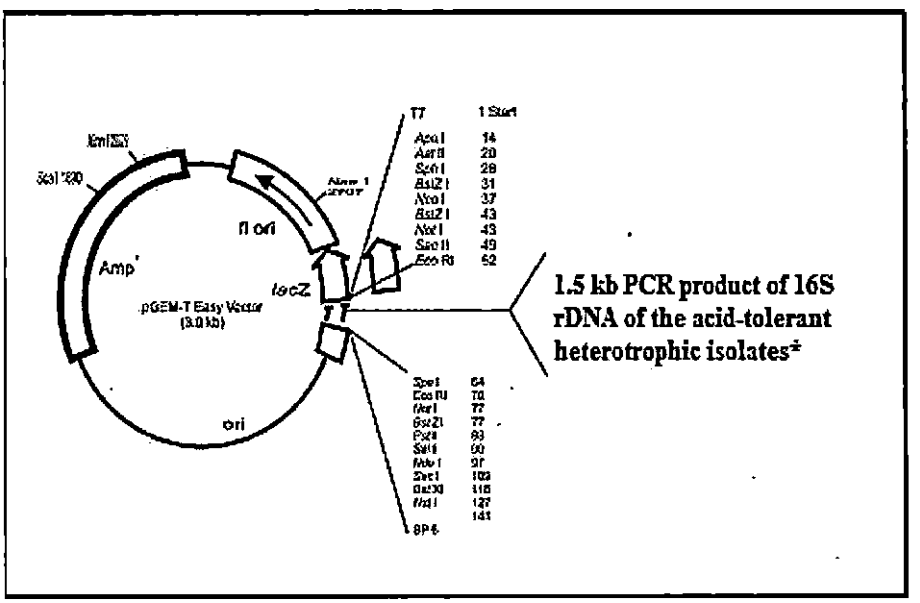


Fig. 4.2: Diagrammatic representation of pGEM-T easy vector containing 1.5 kb PCR product of 16S rDNA of AMD isolates

4.3.2 Nucleotide sequence accession numbers of 16S rRNA gene

The sequences of the 16S rRNA (Fig. 4.3 - 4.15) of GAH1, GAH2, GAH4, GAH5, GAH8, GAH44, GMX1, GMX2, GMX4, GMX5, GMX6, GMX7, GMX8, DK1AH1, and DK2AH2 obtained in this work have been deposited in EMBL, GenBank under the accession numbers AM998532, AM992063, AM992537, AM999539, AM998531, FR686457, FN293172, AM992535, AM992536, AM910818, AM422128, AM422129, AM992062, AM403732, and AM403733 respectively.

```
   1 attgaacgct ggcggcatgc cttacacatg caagtcgaac ggcagcacgg gtgcttgcac
  61 ctggtggcga gtggcgaacg ggtgagtaat acatcggaac atgtcctgta gtgggggata
 121 gcccggcgaa agccggatta ataccgcata cgatctatgg atgaaagcgg gggaccttcg
 181 ggcctcgcgc tatagggttg gccgatggct gattagctag ttggtggggt aaaggcctac
 241 caaggcgacg atcagtagct ggtctgagag gacgaccagc cacactggga ctgagacacg
 301 gcccagactc ctacgggagg cagcagtggg gaattttgga caatgggcga aagcctgatc
 361 cagcaatgcc gcgtgtgtga agaaggcctt cgggttgtaa agcacttttg tccggaaaga
 421 aatccttggt tctaatatag ccgggggatg acggtaccgg aagaataagc accggctaac
 481 tacgtgccag cagccgcggt aatacgtagg gtgcgagcgt taatcggaat tactgggcgt
 541 aaagcgtgcg caggcggttt gctaagaccg atgtgaaatc cccgggctca acctgggaac
 601 tgcattggtg actggcaggc tagagtatgg cagaggggg tagaattcca cgtgtagcag
 661 tgaaatgcgt agagatgtgg aggaataccg atggcgaagg cagccccctg ggccaatact
 721 gacgctcatg cacgaaagcg tggggagcaa acaggattag ataccctggt agtccacgcc
 781 ctaaacgatg tcaactagtt tgttgggatt catttcctta gtaacgtagc taacgcgtga
 841 agttgaccgc ctgggagtac ggtcgcaaga ttaaactcaa aggaattgac ggggaccgca
 901 caagcggtgg atgatgtgga ttaattcgat gcaacgcgaa aaaccttacc taccttgac
 961 gtggtcggaa tcctgctgag aggtgggagt gctcgaaaga gaaccgatac acaggtgctg
1021 catggctgtc gtcagctcgt gtcgtgagat gttgggttaa gtcccgcaac gagcgcaacc
1081 cttgtcctta gttgctacgc aagagcactc taaggagact gccggtgaca aaccggagga
1141 aggtggggat gacgtcaagt cctcatggcc cttatgggta gggcttcaca cgtcatacaa
1201 tggtcggaac agagggttgc caacccgcga gggggagcta atctcagaaa accgatcgta
1261 gtccggattg cactctgcaa ctcgagtgca tgaagctgga atcgctagta atcgcggatc
1321 agcatgccgc ggtgaatacg ttcccgggtc ttgtacacac cgcccgtcac accatgggag
1381 tgggttttac cagaagtggc tagtctaacc gcaaggagga cggtcaccac ggtaggattc
1441 atgactgggg tgaagtcgta acaaggtaac cgtaaatcg
```

Fig. 4.3: Partial 16S rRNA gene sequence of the isolate GAH1 (Accession no. AM998532)

```
   1 agagtttgat cctggctcag attgaacgct ggcggcatgc cttacacatg caagtcgaac
  61 ggcagcacgg gtgcttgcac ctggtggcga gtggcgaacg ggtgagtaat acatcggaac
 121 atgtcctgta gtgggggata gcccggcgaa agccggatta ataccgcata cgatctacgg
 181 atgaaagcgg gggaccttcg ggcctcgcgc tatagggttg gccgatggct gattagctag
 241 ttggtggggt aaaggcctac caaggcgacg atcagtagct ggtctgagag gacgaccagc
 301 cacactggga ctgagacacg gcccagactc ctacgggagg cagcagtggg gaattttgga
 361 caatgggcga aagcctgatc cagcaatgcc gcgtgtgtga agaagccttc gggttgtaaa
 421 gcacttttgt ccggaagaaa tccttggttc taatatagcc gggggatgcg gtaccggaag
 481 aataagcacc ggctaactac gtgccagcag ccgcggtaat acgtaggtgc gagcgttaat
 541 cggaattact gggcgtaaag cgtgcgcagc gtttgctaag accgatgtga aatcccggct
 601 cacctggaac cgcattgtga ctgcagctaa gtatgccaag gggtaaattc ccggtaacat
 661 gaatgctaag atgggagaac atggcaagcc tggcaactga ccatgcaact ggacaaagat
 721 tatcctgatt ccccctaaaa gccaattgtg gatttttctt aaaggctacg actaatccgc
 781 tcactggaca ttgttccagt aatgcagggt atcccgtgca gaaacgggga atgggaaatc
 841 gtgggggca ccccttggca attgcgctta tcaggaaggc gtggaccaac cagaaaaatc
 901 ctggattccc ccctaacgag ttcaactagt gttgggatca tttcttagta acgtagctaa
 961 cgcgtgaagt gacccctggg agtacgtcgc aagattaaaa ctcaaaggat tgacgggacc
1021 cgcacagcgg tggatgatgt ggattattcg atgcacgcga aaaccttacc ttacccttga
1081 catggtcgga atcctgctga gaggtgggag tgctcgaaag agaaccgata cacaggtgct
1141 gcatggctgt cgtcagctcg tgtcgtgaga tgttgggtta agtcccgcaa cgagcgcaac
1201 ccttgtctta gttgctacgc aagagcactc taaggagact gccggtgaca aaccggagga
1261 aggtggggat gacgtcaagt cctcatggcc cttatgggta gggcttcaca cgtcatacaa
1321 tggtcggaac agagggttgc caacccgcga gggggagcta atcccagaaa accgatcgta
1381 gtccggattg cactctgcaa ctcgagtgca tgaagctgga atcgctagta atcgcggatc
1441 agcatgccgc ggtgaatacg ttcccgggtc ttgtacacac cgcccgtcac accatgggag
1501 tgggttttac cagaagtggc tagtctaacc gcaaggagga cggtcaccac ggtaggattc
1561 atgactgggg tgaagtcgta acaaggtaac cgtaaatcg
```

Fig. 4.4: Partial 16S rRNA gene sequence of the isolate GAH2 (Accession no. AM992063)

```
   1 agagtttgat cctggctcag attgaacgct ggcggcatgc cttacacatg caagtcgaac
  61 ggcagcacgg gtgcttgcac ctggtggcga gtggcgaacg ggtgagtaat acatcggaac
 121 gtgtcctgta gtgggggata gcccggcgaa agccggatta ataccgcata cgatctacgg
 181 atgaaagcgg gggatcttcg gacctcgcgc tataggggcg gccgatggcg gattagctag
 241 ttggtgaggt aaaggctcac caaggcgacg atccgtagct ggtctgagag gacgaccagc
 301 cacactggga ctgagacacg gcccagactc ctacgggagg cagcagtggg gaattttgga
 361 caatgggcga aagcctgatc agcaatgccg cgtgtgtgaa gaaggccttc gggttgtaaa
 421 gcacttttgt ccggaaagaa atccctggtc ctaatatggc cgggggatga cggtaccgga
 481 agaataagca ccggctaact acgtgccagc agccgcggta atacgtaggg tgcaagcgtt
 541 aatcggaatt actgggcgta aagcgtgcgc aggcggtgat gtaagaccga tgtgaaatcc
 601 ccgggctcaa cctgggaact gcattggtga ctgcatcgct tgagtatggc agaggggggt
 661 agaattccac gtgtagcagt gaaatgccga acttgcctcc cttgattgcg aggggtaatt
 721 cccgtgacag gaatgtggag agtggggaat cgaggggggag gcagccctgg ttcaattgac
 781 gctcttccgg aagttgggag caaacagatt agattccctg tagtcacgcc ttaacgagtt
 841 caactgggtg tcggtctttc atgactggta acgtagctaa acgcgggagt tgacccctgg
 901 gagtacggtc gcaagattaa actcaaagga attgacggga ccgcccaagc ggtggatgat
 961 tggattaatt cgatgcaacg ggaaaacctt acctaccctt gacatgtacg gaatcctgct
1021 gagaggtggg agtgcccgaa ggggagccgt aacacaggtg ctgcatggct gtcgtcagct
1081 cgtgtcgtga gatgttgggt taagtcccgc aacgagcgca acccttgtcc ctagttgcta
1141 cgcaagagca ctccagggag actgccggtg acaaaccgga ggaaggtggg gatgacgtca
1201 agtcctcatg gcccttatgg gtagggcttc acacgtcata caatggtcgg aacagagggt
1261 tgccaagccg cgaggtggag ccaatcccag aaaaccgatc gtagtccgga tcgcagtctg
1321 caactcgact gcgtgaagct ggaatcgcta gtaatcgcgg atcagcatgc cgcggtgaat
1381 acgttcccgg gtcttgtaca caccgcccgt cacaccatgg gagtgggttt tgccagaagt
1441 ggctagtcta accgcaagga ggacggtcac cacggcagga ttcatgactg gggtgaagtc
1501 gtaacaaggt aaccgtaaat cg
```

Fig. 4.5: Partial 16S rRNA gene sequence of the isolate GAH4 (Accession no. AM992537)

```
   1 cgattagagt ttgatcctgg ctcagattga acgctggcgg catgccttac acatgcaagt
  61 cgaacggcag cacgggtgct tgcacctggt ggcgagtggc gaacgggtga gtaatacatc
 121 ggaacatgtc ctgtagtggg ggatagcccg gcgaaagccg gattaatacc gcatacgatc
 181 tttggatgaa agcgggggac cttcgggcct cgcgctatag ggttggccga tggctgatta
 241 gctagttggt ggggtaaagg cctaccaagg cgacgatcag tagctggtct gagaggacga
 301 ccagccacac tgggactgag acacggccca gactcctacg ggaggcagca gtggggaatt
 361 ttggacaatg ggcgaaagcc tgatccagca atgccgcgtg tgtgaagaag gccttcgggt
 421 tgtaaagcac ttttgtccgg aaagaaatcc ttggttctaa tacagccggg ggatgacggt
 481 accggaagaa taagcaccgg ctaactacgt gccagcagcc gcggtaatac gtagggtgcg
 541 agcgttaatc ggaattactg ggcgtaaagc gtgcgcaggc ggtttgctaa gaccgatgtg
 601 aaatccccgg gctcaacctg ggaactgcat tggtgactgg caggctagag tatggcagag
 661 gggggtagaa ttccacgtgt agcagtgaaa tgcgtagaga tgtggaggaa taccgatggc
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 781 attagatacc ctggtagtcc acgccctaaa cgatgtcaac tagttgttgg ggattcattt
 841 ccttagtaac gtagctaacg cgtgaagttg accgcctggg gagtacggtc gcaagattaa
 901 aactcaaagg aatgacgggg acccgcacaa gcggtggatg atgtggatta attcgatgca
 961 acgcgaaaaa ccttacctac ccttgacatg gtcggaatcc tgctgagagg cgggagtgct
1021 cgaaagagaa ccgatacaca ggtgctgcat ggctgtcgtc agctcgtgtc gtgagatgtt
1081 gggttaagtc ccgcaacgag cgcaaccctt gtccttagtt gctacgcaag agcactctaa
1141 ggagactgcc ggtgacaaac cggaggaagg tggggatgac gtcaagtcct catggccctt
1201 atgggtaggg cttcacacgt catacaatgg tcggaacaga gggttgccaa cccgcgaggg
1261 ggagctaatc ccagaaaacc gatcgtagtc cggattgcac tctgcaactc gagtgcatga
1321 agctggaatc gctagtaatc gcggatcagc atgccgcggt gaatacgttc ccgggtcttg
1381 tacacaccgc ccgtcacacc atgggagtgg gttttaccag aagtggctag tctaaccgca
1441 aggaggacgg tcaccacggt aggattcatg actggggtga agtcgtaaca aggtaaccgt
1501 aa
```

Fig. 4.6: Partial 16S rRNA gene sequence of the isolate GAH5 (Accession no. AM999539)

```
   1 cgattagagt ttgatcctgg ctcagattga acgctggcgg catgctttac acatgcaagt
  61 cgaacggtaa caggtcttcg gatgctgacg agtggcgaac gggtgagtaa tacatcggaa
 121 cgtgcctggt agtgggggat aactactcga aagagtagct aataccgcat gagatctacg
 181 gatgaaagca ggggaccttc gggccttgtg ctaccagagc ggctgatggc agattaggta
 241 gttggtgggg taaaggctta ccaagcctgc gatctgtagc tggtctgaga ggacgaccag
 301 ccacactggg actgagacac ggcccagact cctacgggag gcagcagtgg ggaattttgg
 361 acaatgggcg aaagcctgat ccagcaatgc cgcgtgcagg atgaaggccc tcgggttgta
 421 aactgctttt gtacggaacg aaaagcctgg ggccaatacc cccgggtcat gacggtaccg
 481 taagaataag caccggctaa ctacgtgcca gcagcgcggt aatacgtagg gtgcaagcgt
 541 aatcggaata ctgggcgtaa agcgtgcgca gcggtttgta agacagtggt gaaatcccgg
 601 gctcaacctg ggaactgcca tgtggactgc aggctaagtg cggcaaa
```

Fig. 4.7: Partial 16S rRNA gene sequence of the isolate GAH8 (Accession no. AM998531)

```
   1 attgaacgct ggcggcatgc cttacacatg caagtcgaac ggcagcacgg gtgcttgcac
  61 ctggtggcga gtggcgaacg ggtgagtaat acatcggaac gtgtcctgta gtgggggata
 121 gcccggcgaa agccggatta ataccgcata cgatctacgg atgaaagcgg gggatcttcg
 181 gacctcgcgc tatagggggcg gccgatggcg gattagctag ttggtgaggt aaaggctcac
 241 caaggcgacg atccgtagct ggtctgagag gacgaccagc cacactggga ctgagacacg
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 361 cagcaatgcc gcgtgtgtga agaaggcctt cgggttgtaa agcacttttg tccggaaaga
 421 aatccctggt cctaatatgg ccgggggatg acggtaccgg aagaataagc accggctaac
 481 tacgtgccag cagccgcggt aatacgtagg gtgcaagcgt taatcggaat tactgggcgt
 541 aaagcgtgcg caggcggtga tgtaagaccg atgtgaaatc cccgggctca acctgggaac
 601 tgcattggtg actgcatcgc ttgagtatgg cagaggggg tagaattcca cgtgtagcag
 661 tgaaatgcgt agagatgtgg aggaataccg atggcgaagg cagccccctg ggtcaatact
 721 gacgctcatg cacgaaagcg tggggagcaa acaggattag ataccctggt agtccacgcc
 781 ctaaacgatg tcaactggtt gtcgggtctt cattgacttg gtaacgtagc taacgcgtga
 841 agttgaccgc ctggggagta cggtcgcaag attaaaactc aaaggaattg acggggaccc
 901 gcacaagcgg tggatgatgt ggattaattc gatgcaacgc gaaaaacctt acctaccctt
 961 gacatgtacg gaatcctgct gagaggtggg agtgcccgaa agggagccgt aacacaggtg
1021 ctgcatggct gtcgtcagct cgtgtcgtga gatgttgggt taagtcccgc aacgagcgca
1081 acccttgtcc ctagttgcta cgcaagagca ctccagggag actgccggtg acaaaccgga
1141 ggaaggtggg gatgacgtca agtcctcatg gcccttatgg gtagggcttc acacgtcata
1201 caatggtcgg aacagagggt tgccaagccg cgaggtggag ccaatcccag aaaaccgatc
1261 gtagtccgga tcgcagtctg caactcgact gcgtgaagct ggaatcgcta gtaatcgcgg
1321 atcagcatgc cgcggtgaat acgttcccgg gtcttgtaca caccgcccgt cacaccatgg
1381 gagtgggttt tgccagaagt ggctagtcta accgcaagga ggacggtcac cacggcagga
1441 ttcatgactg gggtgaagtc gtaacaaggt agccgtatcg gaagg
```

Fig. 4.8: Partial 16S rRNA gene sequence of the isolate GAH44 (Accession no. FR686457)

```
   1 agagtttgat cctggctcag attgaacgct ggcggcaggc ttaacacatg caagtcgagc
  61 ggtagcacaa gagagcttgc tctctgggtg acgagcggcg gacgggtgag taatgtctgg
 121 gaaactgcct gatggagggg gataactact ggaaacggta gctaataccg cataacgtcg
 181 caagaccaaa gaggggacc ttcgggcctc ttgccatcag atgtgcccag atgggattag
 241 ctagtaggtg gggtaatggc tcacctaggc gacgatccct agctggtctg agaggatgac
 301 cagccacact ggaactgaga cacggtccag actcctacgg gaggcagcag tggggaatat
 361 tgcacaatgg gcgcaagcct gatgcagcca tgccgcgtgt gtgaagaagg ccttcgggtt
 421 gtaaagcact ttcagcgagg aggaaggtgg tgagcttaat acgctcatca attgacgtta
 481 ctcgcagaag aagcaccggc taactccgtg ccagcagccg cggtaatacg gagggtgcaa
 541 gcgttaatcg gaattactgg gcgtaaagcg cacgcaggcg gtttgttaag tcagatgtga
 601 aatccccggg ctcaacctgg gaactgcatt tgaaactggc aagctagagt ctcgtagagg
 661 ggggtagaat tccaggtgta gcggtgaaat gcgtagagat ctggaggaat accggtggcg
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 841 cgtggcttcc ggagctaacg cgttaaatcg accgcctggg gagtacggcc gcaaggttaa
 901 aactcaaatg aattgacggg ggcccgcaca agcggtggag catgtggttt aattcgatgc
 961 aacgcgaaga accttaccta ctcttgacat ccagagaact ttccagagat ggattggtgc
1021 cttcgggaac tctgagacag gtgctgcatg gctgtcgtca gctcgtgttg tgaaatgttg
1081 ggttaagtcc cgcaacgagc gcaaccctta tcctttgttg ccagcggttc ggccgggaac
1141 tcaaaggaga ctgccagtga taaactggag gaaggtgggg atgacgtcaa gtcatcatgg
1201 cccttacgag tagggctaca cacgtgctac aatggcatat acaaagagaa gcgacctcgc
1261 gagagcaagc ggacctcata aagtatgtcg tagtccggat tggagtctgc aactcgactc
1321 catgaagtcg gaatcgctag taatcgtaga tcagaatgct acggtgaata cgttcccggg
1381 ccttgtacac accgcccgtc acaccatggg agtgggttgc aaaagaagta ggtagcttaa
1441 ccttcgggag ggcgcttacc actttgtgat tcatgactgg ggtgaagtcg taacaaggta
1501 accgtagggg aacctgcggc tggatcacct cctt
```

Fig. 4.9: Partial 16S rRNA gene sequence of the isolate GMX1 (Accession no. FN293172)

```
   1 cagattgaac gctggcggca tgccttacac atgcaagtcg aacggcagca cgggtgcttg
  61 cacctggtgg cgagtggcga acgggtgagt aatacatcgg aacatgtcct gtagtggggg
 121 atagcccggc gaaagccgga ttaataccgc atacgatcta cggatgaaag cgggggacct
 181 tcgggcctcg cgctataggg ttggccgatg gctgattagc tagttggtgg ggtaaaggcc
 241 taccaaggcg acgatcagta gctggtctga gaggacgacc agccacactg ggactgagac
 301 acggcccaga ctcctacggg aggcagcagt ggggaatttt ggacaatggg cgaaagcctg
 361 atccagcaat gccgcgtgtg tgaagaaggc cttcgggttg taaagcactt ttgtccggaa
 421 agaaatcctt ggccctaata cggtcggggg atgacggtac cggaagaata agcaccggct
 481 aactacgtgc cagcagccgc ggtaatacgt agggtgcaag cgttaatcgg aattactggg
 541 cgtaaagcgt gcgcaggcgg tttgctaaga ccgatgtgaa atccccgggc tcaacctggg
 601 aactgcattg gtgactggca ggctagagta tggcagaggg gggtagaatt ccacgtgtag
 661 cagtgaaatg cgtagagatg tggaggaata ccgatggcga aggcagcccc ctgggccaat
 721 actgacgctc atgcacgaaa gcgtggggag caaacaggat tagataccct ggtagtccac
 781 gccctaaacg atgtcaacta gttgttgggg attcatttcc ttagtaacgt agctaacgcg
 841 tgaagttgac cgcctgggga gtacggtcgc aagattaaaa ctcaaaggaa ttgacgggga
 901 cccgcacaag cggtggatga tgtggattaa ttcgatgcaa cgcgaaaaac cttacctacc
 961 cttgacatgg tcggaatccc gctgagaggt gggagtgctc gaaagagaac cggcgcacag
1021 gtgctgcatg gctgtcgtca gctcgtgtcg tgagatgttg ggttaagtcc cgcaacgagc
1081 gcaacccttg tccttagttg ctacgcaaga gcactctaag gagactgccg gtgacaaacc
1141 ggaggaaggt ggggatgacg tcaagtcctc atggccctta tgggtagggc ttcacacgtc
1201 atacaatggt cggaacagag ggttgccaac ccgcgagggg gagctaatcc cagaaaaccg
1261 atcgtagtcc ggattgcact ctgcaactcg agtgcatgaa gctggaatcg ctagtaatcg
1321 cggatcagca tgccgcggtg aatacgttcc cgggtcttgt acacaccgcc cgtcacacca
1381 tgggagtggg ttttaccaga agtggctagt ctaaccgcaa ggaggacggt caccacngta
1441 ggattcatga ctgggg
```

Fig. 4.10: Partial 16S rRNA gene sequence of the isolate GMX2 (Accession no. AM992535)

```
   1 aacgctggcg gcatgcctta cacatgcaag tcgaacggca gcacgggtgc ttgcacctgg
  61 tggcgagtgg cgaacgggtg agtaatacat cggaacatgt cctgtagtgg gggatagccc
 121 ggcgaaagcc ggattaatac cgcatacgat ctacggatga aagcggggga ccttcgggcc
 181 tcgcgctata gggttggccg atggctgatt agctagttgg tggggtaaag gcctaccaag
 241 gcgacgatca gtagctggtc tgagaggacg accagccaca ctgggactga gacacggccc
 301 agactcctac gggaggcagc agtggggaat tttggacaat gggcgaaagc ctgatccagc
 361 aatgccgcgt gtgtgaagaa ggccttcggg ttgtaaagca cttttgtccg gaaagaaatc
 421 cttggctcta atacagtcgg gggatgacgg taccggaaga ataagcaccg gctaactacg
 481 tgccagcagc cgcggtaata cgtagggtgc gagcgttaat cggaattact gggcgtaaag
 541 cgtgcgcagg cggtttgcta agaccgatgt gaaatccccg ggctcaacct gggaactgca
 601 ttggtgactg gcaggctaga gtatggcaga ggggggtaga attccacgtg tagcagtgaa
 661 atgcgtagag atgtggagga ataccgatgg cgaaggcagc cccctgggcc aatactgacg
 721 ctcatgcacg aaagcgtggg gagcaaacag gattagatac cctggtagtc cacgccctaa
 781 acgatgtcaa ctagttgttg gggattcatt tccttagtaa cgtagctaac gcgtgaagtt
 841 gaccgcctgg ggagtacggt cgcaagatta aaactcaaag gaattgacgg ggacccgcac
 901 aagcggtgga tgatgtggat taattcgatg caacgcgaaa aaccttacct acccttgaca
 961 tggtcggaat cccgctgaga ggtgggagtg ctcgaaagag aaccggcgca caggtgctgc
1021 atggctgtcg tcagctcgtg tcgtgagatg ttgggttaag tcccgcaacg agcgcaaccc
1081 ttgtccttag ttgctacgca agagcactct aaggagactg ccggtgacaa accggaggaa
1141 ggtggggatg acgtcaagtc ctcatggccc ttatgggtag ggcttcacac gtcatacaat
1201 ggtcggaaca gagggtcgcc aacccgcgag ggggagctaa tcccagaaaa ccgatcgtag
1261 tccggatcgc actctgcaac tcgagtgcgt gaagctggaa tcgctagtaa tcgcggatca
1321 gcatgccgcg gtgaatacgt tcccgggtct tgtacacacc gcccgtcaca ccatgggagt
1381 gggttttacc agaagtggct agtctaaccg caaggaggac ggtcaccacg gtaggattca
1441 tgactggggt gaagtcgtaa caaggtagcc gtatcggaag g
```

Fig. 4.11: Partial 16S rRNA gene sequence of the isolate GMX4 (Accession no. AM992536)

```
   1 atcctggctc aggatgaacg ctggcggcgt gcctaataca tgcaagtcga gcgaactgat
  61 tagaagcttg cttctatgac gttagcggcg gacgggtgag taacacgtgg gcaacctgcc
 121 tgtaagactg ggataacttc gggaaaccga agctaatacc ggataggatc ttctccttca
 181 tgggagatga ttgaaagatg gtttcggcta tcacttacag atgggcccgc ggtgcattag
 241 ctagttggtg aggtaacggc tcaccaaggc aacgatgcat agccgacctg agagggtgat
 301 cggccacact gggactgaga cacggcccag actcctacgg gaggcagcag tagggaatct
 361 tccgcaatgg acgaaagtct gacggagcaa cgccgcgtga gtgatgaagg ctttcgggtc
 421 gtaaaactct gttgttaggg aagaacaagt acaagagtaa ctgcttgtac cttgacggta
 481 cctaaccaga aagccacggc taactacgtg ccagcagccg cggtaatacg taggtggcaa
 541 gcgttatccg gaattattgg gcgtaaagcg cgcgcaggcg gtttcttaag tctgatgtga
 601 aagcccacgg ctcaaccgtg gagggtcatt ggaaactggg gaacttgagt gcagaagaga
 661 aaagcggaat tccacgtgta gcggtgaaat gcgtagagat gtggaggaac accagtggcg
 721 aaggcggctt tttggtctgt aactgacgct gaggcgcgaa agcgtgggga gcaaacagga
 781 ttagataccc tggtagtcca cgccgtaaac gatgagtgct aagtgttaga gggtttccgc
 841 cctttagtgc tgcagctaac gcattaagca ctccgcctgg ggagtacggt cgcaagactg
 901 aaactcaaag gaattgacgg gggcccgcac aagcggtgga gcatgtggtt taattcgaag
 961 caacgcgaag aaccttacca ggtcttgaca tcctctgaca actctagaga tagagcgttc
1021 cccttcgggg gacagagtga caggtggtgc atggttgtcg tcagctcgtg tcgtgagatg
1081 ttgggttaag tcccgcaacg agcgcaaccc ttgatcttag ttgccagcat ttagttgggc
1141 actctaaggt gactgccggt gacaaaccgg aggaaggtgg ggatgacgtc aaatcatcat
1201 gccccttatg acctgggcta cacacgtgct acaatggatg gtacaaaggg ctgcaagacc
1261 gcgaggtcaa gccaatccca taaaaccatt ctcagttcgg attgtaggct gcaactcgcc
1321 tacatgaagc tggaatcgct agtaatcgcg gatcagcatg ccgcggtgaa tacgttcccg
1381 ggccttgtac acaccgcccg tcacaccacg agagtttgta acacccgaag tcggtggagt
1441 aaccgtaagg agctagccgc ctaaggtggg acagatgatt gg
```

Fig. 4.12: Partial 16S rRNA gene sequence of the isolate GMX5 (Accession no. AM910818)

```
   1 atgcagtcga gcgaatggat taagagcttg ctcttatgaa gttagcggcg gacgggtgag
  61 taacacgtgg gtaacctgcc cataagactg ggataactcc gggaaaccgg ggctaatacc
 121 ggataacatt ttgaaccgca tggttcgaaa ttgaaaggcg gcttcggctg tcacttatgg
 181 atggacccgc gtcgcattag ctagttggtg aggtaacggc tcaccaaggc aacgatgcgt
 241 agccgacctg agagggtgat cggccacact gggactgaga cacggcccag actcctacgg
 301 gaggcagcag tagggaatct tccgcaatgg acgaaagtct gacggagcaa cgccgcgtga
 361 gtgatgaagg ctttcgggtc gtaaaactct gttgttaggg aagaacaagt gctagttgaa
 421 taagctggca ccttgacggt acctaaccag aaagccacgg ctaactacgt gccagcagcc
 481 gcggtaatac gtaggtggca agcgttatcc ggaattattg ggcgtaaagc gcgcgcaggt
 541 ggtttcttaa gtctgatgtg aaagcccacg gctcaaccgt ggagggtcat tggaaactgg
 601 gagacttgag tgcagaagag gaaagtggaa ttccatgtgt agcggtgaaa tgcgtagaga
 661 tatggaggaa caccagtggc gaaggcgact ttctggtctg taactgacac tgaggcgcga
 721 aagcgtgggg agcaaacagg attagatacc tggtagtcca cgccgtaaac gatgagtgct
 781 aagtgttaga gggtttccgc cctttagtgc tgaagttaac gcattaagca ctccgcctgg
 841 ggagtacggc cgcaaggctg aaactcaaag gaattgacgg gggcccgcac aagcggtgga
 901 gcatgtggtt taattcgaag caacgcgaag aaccttacca ggtcttgaca tcctctgaaa
 961 accctagaga tagggcttct ccttcgggag cagagtgaca ggtggtgcat ggttgtcgtc
1021 agctcgtgtc gtgagatgtt gggttaagtc ccgcaacgag cgcaaccctt gatcttagtt
1081 gccatcatta agttgggcac tctaaggtga ctgccggtga caaaccggag gaaggtgggg
1141 atgacgtcaa atcatcatgc cccttatgac ctgggctaca cacgtgctac aatggacggt
1201 acaaagagct gcaagaccgc gaggtggagc taatctcata aaaccgttct cagttcggat
1261 tgtaggctgc aactcgccta catgaagctg gaatcgctag taatcgcgga tcagcatgcc
1321 gcggtaaata cgttcccggg ccttgtacac accgcccgtc acaccacgag agtttgtaac
1381 acccgaagtc ggtggggtaa cctttggag ccagccgcct aaggtga
```

Fig. 4.13: Partial 16S rRNA gene sequence of the isolate GMX6 (Accession no. AM422128)

```
   1 tttgatcctg gctcagattg aacgctggcg gcaggcttaa cacatgcaag tcgagcggta
  61 acaggagaag cttgcttctc gctgacgagc ggcggacggg tgagtaatac ttaggaatct
 121 acctagtagt gggggatagc tcggggaaac tcgaattaat accgcatacg acctacggga
 181 gaaaggggc agtttactgc tctcgctatt agatgagcct aagtcggatt agctagttgg
 241 tggggtaaag gcctaccaag gcgacgatct gtagctggtc tgagaggatg atcagccaca
 301 ccgggactga gacacggccc ggactcctac gggaggcagc agtggggaat attggacaat
 361 gggggcaacc ctgatccagc catgccgcgt gtgtgaagaa ggccttttgg ttgtaaagca
 421 ctttaagcag tgaagaagac tctatggtta atacccatag acgatgacat tagctgcaga
 481 ataagcaccg gctaactctg tgccagcggc cgcggtaata cagagggtgc aagcgttaat
 541 cggaattact gggcgtaaag cgagcgtagg tggcttgata agtcagatgt gaaagccccg
 601 ggcttaacct gggaacggca tctgatactg ttaggctaga gtaggtgaga ggaaggtaga
 661 attccaggtg tagcggtgaa atgcgtagag atctggagga ataccgatgg cgaaggcagc
 721 cttctggcat catactgaca ctgaggttcg aaagcgtggg tagcaaacag gattagatac
 781 cctggtagtc cacgccgtaa acgatgtcta ctagtcgttg gggcccttga ggacttagtg
 841 acgcagctaa cgcaataagt agaccgcctg gggagtacgg ccgcaaggtt aaaactcaaa
 901 tgaattgacg ggggcccgca caagcggtgg agcatgtggt ttaattcgat gaaacgcgaa
 961 gaaccttacc tggtcttgac atatctagaa tcctgcagag atgcgggagt gccttcggga
1021 attagaatac aggtgctgca tggctgtcgt cagctcgtgt cgtgagatgt tgggttaagt
1081 cccgcaacga gcgcaaccct tgtccttagt taccagcggt ttggccggga actctaagga
1141 tactgccagt gacaaactgg aggaaggcgg ggacgacgtc aagtcatcat ggcccttacg
1201 accagggcta cacacgtgct acaatggtag gtacagaggg cagctacaca gcgatgtgat
1261 gcgaatctca aaaagcctat cgtagtccag attggagtct gcaactcgac tccatgaagt
1321 aggaatcgct agtaatcgcg gatcagaatg ccgcggtgaa tacgttcccg ggccttgtac
1381 acaccgcccg tcacaccatg ggagttgatt gcaccagaag tggttagcct aacctttag
1441 gaaggcgatc accacggtgt ggttgatgac tggggtgaag tcgaacaagg
```

Fig. 4.14: Partial 16S rRNA gene sequence of the isolate GMX7 (Accession no. AM422129)

```
   1 cgattagagt ttgatcctgg ctcaggatga acgctggcgg cgtgcctata catgcaagtc
  61 gagcgaactg attagaagct tgcttctaag acgttagcgg cggacgggtg agtaacacgt
 121 gggcaacctg cctgtaagac tgggataact tcgggaaacc gaagctaata ccggatagga
 181 tcttctcctt catgggagat gattgaaaga tggtttcggc tatcacttac agatgggccc
 241 gcggtgcatt agctagttgg tgaggtaacg gctcaccaag gcaacgatgc atagccgacc
 301 tgagagggtg atcggccaca ctgggactga gacacggccc agactcctac gggaggcagc
 361 agtagggaat cttccgcaat ggacgaaagt ctgacggagc aacgccgcgt gagtgatgaa
 421 ggctttaggg tcgtaaaact ctgttgttag ggaagaacaa gtacgagagt aactgctcgt
 481 accttgacgg tacctaacca gaaagccacg gctaactacg tgccagcagc cgcggtaata
 541 cgtaggtggc aagcgttatc cggaattatt gggcgtaaag cgcgcgcagg cggtttctta
 601 agtctgatgt gaaagcccac ggctcaaccg tgggagggtc attggaaact gggggaactt
 661 gagtgcagaa gagaaaagcg gaattccacg tgtagccggt gaaatgcgta gagatgtgga
 721 ggaacaccag tggcgaaggc ggcttttgg tctgtaactg acgctgaggc gcgaaagcgt
 781 ggggagcaaa caggattaga taccctggta gtccacgccg taaacgatga gtgctaagtg
 841 ttagagggtt tccgcccttt agtgctgcag ctaacgcatt aagcactccg cctggggagt
 901 acggtcgcaa gactgaaact caaaggaatt gacgggggcc cgcacaagcg gtggagcatg
 961 tggtttaatt cgaagcaacg cgaagaacct taccaggtct tgacatcctc tgacaactct
1021 agagatagag cgttcccctt cgggggacag agtgacaggt ggtgcatggt tgtcgtcagc
1081 tcgtgtcgtg agatgttggg ttaagtcccg caacgagcgc aaccccttgat cttagttgcc
1141 agcatttagt tgggcactct aaggtgactg ccggtgacaa accggaggaa ggtggggatg
1201 acgtcaaatc atcatgcccc ttatgacctg ggctacacac gtgctacaat ggatggtaca
1261 aagggctgca agaccgcgag gtcaagccaa tcccataaaa ccattctcag ttcggattgt
1321 aggctgcaac tcgcctacat gaagctggaa tcgctagtaa tcgcggatca gcatgccgcg
1381 gtgaatacgt tcccgggcct tgtacacacc gcccgtcaca ccacgagagt ttgtaacacc
1441 cgaagtcggt ggagtaaccg taaggagcta gccgcctaag gtgggacaga tgattggggt
1501 gaagtggag
```

Fig. 4.15: Partial 16S rRNA gene sequence of the isolate GMX8 (Accession no. AM992062)

```
   1 gtcgcacggg cagggcaacc tgtcagtggc ggacgggtga gtaacgcrwr rgratcyatc
  61 cttgggtggg ggacaaccgt gggmaactac sgctaatacc gcatgatccc tgaggggcaa
 121 aggcgaaagt cgcctgagga ggagcctgcg tctgattagg tagttggtgg ggtaaaggcc
 181 taccaagcct gcgatcagta gctggtckgr gaggatgatc agccacactg ggactgagac
 241 acggcccaga ctcctacggg aggcagcagt ggggaatatt ggacaatggg cgaaagcctg
 301 atccagcaat gccgcgtgga tgaagaaggt cttcggattg taaagtcctt ttggcgggga
 361 cgatgatgac ggtacccgca gaataagctc cggctaactt cgtgccagca gccgcggtaa
 421 tacgaagggg gctagcgttg cksggaatga ctggkygtaw wgggcgcgta ggcsgmcgsc
 481 mcagtcaggc gtgaaattcc tgggcwcaac ctggggacyg cgtctgagac gtgttgtctt
 541 gagtatggaa gagggttgtg gaatttccag tgtagaggtg aaattcgtag atattggaaa
 601 gaacaccggt ggcgaaggcg gcaacctggt ccattactga cgctgaggcg cgaaagcgtg
 661 gggagcaaac aggattagat accctggtag tccacgctgt aaacgatgtg tgctggatgt
 721 tggggtgctt agcacttcag tgtcgtagct aacgcggtaa gcacaccgcc tggggagtac
 781 ggccgcaagg ttgaaactca aaggaattga cgggggcccg cacaagcggt ggagcatgtg
 841 gtttaattcg aagcaacgcg cagaacctta ccaggatttg acatggggag taccggtcca
 901 gagatggacc ttcccgcaag gggctcccgc acaggtgctg catggctgtc gtcagctcgt
 961 gtcgtgagat gttgggttaa gtcccgcaac gagcgcaacc ctcgccttca gttgccagca
1021 tgtttgggtg ggyactctga aggaactgcc ggtgacaagc cggaggaagg tggggatgac
1081 gtcaagtcct catggccctt atgtcctggg ctacacacgt gctacaatgg cggtgacagt
1141 gggaagccag gtggtgacac cgagctgatc tcaaaaagcc gtctcagttc ggattgcact
1201 ctgcaactcg agtgcatgaa ggtggaatcg ctagtaatcg cggatcagca tgccgcggtg
1261 aatacgttcc cgggccttgt acacaccgcc cgtcacacca tgggatttgg tttgaccttа
1321 agttggtgcg ctaacccgca agggaggcag ccaaccacgg tcgggtcaga gactggggtg
1381 aagtcgtaac aagggtagcc gta
```

Fig. 4.16: Partial 16S rRNA gene sequence of the isolate DK1AH1 (Accession no. AM403732)

```
   1 cagtcgcacg ggcagggcaa cctgtcagtg gcggacgggt gagtaacgcg wargratcta
  61 tccttgggtg ggggacaacc gtgggmaact acggctaata ccgcatgatc cctgaggggc
 121 aaaggcgaaa gtcgcctgag gaggagcctg cgtctgatta ggtagttggt ggggtaaagg
 181 cctaccaagc ctgcgatcag tagctggtct gagaggatga tcagccacac tgggactgag
 241 acacggccca gactcctacg ggaggcagca gtggggaata ttggacaatg ggcgaaagcc
 301 tgatccagca atgccgcgtg gatgaagaag gtcttcggat tgtaaagtcc ttttggcggg
 361 gacgatgatg acggtacccg cagaataagc tccggctaac ttcgtgccag cagccgcggt
 421 aatacgaagg gggctagcgt tgctcggaat gactgggcgt aaagggcgcg taggcggacg
 481 gcacagtcag gcgtgaaatt cctgggctca acctggggac tgcgtctgag acgtgttgtc
 541 ttgagtatgg aagagggttg tggaatttcc agtgtasagg tgaaattcgt akatattgga
 601 aagaacaccg gtggcgaasg cggcaacctg gtccattact gacgctgagg cgcgactggg
 661 cgtaagggcg cgtaggcgga cggcacagtc aggcgtgaaa ttcctgggct caacctgggg
 721 actgcgtctg agacgtgttg tcttgagtat ggaagagggt tgtggaattt ccagtgtaga
 781 ggtgaaattc gtagatattg gaaagaacac cggtggcgaa ggcggcaacc tggtccatta
 841 ctgacgctga ggcgcgaaag cgtggggagc aaacaggatt agataccctg gtagtccacg
 901 ctgtaaacga tgtgtgctgg atgttggggt gcttagcact tcagtgtcgt agctaacgcg
 961 gtaagcacac cgcctgggga gtacggccgc aaggttgaaa ctcaaaggaa ttgacggggg
1021 cccgcacaag cggtggagca tgtggtttaa ttygaagcaa cgcgcagaac cttaccagga
1081 tttgacatgg ggagtaccgg tccagagatg gaccttcccg caaggggctc ccgcacaggt
1141 gctgcatggc tgtcgtcagc tcgtgtcgtg agatgttggg ttaagtcccg caacgagcgc
1201 aaccctcgcc ttcagttgcc agcatgtttg ggkgggcact ytgaaggaac tgccggtgac
1261 aagccggagg aaggtgggga tgacgtcaag tcctcatggc ccttatgtcc tgggctacac
1321 acgtgctaca atggcggtga cagtgggaag ccaggtggtg acaccgagct gatctcaaaa
1381 agccgtctca gttcggattg cactctgcaa ctcgagtgca tgaaggkgga atcgctagta
1441 atcgcggatc agcatgccgc ggtgaatacg ttcccgggcc ttgtacacac cgcccgtcac
1501 accawrrrrt ttggtttgac cttaagttgg tgcgctaacc cgcaagggag gcagcc
```

Fig. 4.17: Partial 16S rRNA gene sequence of the isolate DK2AH2 (Accession no. AM403733)

4.3.3 16S rRNA phylogeny of acid-tolerant heterotrophic strains

The 16S rRNA gene sequences of the isolates were compared with those in GenBank using the BLASTN program (Atschul *et al.*,1997). The 16S rRNA gene sequences of the isolates, GMX1,GMX2, GMX4, GMX5, GMX6, GMX7, GMX8, GAH1, GAH2, GAH4, GAH5, GAH8, GAH44, DK1AH1, and DK2AH2 produced maximum similarity of 93-99% with the 16S rDNA sequences of the strains of *Serratia marcescens, Burkholderia* sp., *Burkholderia* sp., *Bacillus megaterium, Bacillus cereus, Psychrobacter faecalis, B. megaterium, Burkholderia pyrrocinia, Burkholderia tropica, Burkholderia ambifaria, Comamonas testosteroni, B. tropica, Acidiphilium multivorum,* and *Acidiphilium cryptum* respectively.

With the sequences of the 16S rRNA gene of the isolates GAH4, GAH5, GAH2, GAH1, GMX2, and GMX4 were used to construct a phylogenetic tree by using the neighbor-joining method (Saitou and Nei, 1987) (Fig. 4.18). The sequence of *Neisseria gonorrhoeae* NCTC 83785^T, which belongs to the *ß*-subclass of the *Proteobacteria,*

was used to root the tree. All these isolates were placed as a member of the genus *Burkholderia*. The similarities of the 16S rRNA gene sequence of the isolates GMX2 and GMX4 with those of *Burkholderia* sp. were greater than 96%. The sequence similarities of GMX2 and GMX4 to two closely related species, *B. cepacia* ATCC 17759 and *B. vietnamiensis* LMG 10929^T, were 99%. They were also found to cluster with *B. cepacia* ATCC 17759 and *B. vietnamiensis* LMG 10929^T with a bootstrap support of 69%. Similarly, GAH1 and GAH2 formed a cluster with *B. pyrrocinia* ATCC 51958^T with a bootstrap value of 67%. The sequence similarities of GAH1 and GAH2 to closely related species, *B. pyrrocinia* were 98% and 96% respectively. GAH1 showed 99% and GAH2 showed 96% sequence similarities with *B. ambifaria*. The 16S rRNA gene sequence of the isolate GAH5 was found to closely related with *B. ambifaria* (sequence similarity 99%). It was also found to cluster with *B. ambifaria* with a bootstrap support of 67%. However, the similarities of gene sequence of isolate GAH4 with those of *Burkholderia* were less than 94%. The sequence similarities of GAH4 to closely related species, *B. tropica* CICC 10348 and *B. unamae* were 93% and 92% respectively. Such low sequence similarities suggest that the isolate may be assigned to a novel species of the genus *Burkholderia*.

A neighbour-joining phylogenetic tree (Fig. 4.19) based on 16S rRNA gene sequence comparison clearly showed that strain GAH44^T belongs to the genus of *Burkholderia* and forms a deep branching with *Burkholderia tropica* Ppe8^T. Similar branching was also observed in Maximum Parsimony method. The 16S rRNA gene sequence similarity between strain GAH44^T and its nearest neighbours; *Burkholderia tropica* Ppe8^T, *Burkholderia unamae* MTl-641^T and *Burkholderia bannensis* E25^T was 96%, 95% and 95% respectively and lower than 95% 16S rRNA gene sequence similarity was revealed with other species of the genus *Burkholderia*. Relationships between *Burkholderia garubathanensis* sp. nov. GAH44^T and other known members of the genus *Burkholderia* are also evident in the extended phylogenetic tree (Fig. 4.19).

The sequences of the 16S rRNA gene of the isolates GMX5, GMX8 and GMX6 were used to construct a phylogenetic tree by the method mentioned above (Fig. 4.20). The sequence of *Paenibacillus polymyxa* DSM 36^T, was used as an out-group. All these isolates were placed as a member of the genus *Bacillus*. The similarities of the 16S rRNA gene sequence of the isolates GMX5 and GMX8 with those of *Bacillus*

megaterium were 99%. Also they were found to cluster with *B. megaterium* with a bootstrap support of 100%. Similarly, GMX6 showed sequence similarity of 99% with *Bacillus cereus*. It was also clustered with *B. cereus* with a bootstrap value of 78%. Therefore, it was assumed that the isolates GMX5 and GMX8 were strains of *B. megaterium* and GMX6 as a strain of *B. cereus*.

A phylogenetic tree for the isolate GAH8 was constructed by neighbor-joining method (Fig. 4.21). The sequence of *Paracoccus versutus* ATCC 25364^T, which belongs to the *ß*-subclass of the *Proteobacteria*, was used to root the tree. The isolate was placed as member of the genus *Comamonas*. The 16S rRNA gene sequence of the isolate GAH8 was found to closely related with *Comamonas testosteroni* ATCC 11996^T (sequence similarity 99%). It was also found to cluster with *C. testosteroni* ATCC 11996^T with a bootstrap support of 100%. Hence, it was assumed that the isolate GAH8 was the strain of *C. testosteroni*.

Similarly, a phylogenetic tree was obtained with neighbor-joining method (Fig. 4.22), showed that the isolate GMX7 forms a separate cluster with *Psychrobacter faecalis*. The sequence of *Acinetobacter baumanii* , which belongs to the *gamma*-subclass of the *Proteobacteria*, was used to root the tree. The 16S rRNA gene sequence of the isolate GMX7 was found to closely related with *P. faecalis* DSM 14664^T (sequence similarity 99%). It was also found to cluster with *P. faecalis* DSM 14664^T with a bootstrap support of 100%. Hence, it was assumed that the isolate GMX7 was the strain of *P. faecalis*.

A phylogenetic tree for the isolates DK1AH1 and DK2AH2 was constructed by neighbor-joining method (Fig. 4.23). The sequence of *Acidithiobacillus ferrooxidans* (AF465604), which belongs to the *α*-subclass of the *Proteobacteria*, was used to root the tree. The isolate was placed as member of the genus *Acidiphilium*. The 16S rRNA gene sequence of the isolate DK1AH1 was found to closely related with *Acidiphilium multivorum* (sequence similarity 98%) and DK2AH2 with *Acidiphilium cryptum* (sequence similarity 98%). It was also found to cluster with *Acidiphilium cryptum* with a bootstrap support of 100%. Hence, it was assumed that the isolate DK1AH1and DK2AH2 were the strains of *Acidiphilium multivorum* and *Acidiphilium cryptum* respectively.

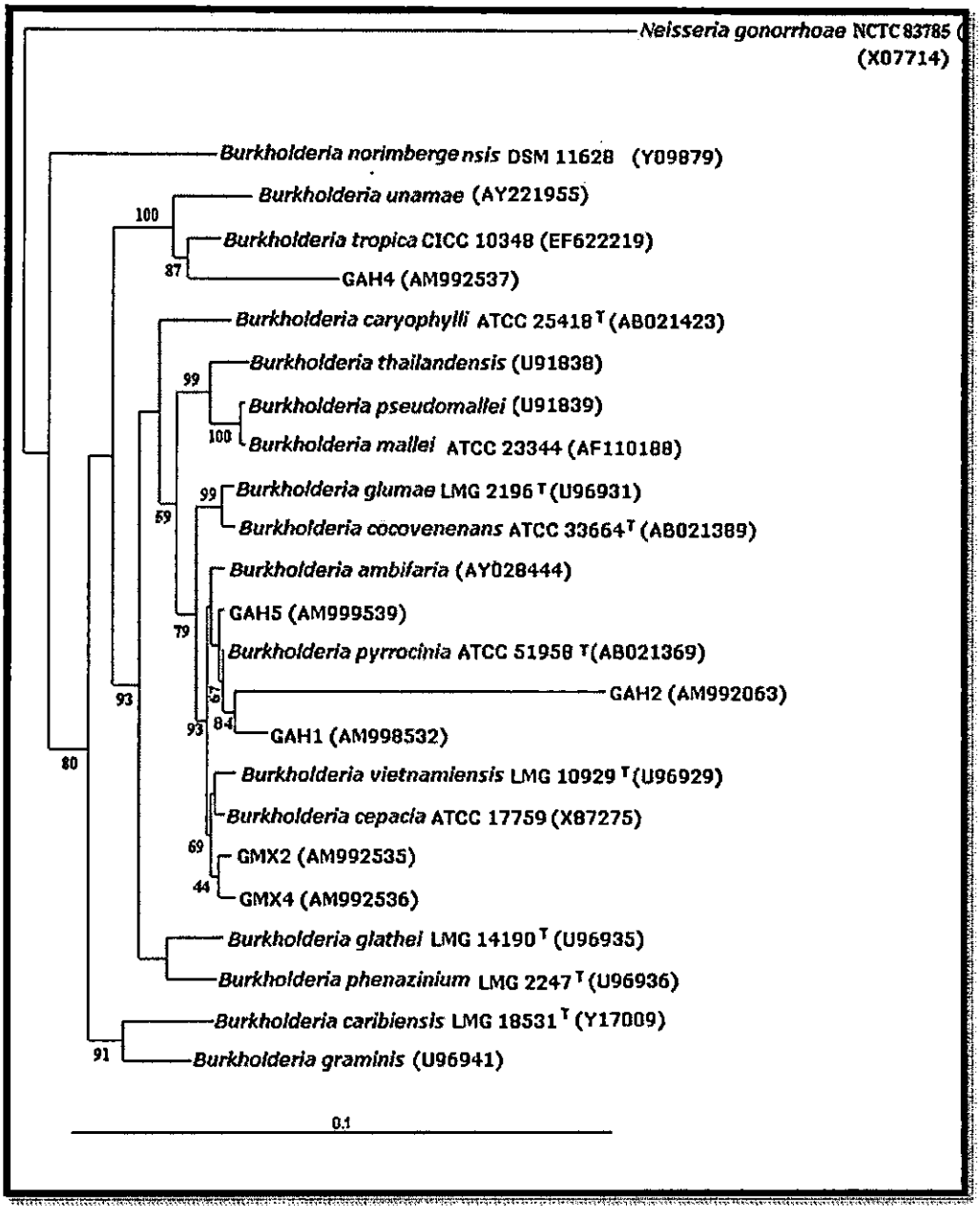

Fig. 4.18: Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing systematic position of GAH1, GAH2, GAH4, GAH5, GMX2, and GMX4 within members of the family Burkholderiaceae under *Beta-proteobacteria*. Bootstrap values are calculated from 100 replicates. The scale bar indicates 0.1 inferred nucleotide substitutions per site.The 16S rRNA gene sequence of *Neisseria gonorrhoeae* was used as an outgroup reference.

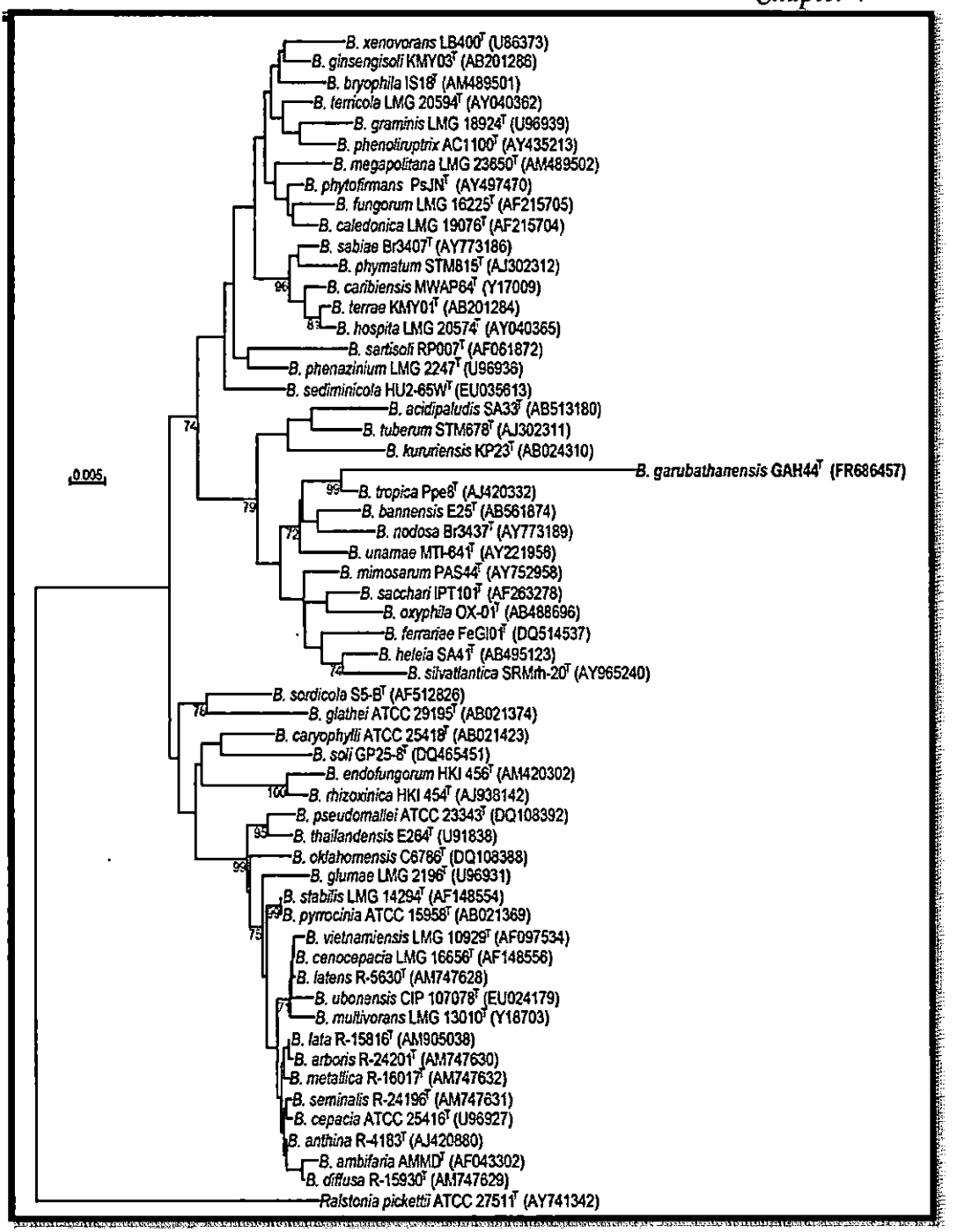

Fig. 4.19: Rooted NJ tree based on 16S rRNA gene sequences showing the phylogenetic positions of GAH44 within related taxa. Bootstrap values (expressed as percentages of 1000 replications). The phylogenetic tree was rooted using *Ralstonia pickettii* ATCC 27511^T as an outgroup. Bar, 0.005 substitutions per nucleotide.

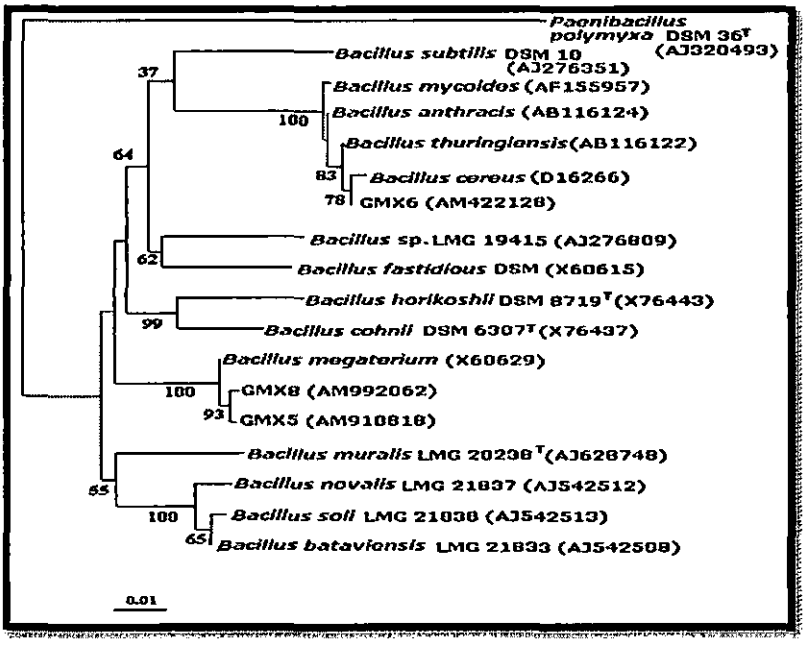

Fig. 4.20: NJ phylogenetic tree showing systematic positions of GMX5, GMX6, and GMX8 within members of the family Bacillaceae under Firmicutes. Bootstrap values are calculated from 100 replicates. The scale bar indicates 0.01 inferred nucleotide substitutions per site.The 16S rRNA gene sequence of *Paenibacillus polymyxa* was used as an outgroup.

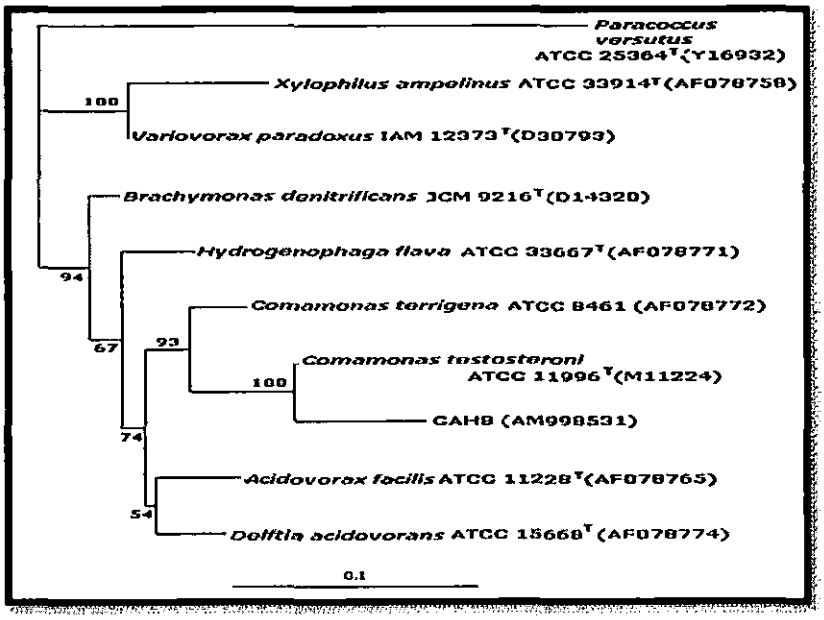

Fig. 4.21: NJ phylogenetic tree based on 16S rRNA gene sequences showing systematic position of GAH8 within members of the family Comamonadaceae of *Beta-proteobacteria*. Bootstrap values are

calculated from 100 replicates. The scale bar indicates 0.1 inferred nucleotide substitutions per site. *Paracoccus versutus* was used as an outgroup.

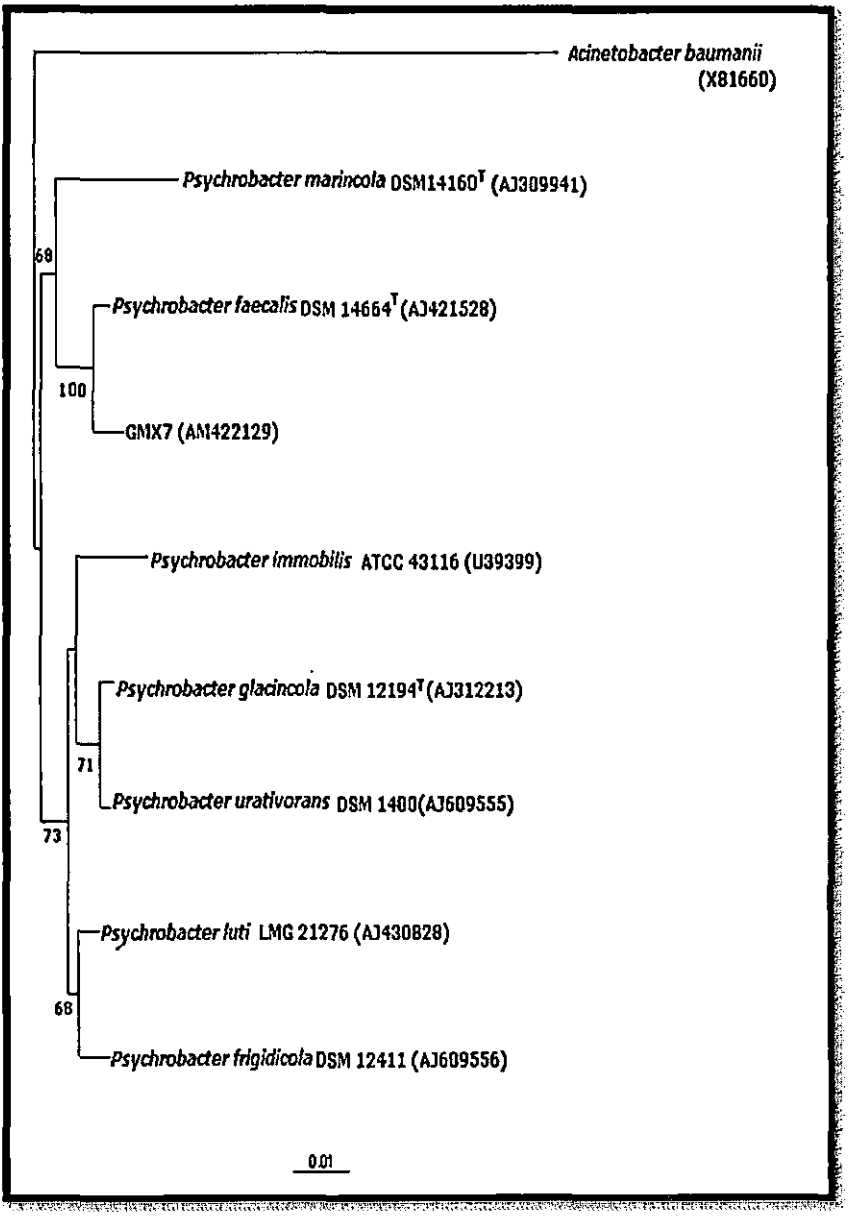

Fig.4.22: Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing systematic position of GMX7 within members of the family Moraxellaceae under *Gamma-proteobacteria*. Bootstrap values are calculated from 100 replicates and above 50% are represented at the nodes. The scale bar indicates 0.01 inferred nucleotide substitutions per site. The 16S rRNA gene sequence of *Acinetobacter baumanii* was used as an outgroup reference.

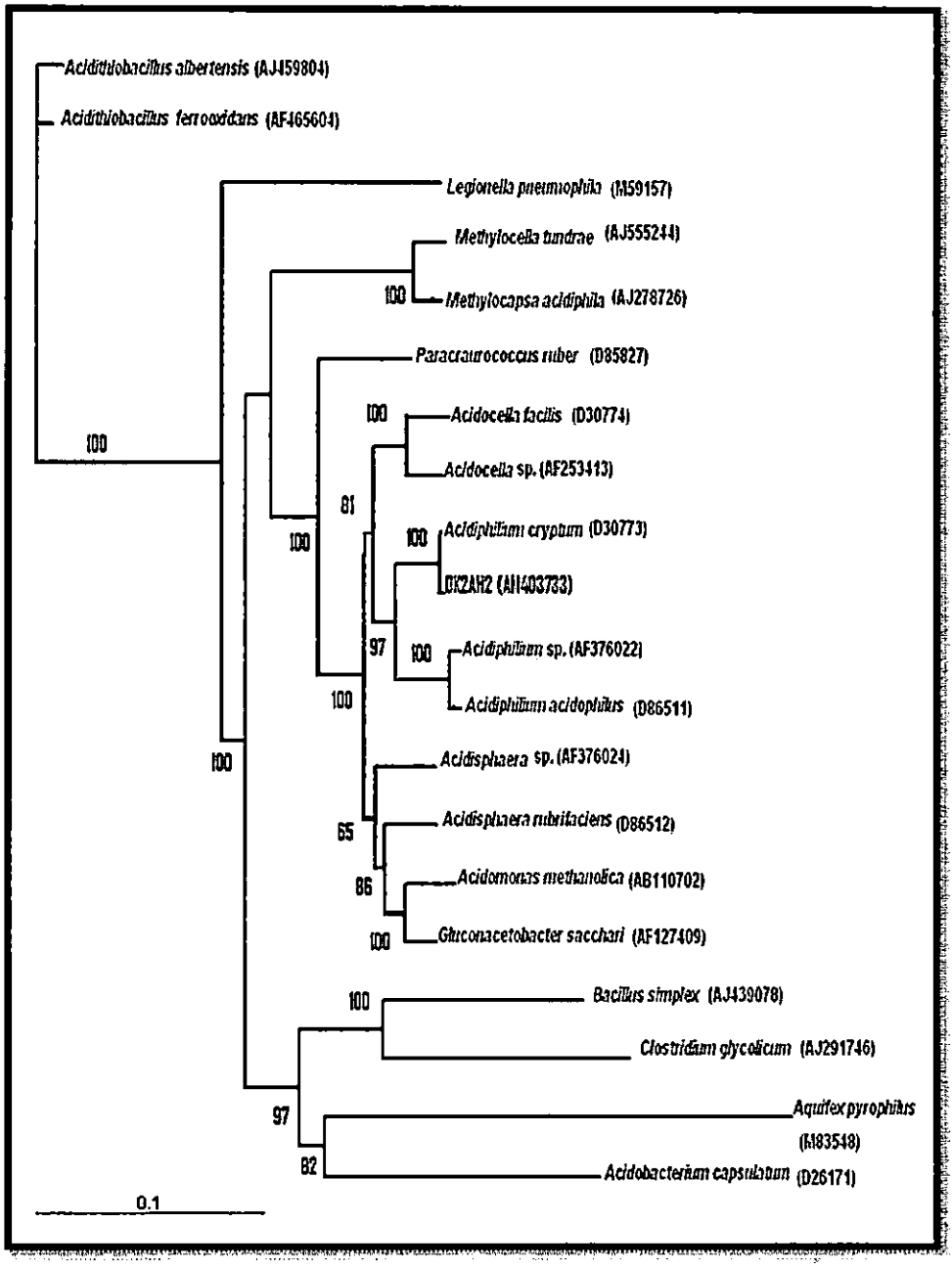

Fig. 4.23: Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing systematic position of DK2AH2 within members of the family Acetobacteraceae under *Alpha-proteobacteria.* Bootstrap values are calculated from 100 replicates and above 50% are represented at the nodes. The scale bar indicates 0.1 inferred nucleotide substitutions per site.The 16S rRNA gene sequence of *Acidithiobacillus ferrooxidans* and *A. albertensis* was used as an outgroup reference.

According to previous studies if any strain, showing 3% or more than 3% 16S rRNA gene sequence dissimilarity with their neighbours, it can be assigned as separate species without going to DNA–DNA hybridization. Recently, this threshold value has been increased to 98.7 % (Stakebrandt and Ebars, 2006). As the level of similarity between *Burkholderia garubathanensis* sp. nov. strain GAH44^T and the other *Burkholderia* species were lower than 97 %, therefore this strain GAH44^T can be easily considered to a separate genospecies without the need of DNA–DNA hybridization (Stackebrandt and Goebel, 1994; Stackebrandt *et al.*, 2002; Stackebrandt and Ebers, 2006).

GAH44 could be distinguished from closely related species of *Burkholderia* in the phylogenetic trees (Fig.4.19) on the basis of physiological and biochemical characteristics (Table 4.1 and 4.2). The results of physiological and biochemical tests, allowed phenotypic differentiation of this strain from described *Burkholderia* species.

For determination of the DNA base composition, the deoxyribonucleic acid was degraded enzymatically into nucleosides as described earlier (Mesbah and Whitman, 1989). The obtained nucleoside mixture was then separated using a Waters Breeze HPLC system and XBridge Shield RP18 column thermally stabilised at 37 °C. Acetonitrile (1.5%, v/v) was used as solvent with 0.02 M $NH_4H_2PO_4$ (pH 4.0). The system was calibrated with non methylated lamda phage DNA (Sigma) and *Escherichia coli* LMG 2093 DNA. The total guanine plus cytosine content of DNA of the type strain GAH44^T was 65.0 mol%, which is falling in the range reported previously for members of the genus *Burkholderia* (Chen *et al.*, 2006).

The cellular fatty acid were analysed after an incubation period of 24 hr at 28 °C. A loopfull of well grown cells were harvested. The fatty acid methyl esters were prepared according to previously described protocol (Vandamme *et al.*, 1992) and separated; and identified using the Sherlock Microbial Identification System (version 3.1, MIDI Inc.). The fatty acid components (only >1% are given here) detected in strain GAH44^T are $C_{14:0}$ (4.5%); summed feature 2 ($C_{14:0}$ 3-OH and/or iso-$C_{16:1}$ I or both, 5.4 %); Summed feature 3 comprises $C_{16:1}$ ω7c and/or iso-$C_{15:0}$ 2-OH or both, 7.1 %; $C_{16:0}$ (22.8 %, 2nd major component); $C_{16:0}$ 2-OH (2.9 %); $C_{16:1}$ 2-OH (2.2 %); $C_{16:0}$ 3-OH (6.3 %); $C_{17:0}$ cyclo (12.8 %); $C_{18:1}$ ω7c (23.6 %, major component); $C_{18:0}$ (1.6 %) and

$C_{19:0}$ ω8c (6.6 %). Considerable differences in fatty acid profile were observed between strain GAH44^T and the other type strains of *Burkholderia* species (Table 4.2).

Strain GAH44^T could be differentiated phenotypically and genotypically from recognized species of the genus *Burkholderia* and hence, we suggested it to be a novel species, for which the name *Burkholderia garubathanensis* sp. nov. was proposed.

Table. 4.1: Differences in the assimilation of carbon sources by the strain *B. garubathanensis* GAH44^T and phylogenetically related *Burkholderia* species.

Species: 1, *B. garubathanensis* GAH44^T; 2, *B. tropica* Ppe8^T; 3, *B. unamae* MTI-641^T; 4, *B. ferrariae* FeGl01^T; 5, *B. bannensis* E25^T.

Carbon source	**1**	**2**	**3**	**4**	**5**
Esculin	-	±	±	-	+
Melibiose	-	±	-	-	-
Rhamnose	+	+	+	-	+
Ribose	-	+	-	+	+
Lactose	-	+	-	-	-
Salicin	-	+	-	-	±
Trehalose	-	+	+	+	+
Dulcitol	-	-	-	+	ND
Maltose	-	-	-	-	+
D-Raffinose	-	-	-	-	-
Sucrose	+	-	-	-	-
Xylitol	-	-	-	-	-
L-xylose	+	-	-	-	±
ß-Galactosidase	-	+	+	+	-

Table. 4.2: Phenotypic characteristics that differentiate *Burkholderia garubathanensis* GAH44^T from phylogenetically related species

Species: 1, *B. garubathanensis* GAH44^T; 2, *B. tropica* Ppe8^T; 3, *B. unamae* MTI-641^T; 4, *B. ferrariae* FeGl01^T; 5, *B. bannensis* E25^T.

Characteristic	1	2	3	4	5
Growth on:					
BAc agar medium	+	+	+	ND	ND
BSE agar medium 29 °C	+	+	+	ND	ND
MacConkey medium at 29 °C	+	+	±	–	ND
MacConkey medium at 37 °C	+	–	–	ND	ND
G+C content (mol%)	65.0	63.5	63.5	62.7	65.0
Fatty acid content (%)□					
16 : 0	22.8	ND	18.0	18.0	44.3
17 : 0 cyclo	12.8	ND	7.4	18.9	14.6
Summed feature 3#	7.1	ND	14.9	1.9	8.6
Oxidase	-	+	+	+	+
Catalase	+	+	+	+	+
Urease	–	+	+	–	–
Gelatin hydrolysis	–	–	–	–	–
Nitrate reduction	+	+	+	+	+

#Summed feature 3 comprises $C_{16:1}$ ω7c and/or iso-$C_{15:0}$ 2-OH or both for strain GAH44^T (*B. garubathanensis* sp. nov.), *B. unamae* (Caballero-Mellado *et al.*, 2004), *B. ferrariae* (Valverde *et al.*, 2006), and *B. bannensis* (Aizawa *et al.*, 2010 in press).

Description of *Burkholderia garubathanensis* sp.nov.

Burkholderia garubathanensis (ga.ru'ba.tha.nen.sis. M.L. adj. garubathanensis, referring to Garubathan, from where the organism was isolated).

Cells are Gram-negative, small rod shaped, non-spore-forming and motile. Colonies are smooth, round, convex and off-white,1-1.5 mm in diameter with entire margins after 2 days of incubation at 30 °C on DSMZ 269 (pH 3.0) agar plates. Growth occurs at 15-40 °C with optimum growth at 30-32 °C. The pH range for growth is 3-10, with optimum growth at pH 6. The strain is facultatively oligotrophic in nature as it showed growth on diluted (0.1x, 0.01x, 0.001x) DSMZ 269 and LB media; undiluted R2A and LB. Lipase and catalase activity is observed. No indole production, gelatine hydrolysis, esculin hydrolysis, β- galactosidase, caseinase, and amylase activity is observed. Sucrose, D-mannitol, D- adonitol, myo- inositol, L- xylose, D- sorbitol, L- arabinose, L-rhamnose, D- xylose, cellobiose, D- mannose, fructose, dextrose, D- arabinose, and malonate are utilized as sole carbon sources; lactose, dulcitol, salicin, raffinose, maltose, trehalose, α- methyl- D- glucoside, melibiose, galactose, inulin, Na- gluconate, glycerol, glucosamine, ribose, melezitose, mannoside, xylitol, and sorbose are not. The strain GAH44^T is susceptible to antibiotics (in $\mu g ml^{-1}$) azithromycin,10; kanamycin,10; netilmicin,1; streptomycin,5; sulfamethoxazole,10; cefepime,10; cefotaxime,10; ampicillin,50; ciprofloxacin,5; livofloxacin,5; chloramphenicol,150; oxytetracycline, 2. Additional characteristics are mentioned in Table 4.1 and 4.2. The major isoprenoid quinone is Q-8, as depicted for the genus. The major components in fatty acids are $C_{18:1}$ ω7c, $C_{16:0}$, and $C_{17:0}$ cyclo and the G+C content of the DNA is 65 mol%.

The type strain is GAH44^T (= DSMZ 23993^T = LMG 26026^T), which was isolated from acid mine drainage in Garubathan, West Bengal, India.

4.3.4 Amplification, cloning and sequencing of *sox*B gene in thiosulfate metabolizing strains

PCR amplicons generated with soxB primers from GAH1, GAH2, GAH4, GAH5, GMX5, and GMX7 corresponded to the expected sizes (except GMX6) (Table 4.3) in suitable quantity which could be purified, cloned and sequenced. GMX1 and GMX2 could not generate amplicons, though thiosulfate metabolizing activity was evident among them (discussed in chapter 2). Strains GAH2, GAH4, GMX5, and GMX7 could generate amplicons with both primer sets (I and II) while GAH1 could generate the amplicon with primer set I; GAH5 and GMX6 with primer set II only. The PCR product thus obtained was purified and cloned in pGEM T-easy vector (similarly, as described in section 4.3.1) prior to sequencing.

Table. 4.3: PCR amplification results of *sox*B gene fragments from acid-tolerant thiosulfate metabolizing strains isolated from AMD

Strains	*PCR product obtained with primer set*		*Size (s) of soxB amplicon (bp)*
	soxB 432f soxB 1446r (Set-I)	**soxB 693f soxB 1446r (Set-II)**	
GAH1	+	-	900#
GAH2	+	+	900*, 700*
GAH4	+	+	1100*, 700*
GAH5	-	+	600#
GMX1	-	-	-
GMX2	-	-	-
GMX5	+	+	1100, 900
GMX6	-	+	400
GMX7	+	+	900□, 700□

*, Sequence obtained produced significant alignment with reported *sox*B gene sequences; #, sequence obtained matched with ABC transporter gene(s); □, sequence obtained matched with sulfate/thiosulfate ABC transporter gene(s).

```
  1 tccgcagact ggaccttccg gcgcaccagg acgaggagct gcaggaactg gtcgacgata
 61 tccgcgaaaa cgaaaagccg gatgtggtgg tggtgatctc ccacaacggt atggatgtgg
121 accttcaaaa tggcctcgcg cgtgaccggc atcgatgcca tcatgggggg gcacacccac
181 gacgcatgcc cgcaccgagc atcgtcaaga accccggcgg ccagaccctg gtgaccaacg
241 ccggctccgg caagttcctc ggcgtgctgg aattcgacgc cgaagtccct gggttcgcga
301 cggcaaggtg caggacttac gactgcccgc taccgcctgc tgccggtgtt ctccaacgat
361 cgacccggat gcggagatgc aggcctacat cgacgaggtg cgcaagccct atcggccagc
421 tcaacgagga gccggcggtg gccggcgacc tgctctacga caaacggctt
```

Fig. 4.24 : Partial *sox*B gene sequence of *Burkholderia* sp. GAH2 for sulfate thioesterase/sulfate thiohydrolase. Accession no. FN995257.

```
  1 rrldlpahqd eelqelvddi renekpdvvv vishngmdvd lqnglardrh rchhggahpr
 61 rmpapsivkn pggqtlvtna gsgkflgvle fdaevpgfat arcrtydcpl ppaagvlqrs
121 trmrrcrpts trcaspigql neepavagdl lydkrl
```

Fig. 4.25 : Translated amino acid sequence of partial *sox*B gene of *Burkholderia* sp. GAH2. Accession no. CBO04274.

```
  1 ggcgtttccc tataccccat gattgccaac ccctcccgct tcattcccga ctggaccttc
 61 ggcatcaagg acgaggagct gcaggaactg gtcgacgata tccgcgaaaa cgaaagagcc
121 ggatgtggtg gtggtgatct cccacaacgg tatggatggg tggacctgaa gatggcctct
181 attcgtcagc ggcgtggacg tcatcctccg gcggccatac ccacgacggc atgcccgcac
241 cgagcaatcg tcaagaaccc cggcggccag accctggtga ccaacgccgg ctccaacggc
301 aagttcctcg gcgtgctgga cctggatgtt cgcgacggca aggtgcagga cttccgctac
361 cgcctgctgc cggtgttctc caacctgatc gacccggatg cggagatgca ggcctacatc
421 gacgaggtgc gcaagcccta tctcggccag
```

Fig. 4.26 : Partial *sox*B gene sequence of *Burkholderia* sp. GAH4 for sulfate thioesterase/sulfate thiohydrolase. Accession no. FN995256.

```
  1 gvslypmian psrfipdwtf gikdeelqel vddirenera gcgggdlpqr ygwvdlkmas
 61 irqrrgrhpp aaipttacph raivknpggq tlvtnagsng kflgvldldv rdgkvqdfry
121 rllpvfsnli dpdaemqayi devrkpylgq
```

Fig. 4.27 : Translated amino acid sequence of partial *sox*B gene of *Burkholderia* sp. GAH4. Accession no. CBO04273.

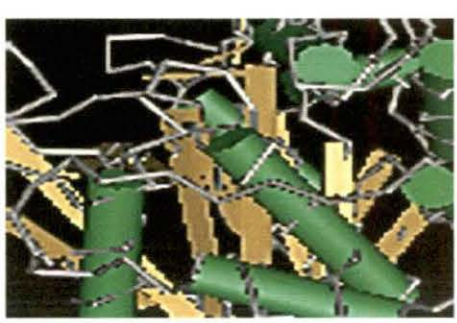

Fig. 4.28: The amino acid sequence of *sox*B gene of GAH2 and GAH4 showing the conserved domain of metallophosphatase superfamily.

Metallophosphatase superfamily, metallophosphatase domain

Metallophosphatases (MPPs), also known as metallophosphoesterases, phosphodiesterases (PDEs), binuclear metallophosphoesterases, and dimetal-containing phosphoesterases (DMPs), represent a diverse superfamily of enzymes with a conserved domain containing an active site consisting of two metal ions (usually manganese, iron, or zinc) coordinated with octahedral geometry by a cage of histidine, aspartate, and asparagine residues. This superfamily includes: the phosphoprotein phosphatases (PPPs), Mre11/SbcD-like exonucleases, Dbr1-like RNA lariat debranching enzymes, YfcE-like phosphodiesterases, purple acid phosphatases (PAPs), YbbF-like UDP-2,3-diacylglucosamine hydrolases, and acid sphingomyelinases (ASMases). The conserved domain is a double beta-sheet sandwich with a di-metal active site made up of residues located at the C-terminal side of the sheets. This domain is thought to allow for productive metal coordination.

```
  1 catgtccccc catggaggag acatgacacc gcgcgcgtga aatcgattag ctcgcgccgc
 61 actgtccact gccgaacttt cggagacggg catatcaacc atcggcgctc gtagcgaaga
121 aggtacgttc tatcgagtga tagcgacact cgatgcgcag accgttactg tatatggccg
181 gccgcaatct ctgcagacag gcatgactct ccaggccgat atccttcgag aaagaaggcg
241 cctctacgaa tgggtgcttg atccgctcta tagcctcact ggaaaattct gagttttttt
301 cgtcttatgt cacttcttga tcgtctatca ttcggcgtag gtcgcaagct gttgatgttg
361 cttcagacgg aggcaactga atgtgggatt gcgtgcctcg caatgattgc gagttatcac
421 ggccacagcg tagatattcc ggagcttcga aaccgtttcc cgatatcctt aaaaggagca
481 gggctatctc gcgtcatcga gatttcgaac agtctcaatt tgagagcgcg cgcggtgaag
541 ctggaccttg atcagctcac gcagttgcga gtgccgtgca ttctgcattg gaacttcaat
601 cactttgtta tactgaaaga agtgtccgga aaggggcga taatccacga tcctgctcaa
661 ggagtgcgaa aaatgtcact cgaggaggta tctcaatcat ttactggcgt cgcgctggag
721 ctatggccaa cgggtgaatt tcagcaacgt gacgcggcac cagcaatccg acttcgatcc
781 ttgatcggct cagtta
```

Fig. 4.29 : Partial ABC transporter gene for ATP binding (soxB amplicon of size 600bp) of *Burkholderia* sp. GAH5. Accession no. FR837594.

```
  1 lsffrlmsll drlsfgvgrk llmllqteat ecgiaclami asyhghsvdi pelrnrfpis
 61 lkgaglsrvi eisnslnlra ravkldldql tqlrvpcilh wnfnhfvilk evsgkgaiih
121 dpaqgvrkms leevsqsftg valelwptge fqqrdaapai rlrsligsv
```

Fig. 4.30 : Translated amino acid sequence of partial ABC transporter gene of *Burkholderia* sp. GAH5. Accession no. CCA30198.

```
   1 cagtgctggc cttaggattc ggtcttctct tcgtgataca acaagcgact agtgcgatt
  61 gcgcgtgggt gttgatgtat tttagcacca cattaaacgt acaatggcgg aataatgtt
 121 tcgcacatct tatcagtctg ccaactagat atttcgaaaa gagacatctg ggtgatgtt
 181 tttcgagatt cgggtcggtc gacgtaattc aacaaactct aacgacgtcg tttatagg
 241 ctgtcattga cggtctgatg tccgttgtca ctttggcgat gatgtttgta tatagctg
 301 agcttggaat tatcgcgatc gtgaccgtgt taatttacat tttccttcgg tcgatatg
 361 atctcccgct caaaaacgct tcagaagaac aaataataag gattgcaaaa caagaaag
 421 atttcctgga gacaattcgt gggataaagt ctattaaatt atttaatagg caaaatga
 481 ggtgtgccgg atggattaag ttgtttattg agcaaattaa tgcaagtctt cgtgttca
 541 aattggaact tctgtatcat caaattaata gtcttatttt cggtgcagtt ggactgat
 601 ttgtgtggtt gggggcgagg atggtgatgg atggttactt tactgttggg gcgctgat
 661 catttagttc ctataagggg cagtttgata atagagtcgg aagtcttatc gataaatt
 721 ttgaggtgaa acttctaagg caacacggtg gggacatg
```

Fig. 4.31 : Partial gene sequence (soxB generated amplicons) of *Burkholderia* sp. GAH1 for ABC transporter gene. Accession no. FR837593.

```
   1 vlalgfgllf viqqatsair awvlmyfstt lnvqwrnnvf ahlislptry fekrhlgd
  61 srfgsvdviq qtlttsfiga vidglmsvvt lammfvyswk lgiiaivtvl iyiflrsi
 121 lplknaseeq iiriakqesn fletirgiks iklfnrqner cagwiklfie qinaslrv
 181 lellyhqins lifgavgliv vwlgarmvmd gyftvgalia fssykgqfdn rvgslidk
 241 evkllrqhgg dm
```

Fig. 4.32 : Translated amino acid sequence of partial ABC transporter gene of *Burkholderia* sp. GAH1. Accession no. CCA30197.

cl00549: **ABC_membrane Superfamily**

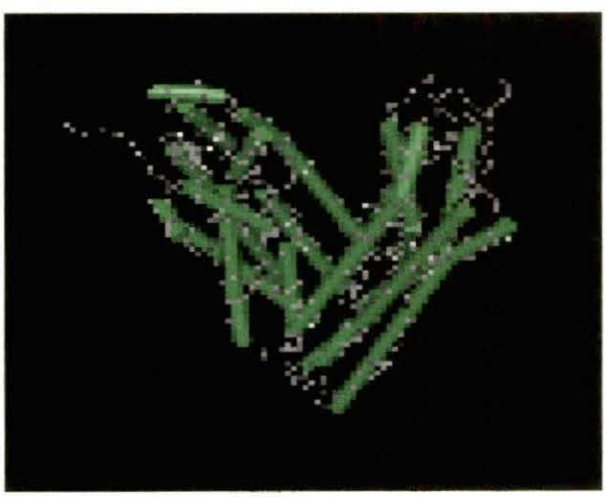

Fig. 4.33: The amino acid sequence of ABC transporter gene of GAH1 showing the conserved domain of ABC transpoter family.

ABC transporter transmembrane region

This family represents a unit of six transmembrane helices. Many members of the ABC transporter family (pfam00005) have two such regions.

```
GMX7  6        CTCCGCCGTGCTGTCCGCAAGTATCGGTTTGGGATTATTTACCGCCGCGCGTATCGTCGA  65
               ||| ||| ||||||| || ||||||||| | || |||||||||||| || ||||| || ||
ABCtr 406237   CTCAGCCATGCTGTCAGCCAGTATCGGTCTTGGTTTATTTACCGCAGCTCGTATTGTTGA  406296

GMX7  66       ACAAGTACGTACGGGTATCGAATCCTTACCTGAAGGGCAAATCAACGCCGCTTATGCGCT  125
               |||||||||||| |||||||| ||  ||||| ||||||| ||||| || || || |||||
ABCtr 406297   ACAAGTACGTACTGGTATCGAGTCACTACCTCAAGGGCAGATCAATGCTGCCTACGCGCT  406356

GMX7  126      CGGTTTTACGATTCCGCAAGCGTATAAAGAGGTGCTACTGCCGCAGGCGTTTCGGATCAT  185
                || ||||  || |||||||| |||||||| || || ||||| || ||||||||||| ||
ABCtr 406357   TGGCTTTAGTATCCCGCAAGCCTATAAAGAAGTACTGCTGCCACAAGCGTTTCGGATTAT  406416

GMX7  186      CTTGCCGCCGCTCAGCTCTGAGCTGACCAACTGCTTTAAAAACGCTTCGGTGGCGTCGCT  245
               ||| ||||| || ||||| |||||||||||||||||||||||||| || || |||||  |
ABCtr 406417   CTTACCGCCACTGAGCTCAGAGCTGACCAACTGCTTTAAAAACGCCTCAGTTGCGTCTTT  406476

GMX7  246      CGTTGGCGTGATGGAGCTGATTAGCCAAACCAAAACCATCAGTGAATATACCCAAAACAG  305
                ||||| || |||||||||| || || |||||||||||||||||||||| |||||||
ABCtr 406477   GGTTGGGGTAATGGAGCTGATCAGTCAGACCAAAACCATCAGTGAATATACTCAAAACAG  406536

GMX7  306      CCTCGAGATTTATACTTATGCGACCATTATCTATCTGGTCTTTAATCTATCGTTAATTGC  365
                || |||||||||| |||||||||||||| |||||||| |||||||| || ||||||||
ABCtr 406537   TCTTGAGATTTATACCTATGCGACCATTATTTATCTGGTGTTTAATCTGTCTTTAATTGC  406596

GMX7  366      CATTACGG  373
               |||||  ||
ABCtr 406597   CATTATGG  406604
```

Fig. 4.34: BlastN result of *Psychrobacter testosteroni* GMX7 (*sox*B amplicon of size 700bp) nucleotide sequence showing 85% homology with the '+' strand of *Psychrobacter cryohalolentis* amino acid ABC transpoter gene.

```
GMX7  56       TTTGTTATTGGCAACAGCAAATACTCGGTGAGTCTATGTTTTCGCGTCATGAGCTGATTC  115
               ||| | |||||||||||||| ||| | | |||||| ||||||||||||||||| |  |||
hypo  2965617  TTTCTCATTGGCAACAGCAGATATTAGATGAGTCATTGTTTTCGCGTCATGAGTTAGTTC  2965558

GMX7  116      GTACATTGTATCGCCATCCTAAACAAGAACAGCAATTACAACAACAAGGCTATGTCGTCC  175
               |||| || |||   ||||| || ||||  ||||| || || ||||||||||||| ||||
hypo  2965557  GTACTTTATATAAGCATCCCAAGCAAGCGCAGCAGTTGCAGCAACAAGGCTATGTTGTCC  2965498

GMX7  176      ATCACCATTCTTATGAGTCTTTAGGTCGGCATTATGTGCTGATATTTTATGGGCAGGATG  235
               | || |||||||| |||| ||| || |||||||||| || |||||||||| || ||||
hypo  2965497  ACCAACATTCTTACGAGTGTTTGGGGCGGCATTATGTACTAATATTTTATGGACAAGATG  2965438

GMX7  236      CAAAGGGGCAACATAGTAAAAAGACCATTCAACCAAATCTTGCAAAAATCGCTTTAGATG  295
               || | ||  | ||| |||| | | || |  |||||| | || ||||||||||||||||
hypo  2965437  CAGAAGGAAAGCATGGTAAGTCGGCTATACGCCCAAATTTAGCGAAAATCGCTTTAGATG  2965378

GMX7  296      TTGCCACGCGCTTACCGCTAGCGGAGCTGCACCACGTGATTGTACTGGGTATAGATTTTA  355
               | || || || ||||| | || || || || || ||| ||||| ||||||||||||||||
hypo  2965377  TGGCGACACGGTTACCAATGGCAGAACTACATCATGTGGTTGTATTGGGTATAGATTTTA  2965318

GMX7  356      TTGCTGAGGCGAATGATACGTTTATTGATATCGATTTGTGGATTCAGCCCGTTGCTGCCA  415
               |    |||||  | ||| | ||||| ||||| ||||| |||||||||| |||  | |||
hypo  2965317  TAATAGAGGCTGAAGATGCTTTTATGGATATTGATTTATGGATTCAGCCAGTTAATCCCA  2965258

GMX7  416      TGAGTCAGCGCGAGCGTCAGCTGACCAAGAAAATCCAGCAACTGCAGCA  464
               ||| ||||||||||| || ||||| || ||  | ||||| |||||||
hypo  2965257  TGACCCAGCGCGAGCGCCAACTGACTAAAAAGGTTCAGCAGTTGCAGCA  2965209
```

```
GMX7   500        TTAAAAACGCTACGACACTACTGGTATAAGGTTTTTGCAGTCTCTGTCTGCGTAGGCACA  559
                  ||||||  | ||||  ||||||||  |     | ||||||| ||||||||||||||||||
Thio   1702206    TTAAAAGAG-TACGGAACTACTGGGGTTCATTATTTGCAGCCTCTGTCTGCGTAGGCACA  1702148

GMX7   560        GCAAGCAAGAGAAA  573
                  ||||||||||| |||
Thio   1702147    GCAAGCAAGAAAAA  1702134
```

Fig. 4.35: BlastN result of *Psychrobacter testosteroni* GMX7 (*sox*B amplicon of size 900bp) nucleotide sequence showing 78% homology with the '-' strand of *Psychrobacter cryohalolentis* hypothetical protein and 82% with the 388bp at 5′ side: thiosulfate-binding protein.

```
GMX5   1          AAGCAGAATCAGCTGAGGTGACAGAGGCAGGTCTTTATGAAATGGCTCCTACAGAATCAG  60
                  ||||||| ||||||||| |||||||||||||| ||||||||||||||||||||||||||||
hypo   2256894    AAGCAGAGTCAGCTGAGCTGACAGAGGCAGGTTTTTATGAAATGGCTCCTACAGAATCAG  2256953

GMX5   61         TGCCGTTTTATACGGTCGAAACACATGGTTATAGTACGCTTCGCCTGCCGGCACCCCCTG  120
                  ||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
hypo   2256954    TGCCGTTTTATACGGTCGCAACACATGGTTATAGTACGCTTCGCCTGCCGGCACCCCCTG  2257013

GMX5   121        GATTAGACAGCGCGTACGTTGAACTGGTGATGCATGCAGATCAAGGTCCGTTTGAAGATG  180
                  |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
hypo   2257014    GATTAGACAGCGCGTACGTTGAACTGGTGATGCATGCAGATCAAGATCCGTTTGAAGATG  2257073

GMX5   181        AAAAGTACAGCTGGATTCCTCAAGTCATGCATCAAGTAGGAAGCTTTGCGGTTAACAATA  240
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
hypo   2257074    AAAAGTACAGCTGGATTCCTCAAGTCATGCATCAAGTAGGAAGCTTTGCGGTTAACAATA  2257133

GMX5   241        TGAATTGGATTGGTCAGTGGATGGTATTTCCAAATCAAGAGCTGGATCGCTACGTGGATA  300
                  ||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
hypo   2257134    TGAATTGGATTGGTCAGTGGATGGTGTTTCCAAATCAAGAGCTGGATCGCTACGTGGATA  2257193

GMX5   301        CCTATGAACGAACGTTAACAGGTGAAAAAGTGAGGCTGCCATTACAGGTTCAACCGTATT  360
                  ||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
hypo   2257194    CCTATGAACGAACGTTAACAGGTGAAAAAGTGAAGCTGCCATTACAGGTTCAACCGTATT  2257253

GMX5   361        CACCAGAAAGCGGCTTTTGTGGTGTTATGGTCGTGCCTCCTTTGCCGCAGTGCCAAGAGG  420
                  ||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
hypo   2257254    CACCAGAAAGCGGCTTTTGTGGTGTTATGGTCGTGCCTCCTTTGCCCCAGTGCCAAGAGG  2257313

GMX5   421        CATTTATGATGCCGTATCTTGAAAACGGAAAAGAAACCCACGGAGAGTGGCCAGTCTATT  480
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
hypo   2257314    CATTTATGATGCCGTATCTTGAAAACGGAAAAGAAACCCACGGAGAGTGGCCAGTCTATT  2257373

GMX5   481        TCCATACGCCTCTCCCTTTGTACGAAGAAGAAATGGAGTACTATTTCCAGCATGGACAAG  540
                  |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
hypo   2257374    TCCATACGCTTCTCCCTTTGTACGAAGAAGAAATGGAGTACTATTTCCAGCATGGACAAG  2257433

GMX5   541        AAGCGTTGCTAGAAAAGATGATGGAAAATGGCATCGAACACTTATTTAACTTACATAGAC  600
                  |||| |||||||||||||||||||||||||||||||||||||||||| ||||||||||
hypo   2257434    AAGCTTTGCTAGAAAAGATGATGGAAAATGGCATCGAACACTTATTTAATTTACATAGAC  2257493

GMX5   601        CTAATACGTTTAAGGAAAAAAGAAAAGGCTTCTTCGGCAGATTTAATGAGTGATAAATAA  660
                  |||||||||||||||||||||||||||||||| |||||||||||||| ||||||||||||
hypo   2257494    CTAATACGTTTAAGGAAAAAAGAAAAGGCTTTTTCGGCAGATTTAATAAGTGATAAATAA  2257553

GMX5   661        AAAGAGGCTGGGACAAAAGTATGTTAGACGAAGTGAAAAACGAACCATTTATCAACCATG  720
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
hypo   2257554    AAAGAGGCTGGGACAAAAGTATGTTAGACGAAGTGAAAAACGAACCATTTATCAACCATG  2257613

GMX5   721        TTGATGAGTGG  731
                  ||||||||||
hypo   2257614    TTGATGAGTGG  2257624
```

Fig. 4.36: BlastN result of *Bacillus megaterium* GMX5 (*sox*B amplicon of size 1100bp) nucleotide sequence showing 98% homology with the '+' strand of *Bacillus megaterium* hypothetical protein.

4.3.5 Sequence analysis of the soxB amplicons generated with two sets of degenerate soxB primers

BlastN was conducted with all the sequences obtained from soxB amplicons. The nucleotide sequences were determined using T7 and SP6 primers. Partial *sox*B gene sequences of *Burkholderia* sp. GAH2, and GAH4, showed 73% and 77% homology for sulfate thioesterase/sulfate thiohydrolase gene respectively. The nucleotide and translated amino acid sequences were deposited in the Genebank and accessioned (Fig. 4.24-4.27). The other two *Burkholderia* sp. GAH5 and GAH1 though produced soxB amplicons could not showed any homology with sulfate thioesterase/sulfate thiohydrolase gene. GAH5 and GAH1 showed 70% and 64% homology with ABC transpoter gene for ATP binding protein respectively (Fig. 4.28-4.33). Similarly, *Psychrobacter testosteroni* GMX7 showed 85% homology with ABC transporter gene (Fig. 4.34). A 900 bp soxB amplicon generated by GMX7 showed 78% and the 388 bp at 5′ side 82% homology with *P. cryohalolentis* hypothetical protein and thiosulfate binding protein respectively (Fig. 4.35). Among *Bacillus* sp., sequence obtained from soxB amplicon of GMX6 did not produce significant result. However, GMX5 showed 98% homology with *B. megaterium* hypothetical protein (Fig. 4.36).

4.3.6 Phylogenetic analysis of *sox*B gene in thiosulfate metabolizing strains

The sequences derived from GAH2 and GAH4 revealed 93% and 97% amino acid similarity to the *sox*B sequence of *Allochromatium vinosum* respectively. To our knowledge this is the first study to report the *sox*B gene in *Burkholderia* spp. Phylogenetic trees were calculated from aligned amino acid and nucleotide sequences to infer reliable branching orders (Fig. 4.37). Within the phylogenetic tree, previously reported thiosulfate oxidising strains of *Betaproteobacteria*, labelled as *Betaproteobacteria* I, formed a separate cluster with strong bootstrap support and similarity values of 52.7% to 72.6% for the amino acid data. *Burkholderia* strains, GAH2 and GAH4 with 84% similarity at the amino acid level, formed a separate phylogenetic branch, which was labelled as *Betaproteobacteria* II. Other branches representing members labelled as *Gammaproteobacteria* I, *Alphaproteobacteria* II, *Alphaproteobacteria* I, Chlorobia and *Gammaproteobacteria* II strongly substantiated with phylogenetic trees published earlier (Anandham et.al., 2008; Petri et.al., 2001).

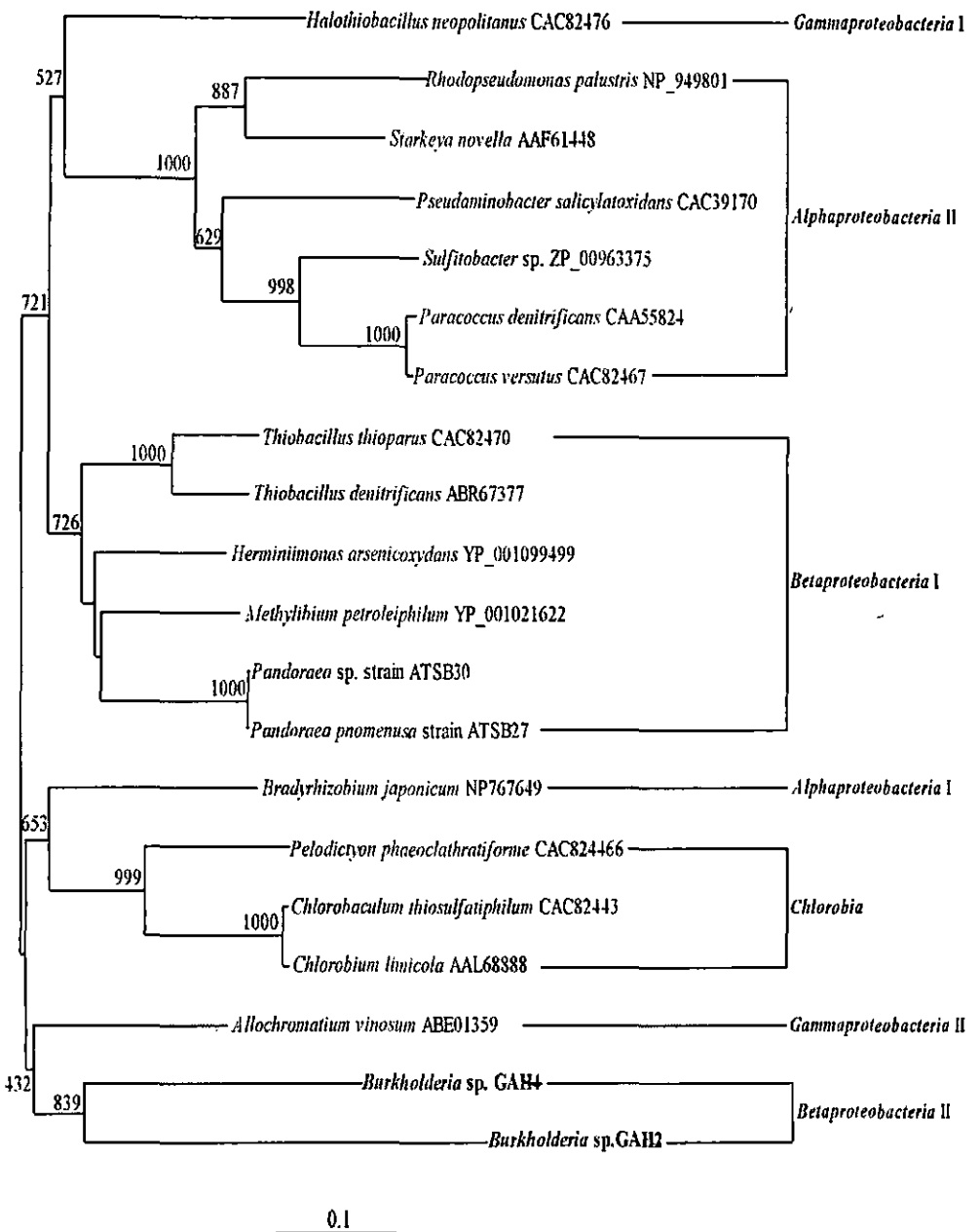

Fig. 4.37: Neighbour-joining phylogenetic trees of thiosulfate metabolising, strains GAH2, and GAH4 based on amino acid sequences of SoxB. Bootstrap values were obtained from 1000 resamplings. More than 50% are indicated on the nodes. The scale bar represents the sequence divergence. Sequences obtained from this study are shown in bold.

4.4 Discussion

16S rRNA sequence comparisons indicated phylogenetic diversity among the isolates studied. The 16S phylogeny of the fifteen acid-tolerant, thiosulfate metabolising bacteria isolated from AMD samples of Garubathan, India, fell into the genus *Burkholderia* , *Comamonas*, *Serratia*, *Psychrobacter*, *Acidiphilium* and *Bacillus* (Fig. 4.3-4.17) of the sub-class *Beta-proteobacteria*, *Gamma-proteobacteria*, *Alpha-proteobacteria* and *Firmicutes*. Despite extremities, a diverse range of bacteria, including representatives of *Proteobacteria*, *Nitrospirae*, *Firmicutes*, and *Actinobacteria*, inhabit AMD environments. Besides the most studied group of bacteria under *Gamma-proteobacteria*, bacteria under *Alpha-* and *Beta-proteobacteria* were also identified in the acid mine environments (Baker and Banfield, 2003; Valverde *et al.*, 2006; Xiao *et al.*, 2009). Reduced sulfur species of sulfidic mineral origin in Garubathan AMD sites of Darjeeling Himalayas favor abundant microbial populations of sulfur oxidizers (both mesophilic and psychrotolerant), including autotrophic *Acidithiobacillus* species as well as heterotrophic sulfur-oxidizing strains. Similar diversity of thiosulfate oxidizing bacteria has been reported from marine sediments and hydrothermal vents (Teske *et al.*, 2000).

High sequence similarities of the 16S rRNA gene sequence of the two strains with *Burkholderia* species and clustering of them with *Burkholderia pyrrocinia* in the phylogenetic tree (Fig. 4.18) suggest that the isolates GAH2 and GAH5 can be assigned as novel acid- and metal-tolerant (see chapter 3) thiosulfate-oxidizing strains of *Burkholderia pyrrocinia.* . In the phylogenetic trees, isolates GMX5 and GMX8 clustered with *Bacillus megaterium* with a bootstrap support of 100%. Another Gram positive heterotrophic strain, GMX6, clustered with *B. cereus* with a bootstrap support of 78%. Isolation and identification of acid-tolerant bacteria from AMD samples from Garubathan, India, revealed diversity of the strains in terms of phylogenetic affiliation and thiosulfate chemolithotrophy. These strains may provide genetic resources for the development of novel biotechnological processes.

The *soxB* gene encodes the SoxB component of the periplasmic thiosulfate-oxidizing Sox enzyme complex, which has been proposed to be widespread among the various phylogenetic groups of sulfur-oxidizing bacteria (SOB) that convert thiosulfate to sulfate with and without the formation of sulfur globules as intermediate. Thiosulfate oxidation pathways operating in sulfur-oxidising bacteria have been tentatively grouped in three categories (Meyer *et al.*, 2007): (a) pathway involving breakdown of thiosulfate to polythionate intermediates by thiosulfate dehydrogenase and tetrathionate hydrolase, which is common in extremophilic sulfur oxidisers (*Acidithiobacillus*, *Thermothiobacillus*, and *Halothiobacillus*) (Kelly *et al.*, 1997); (b) pathway for direct conversion of thiosulfate to sulfate without sulfur globule formation by multienzyme complex (Sox) system, active in photo- and chemotrophic alphaproteobacteria (Friedrich *et al.*, 2001; Mukhopadhyaya *et al.*, 2000); (c) branched thiosulfate oxidation pathway involving formation of sulfur globules operating in sulfur-storing bacteria (Hensen *et al.*, 2006). Sox enzyme system is present in diverse thiosulfate oxidizing bacteria.

Two pairs of degenerate soxB primers (Petri *et al.*, 2001; Meyer *et al.*, 2007) were used to amplify gene sequences. Two of the sequences obtained from the PCR products allowed a first view of the distribution of this gene among sulfur oxidizing *Beta-proteobacteria* and its phylogenetic relationships. Phylogenetic trees constructed with diverse *soxB* gene homologues available in the databases revealed that *Gamma-proteobacteria* and *Alpha-proteobacteria* were not monophyletic and formed at least four and two clusters respectively, while *Beta-proteobacteria* were shown to be monophyletic (Anandham *et al.*, 2008; Meyer *et al.*, 2007; Petri *et al.*, 2001). With introduction of two novel *soxB* gene homologues of *Burkholderia* spp. (strains, GAH2 and GAH4) in the database, the SoxB phylogenetic tree presented in this report has established for the first time that *Beta-proteobacteria* were not monophyletic but formed at least two distinct groups (Fig. 4.37). *Thiobacillus thioparus*, *T. denitrificans*, *Herminimonas arsenicoxydans*, *Methylibium petroleiphilum*, and *Pandorea* spp. formed a distinct cluster (cluster I), congruent to the cluster obtained in an earlier study (Anandham *et al.*, 2008) and the newly described *Burkholderia* strains GAH2 and GAH4 formed a new group among *Beta-proteobacteria* (cluster II). The *soxB* gene

homologues of GAH2 and GAH4 were closest to *soxB* homologue of phototrophic sulfur-oxidising *Allochromatium vinosum* ABE01359. A partial *sox* gene cluster constituted of *soxB* and *soxXA*, detected in by PCR assay, was predicted to be essential for reduced sulfur compound oxidation in *A. vinosum* (Friedrich *et al.*, 2001). Inactivation of *fcc*A and *aprBA* genes in *A. vinosum*, which codes for cytochrome subunit of flavocytochrome c-sulfide dehydrogenase and adenosine-5′-phosphosulphate reductase respectively, left hydrogen sulfide and sulfite oxidation unaffected (Dahl, 1996; Reinartz *et al.*, 1998). Event of lateral *soxB* gene transfer is therefore the most reasonable explanation for the inferred close relationship of *soxB* from thiosulfate -metabolising *Burkholderia* spp. (strains GAH2 and GAH4) and *A. vinosum* species that are distantly related on the basis of the 16S rRNA gene phylogeny.

Interestingly, amplicons generated by degenerate soxB primers produced sequence similar to the genes for ABC transport protein (ATP binding) in GAH1, GAH5 and GMX7 (Fig. 4.29-4.34). Part of gene sequence in GMX7 (Fig. 4.35) was found to possess truncated portion of thiosulfate binding protein. ABC systems constitute probably the largest superfamily of proteins ever detected in living organisms. Most ABC systems are primary transporters that transport unidirectionally molecules through membranes (Schneider and Hunke 1998). The ABC-type drug transporters also belong to the ABC superfamily, the members of which all contain a highly conserved ATP-binding cassette (Higgins 1992; Van Veen *et al.*, 2001). They utilize the energy released by ATP-hydrolysis to pump cytotoxic compounds out of the cell. The substrates handled by these transporters are extraordinarily varied, ranging from small molecules (ions, carbohydrates, amino acids, antibiotics) to macromolecules (polysaccharides, proteins). Purified ABC proteins or modules have been shown to bind and hydrolyze ATP (Dayan *et al.* 1996; Higgins *et al.* 1985; Ko *et al.* 1994; Morbach *et al.* 1993; Muller *et al.* 1994; Shimabuku *et al.* 1992; Thiagalingam and Grossman 1993), making reasonable the hypothesis that they couple the energy of ATP hydrolysis to the movement of substrates by an unknown mechanism.

As the acid-tolerant heterotrophic isolates GAH1, GAH5 and GMX7 isolated from AMD possess facultative thiosulfate chemolithoautotrophic property, the sequence obtained for ATP binding ABC transporters, probably participate in the sulfur metabolism. A number of transport and binding genes appear to play roles in sulfur

metabolism of *A. ferrooxidans*. Recently in one of the study it was seen that the the expression level of transport and binding protein encoding genes for ABC transporter was up-regulated when *A. ferrooxidans* culture was grown on sulfur (Xia *et al.*, 2010). Candidate genes potentially encoding uptake of inorganic sulfate were discovered, including a sulfate permease belonging the SulP family of the major facilitator superfamily of transporters (MFS) and a possible sulfate/thiosulfate/molybdenate ATP binding cassette (ABC)-type transporter in *A. ferrooxidans* (Valdés *et al.*, 2003).

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CHAPTER 5

Characterization of an indigenous plasmid in *Acidiphilium* sp. DK2AH2 and attempt to ascribe its function in relation to metal tolerance

5.1 Introduction

Plasmids are circular double stranded molecule of DNA that exists independently of chromosomal DNA in bacterial cell. These can range in size from several hundred base pairs to several thousand kilobases (Cook *et al.*, 2001). Studies on bacterial plasmids have contributed significantly to our understanding of the mechanisms and regulation of DNA replication, gene expression, and recombination, as well as other areas of prokaryotic biology. To date, many species of bacteria isolated from diverse habitats are known to contain plasmid DNA. Some plasmids are stable and can be maintained through successive generations by being partitioned to each daughter cell during cell division. This allows each cell to receive at least one plasmid copy (Yoo *et al.*, 2001).

5.1.1 Types of Plasmid

There are several types of plasmid present in bacterial cell, which are as follows:

- **Conjugative or F- Plasmid:** This type of plasmids has the ability to transfer the sex factor, gene pickup and production of Hfr Strain. This plasmid helps in bacterial conjugation.
- **R-Plasmid:** This plasmid is a drug resistant plasmid, generally they are antibiotic plasmid. The R- plasmid was first discovered in a strain of *Shigella* in late 1950s in Japan (Partridge and Hall, 2004). Plasmid mediated drug resistant has serious medical implications.
- **Enteric Plasmid:** This type of plasmids produces either heat-stable toxin (ST) or both heat stable or heat labile (ST and LT) toxin. No plasmid coding for LT along has been found.
- **Hly Plasmid:** It codes for the production of α-heamolysins compounds which causes the lyses of the red blood cells.
- **K-Plasmid:** This plasmid specifies the production of species specific antigens, which are large filamentous protein covering the fine pili.

- **Col Plasmid:** Bacteriocins are protein antibiotics produced by bacteria. They are lethal to other bacteria of the same genus or related genus. Certain strains of *E.coli* synthesize bacteriocin (colicins).

- **Degradative Plasmid:** Some microbes have the ability to degrade the organic matter. This degradative enzyme may sometimes be coded by plasmids (Top and Springael, 2003). This type of plasmid is called degradative plasmid. It is found mainly in *Pseudomonas.*

- **Heavy Metal Resistant Plasmid:** Some strains of bacteria, have plasmids which enable the host to tolerate toxic concentration of heavy metal ions. Eg., *Pseudomonas* and *Staphylococcus aureus.*

- **Tumor inducing Plasmid:** A large plasmid in *Agrobacterium tumefaciens* is found to cause the disease crown gal in dicotyledonous plant. These type of plasmids are called Ti-plasmid.

- **Cryptic Plasmid:** Low molecular weight circular DNA molecule has been reported in many genera of bacteria for which no biological role has been established. Therefore these are regarded as Cryptic plasmids.

5.1.2 Plasmid Curing

Bacterial plasmids have a major impact on metabolic function. Bacterial plasmids can be eliminated from bacterial species grown as pure or mixed bacterial cultures in the presence of sub-inhibitory concentrations of non-mutagenic heterocyclic compounds (Spenglar *et al.*, 2006). The anti-plasmid action of the compounds depends on the chemical structure of amphiphillic compounds having a planar ring system with substitution in the L-molecular region. A symmetrical pi-electron conjugation at the highest occupied molecular orbitals favours the anti-plasmid effect. The anti-plasmid effect of heterocyclic compounds is expressed differentially in accordance with the structural form of the DNA to which they bind (Molnár *et al.*, 2003). In this manner "extrachromosomal" plasmid DNA that exists in a superhelical state binds more compound than its linear or open-circular form; and least to the chromosomal DNA of the bacterium, that carries the plasmid. It can also be noted that these compounds are

not mutagenic and their anti-plasmid effects correlate with the energy of HOMO-orbitals.

Plasmid elimination is considered also to take place in ecosystems containing numerous bacterial species. This opens up a new perspective in rational drug design against bacterial plasmids. The inhibition of conjugational transfer of antibiotic resistance plasmid can be exploited to reduce the spread of antibiotic resistance plasmid in the ecosystem. Inhibition of plasmid replication at various stages, as shown in the "rolling circle" model (replication, partition, conjugal transfer) may also be the theoretical basis for the elimination of bacterial virulence in the case of plasmid mediated pathogenicity and antibiotic resistance. The large number of compounds tested for anti-plasmid effects provides opportunities for QSAR studies in order to find a correlation between the anti-plasmid effect and the supramolecular chemistry of these plasmid curing compounds (Molnár *et al.*, 2003). Plasmid elimination *in vitro* provides a method of isolating plasmid free bacteria for biotechnology without any risk of inducing mutations.

Bacterial plasmids are known to harbor genes for: (i) resistances to antibiotics and metals (Foster, 1983; Silver, 1996), (ii) catabolic pathways such as lactose utilization and degradation of hydrocarbons (Chassy *et al.*, 1978; Ghosal *et al.*, 1985), and (iii) biosynthesis of certain antibiotics, etc (Stanisich, 1984). In many cases the characteristics of the host organism conferred by the plasmids remain vague, and such cryptic plasmids are plentiful in nature. Curing of this cryptic plasmid from a bacterial strain is a method to substantiate the relationship between a genetic trait and carriage of that specific trait in the plasmid. Various methods involving chemical and physical agents have been developed to eliminate plasmids. Protocols for curing plasmids consist frequently of exposure of a culture to sub-inhibitory concentrations of some chemical agents, e.g. acridine orange, acriflavine, and sodium dodecyl sulfate or to a super-optimal temperature followed by selection of cured derivatives.

The DNA intercalating agents such as Acridine orange and ethidium bromide are the most commonly used because they are found to be effective against plasmids in a wide variety of genera (Trevors, 1986). Although all of these agents have been used to enhance the recovery of plasmid less derivatives of various bacteria, they are

individually effective only against some plasmids and their likely response is unpredictable. The elimination of a plasmid (curing) from a bacterial culture is the best method to substantiate the relationship between a genetic trait and carriage of specific plasmid by the culture as the phenotypic characters which are associated with the plasmid are not expressed in cured derivatives but on the re-introduction of the plasmid in to the cured strain the lost phenotype is re-appeared. The efficiency of curing can also vary widely depending on the plasmid and the particular bacterial host carrying it. In most instances, the underlying mechanism of curing is not known. The agent may interfere directly with plasmid replication as occurs with the heat induced curing of certain temperature sensitive palsmids or curing of them by acridines or ethidium bromide (Terawaki *et al.*, 1976). Alternatively, curing may interfere with the growth of plasmid carrying bacteria thereby allowing spontaneously arising plasmidless segregants to become predominant. This occurs in certain instances of curing by acridines, sodium dodecyl sulfate and urea.

Methylene blue enhanced the plasmid curing efficiency of chlorpromazine, imipramine and amitriptyline with strains of *Escherichia coli* K12 carrying F-prime *lac* or the resistance factor R-144 (Molnár *et al.*, 1980). In contrast, methylene blue inhibited the elimination of plasmids by acridine orange and ethydium bromide at all concentrations tested. Two metabolic derivatives of chlorpromazine, chlorpromazine sulfoxide and 7.8-dioxochlorpromazine had no plasmid curing effect even in the presence of methylene blue. Amitriptyline, 7,8-dioxochlorpromazine and acridine orange were effective inhibitors of the conjugal transfer of the resistance plasmid, R-144, whilst methylene blue, chlorpromazine sulphoxide, and imipramine had only slight effects (Molnár *et al.*, 1980). A mechanism of plasmid curing by surface action of the drugs is suggested as an alternative to direct intercalation of the drugs into plasmid DNA.

5.1.3 Advantages of plasmid curing methods

- Plasmid displacement methods allow researchers to understand the basis for pathogenicity and antibiotic resistance, resulting in better treatment, vaccination and prevention of infection.
- Pathogenic traits that cause infection and disease are often plasmid encoded.

- Plasmid displacement methods are used to determine whether specific traits are plasmid mediated.
- Bacterial plasmids contain genes that code for additional traits such as antibiotic resistance, heavy metal resistance and biofilm formation.

5.1.4 Plasmid mediated metal resistance

Over the ages, all living systems have evolved to use some metals as vital constituents while they have learned to grapple with some others, which are toxic (Ghosh *et al.*, 1997). The primary source of metals in all ecosystems is the underlying bedrock of the planet. Though many metals are essential, all metal ions are toxic at some level. Heavy metal cations with high atomic masses tend to bind strongly to sulfide groups. The divalent cations of cobalt, nickel, copper and zinc are medium 'sulfur lovers' and these metals (known as trace elements) have essential functions at low concentrations but are toxic at high concentrations (Nies *et al.*, 1989).

The intracellular concentrations of these metals must be finely adjusted to avoid either metal deprivation or metal toxicity and careful homeostasis is necessary (Silver and Phung, 1996). In contrast, homeostasis of the purely toxic metals is simple; the cell quickly eliminate them. Of all life forms, bacteria are not only the oldest, but they also inhabit the greatest diversity of habitats, form a major proportion of the earth's global biomass and have the greatest capacity to sorb metals from the solution, on a biomass to dry weight metal basis (μg/mg dry wt.). Thus they provide the ideal system to study the metal-microbe interactions (Nies, *et al.*, 1989; Springael, *et al.*, 1993; Suzuki *et al.*, 1997). Like all other living organisms microbial communities adapt themselves to the metal concentrations encountered. Two major strategies adopted by the microorganisms to protect themselves against metal toxicity are *avoidance* and *sequestration.* These strategies are reflected in resistance phenotype to one or several metals. A report on plasmid curing from acidophilic bacteria *Acidocella* strain GS19h of acidic mine environment is available (Ghosh *et al.*, 2000). The culture resulted in the elimination of plasmids, suggesting that curing may be achieved in the case of acidophilic bacteria

applying physical or chemical methods. Plasmid-mediated inheritance of metal resistance in this bacterium is also proved.

The obligate acidophilic, metal resistant bacteria *Acidiphilium* sp. DK2AH2 can resist about 450 mM of nickel. The aim of this study is to find the relationship between the metal resistance of DK2AH2 with its plasmid. The molecular characterization of plasmid would certainly reveal considerable information regarding metal resistance mechanisms and their use in constructing genetically engineered high metal tolerating bioleaching bacteria.

5.2 Materials and methods

5.2.1 Determination of metal tolerance profile of DK2AH2

The detailing of the metal tolerance profile is described in chapter 3 under section 3.2.3 and MTC of DK2AH2 is presented in Table 3.2.

5.2.2 Reagents used for plasmid preparation

- Alkaline Solution I:

  50 mM glucose
  25 mM Tris-Cl (pH-8.0)
  10 mM EDTA (pH-8.0)

  Sol. I was prepared from standard stocks in batches of a 100 ml; it was autoclaved for 15 min, at 15 Psi on liq-cycle and stored at 4 ^{0}C.

- Alkaline solution II:

  0.2 (N) NaOH (freshly diluted)
  1% (w/v) SDS

  It was prepared freshly and stored at room temperature.

- Alkaline Solution III:

5(M) KCH_3COO	60.0 ml
CH_3COOH	11.5 ml
H_2O	28.5 ml

The resulting solution is 3 (M) with respect to potassium and 5(M) with respect to acetate. It was stored at 4 ^{0}C.

5.2.3 Isolation of plasmid DNA (Maxi preparation)

Plasmids were routinely isolated by the procedure of Brinboim and Dolly (Brinboim and Dolly, 1979) with slight modifications. Cells of AMD strains harbouring plasmids were grown for 48-56 hours in 1.5 L modified DSMZ 269 medium. The cells were then harvested and resuspended in solution I (1 ml), mixed vigorously and kept at room temperature for 30 min. Solution II (2 ml) was added to it and mixed gently by inverting the tube several times and was kept at room temperature for 15 min. for complete lysis. Chilled solution III (1.5 ml) was added to it and mixed gently by inverting the tube. The mixture was incubated in ice for 30-35 min and then centrifuged at 10, 000 rpm for 20 min at 4 °C. The clear supernatant was taken in a fresh tube. RNaseA (20 μg ml^{-1}) was added and incubated at 37 °C for 2 hours. It was then extracted once by equal volume of phenol: chloroform (1:1) and chloroform respectively. To the aqueous phase 0.7 volume of isopropanol was added and mixed well. The tube was incubated at room temperature for 15 min followed by centrifugation at 12, 000 rpm for 20 min at room temperature. The pellet thus obtained was washed with 70 % ethanol, air-dried and suspended in minimum volume of TE (10 mM: 0.1 mM) buffer.

5.2.4 Isolation of plasmid DNA (Mini preparation)

For screening the recombinant clones from a transformation experiment, individual colonies were incubated in 3 ml LB broth (mini scale isolation) with appropriate antibiotics and culture were grown to saturation at 37 °C overnight. 1.5 ml of culture was taken in individual microfuge and pelleted at 10, 000 rpm for 5 min. The pellet was suspended in 100 μl of solution I, followed by 200 μl of solution II. The suspension was mixed by inverting the tubes and was incubated at room temperature for 5 min for complete lysis. Chilled solution III (150 μl) was added to the tubes and mixed gently. Tubes were chilled on ice for 10 min followed by centrifugation at 10, 000 rpm for 15-20 min at 4 °C. The clear supernatant was transferred to the fresh eppendorfs and 0.7 volume isopropanol was added. Plasmid DNA was pelleted immediately by

centrifugation at 10, 000 rpm for 20 min at room temperature. Pellets were rinsed with 70 % alcohol, air-dried and suspended in 30 µl of TE buffer (pH 8.0) each.

5.2.5 Agarose gel electrophoresis

0.8 % agarose (or less based on the increasing size of DNA fragment to be resolved) in 1X TAE buffer supplemented with 1µg ml^{-1} ethidium bromide was melted in a microwave or boiling water bath and then cooled to 50-60 °C before pouring in a casting tray fitted with a Teflon comb forming wells. DNA loading buffer (1X) was mixed to the sample DNA prior to loading in the wells. Electrophoresis was performed in horizontal electrophoresis tank using 1X TAE buffer containing 1µg ml^{-1} ethidium bromide for 1-2 hours. DNA could be directly viewed in a UV transilluminator.

5.2.6 Plasmid curing by acridine orange treatment

The curing of plasmid using acridine orange was carried out as described by Caro *et al.*, 1984. The concentration range of mutagen was 50 µgml^{-1} to 500 µgml^{-1} with increments of 50 for acridine orange. The protocol for plasmid curing, using the acridine orange is as follows:

- 25ml of modified DSMZ 269 medium was inoculated with the *Acidiphilium* sp. DK2AH2 from fresh slant in a 250 ml flask and incubated on a rotary shaker (120 rev min^{-1}) at 30 ^{0}C for 24 hr.
- To 100 ml of modified DSMZ 269 medium containing 100 µg ml^{-1} acridine orange, the overnight grown pure DK2AH2 culture was inoculated so as to obtain an initial cell density of $10^4 \pm 10^5$ cells ml^{-1}.
- This was incubated on a rotary shaker (120 rev min^{-1}) at 40 °C for 48 hr.
- Cell broth was then suitably diluted and spread on modified DSMZ 269 agar plates and incubated at 28-30 °C for 48 hr till generation of the colonies.

5.2.7 Recovery of plasmid cured colonies

- Well grown discrete colonies of acridine orange treated DK2AH2 strain were selected using sterile toothpicks and placed on master plates which was prepared of modified DSMZ 269 medium. The master plates were then incubated at 30 °C for 48 hr.

- Colonies on master plates were replica-plated onto modified DSMZ 269 agar plates supplemented with metal salts [Zn(II), As(III), Ni(II), Co(II), and Cu(II)] and incubated for 48 hr at 28-30 °C. Colonies unable to grow in metal containing plates were picked up from the master plate and checked further for the disappearance of plasmid DNA cured (by alkaline lysis method described previously). Plasmid-containing (uncured) culture as positive control was included in every batch of plasmid isolation.

5.2.8 Protein profiling of cured (DK2C) and uncured (DK2AH2) cultures

Protein profiling by SDS-PAGE was done following the protocol described in details in the chapter 1 under section 1.2.5.1.

5.2.9 Detection of biofilm formation in DK2AH2 and DK2C cultures

In the present study biofilm formation of DK2AH2 and DK2C cultures were investigated. The screening was done by flask method (Southey-Pillig *et al.*, 2005). The strains formed biofilm in 100 ml flasks.

Materials used:-

- DK2C culture
- DK2AH2 culture
- Crystal Violet stain
- Decolourizer (70% alcohol)

Procedure:-

- In two separated 500 ml conical flasks [containing modified DSMZ 269 medium (pH 3)]the cultures DK2AH2 and DK2C were inoculated and incubated for 4-5 days.

- After proper heavy growth the cultures were discarded and the conical flasks were heated in hot air oven for 15 min. .

- After drying, the bottom of the flasks were flooded with 1% crystal violet to stain the cells that were present on the biofilm attached to the bottom of the flasks.

- After 10 mins of incubation stains were washed with distilled water and the flasks were air dried.

- To the dried flasks decolourizer (70% alcohol) was added.

- After a short incubation period the cells were examined in colorimeter at 470 nm.

5.2.10 Restriction digestion of the plasmid DNA

The reaction buffer used for the restriction enzymes in both single and double digestions were as specified by the supplier. A typical 50 µl reaction mixture contained: DNA of interest, 1-2 µg (1-5 µl or more); 10X digestion buffer (5 µl, 1X); 100X BSA (0.5 µl, 1X when required); restriction enzyme 10 units; sterile double distilled water upto 50 µl. The digestion mixture was mixed well and briefly centrifuged. It was incubated at optimum temperature (generally 37 °C) as specified by the vendors for 2-4 hours. The reaction was stopped by heat inactivation at 65 °C for 10 min. In addition to inhibiting the activity of the present enzyme, this step is necessary to minimize the negative effects on the performance of the following enzymatic reactions.

5.2.11 Elution of plasmid DNA from low melting agarose gel

1.5% low melting agarose purchased from SRL Company, India was used to isolate restricted fragments of plasmid DNA of about 1 µg or less. The gel was run for a required period of time under cold condition. After completion of the run, required DNA band was sliced with the help of sterile scalpel and taken in an eppendorf tube. The gel was melted by keeping in the water bath at 65 °C for 5 minutes to which an equal volume 1X TE was added. The mixture was freeze-thawed thrice and the agarose particles were then removed by phenol extraction. Finally, sliced plasmid DNA was precipitated in isopropanol and was re-suspended in 0.1 X TE buffer and stored at -20 °C.

5.2.12 Ligation of the restricted fragments into pBS vector

0.1 µg of the vector DNA (pBS) was transferred to a sterile microfuge tube. Equimolar amount of restricted fragments of DNA was added to it. Sterile double distilled water was added to make the volume to 8.5 µl and the solution was warmed to 45 °C for 5 minutes to melt any cohesive termini that have re-annealed. Then the mixture was chilled to 0 °C. To the mixture 1 µl of 10X T4 DNA ligase buffer [Tris.HCl - 600 mM; $MgCl_2$ – 50 mM, Dithioerythritol (DTT) - 10 mM and ATP - 10 mM (pH 7.5)] and 0.1 Weiss unit T4 DNA ligase was added. The reaction was incubated for 4 hours at 16 °C. 1-2 µl of each of the ligation mixture was used to transform competent *E. coli* cells.

5.2.13 Preparation of competent cells and transformation of *E. coli* cells

Detailed description of the method is given in chapter 4 under section 4.2.5.1 and 4.2.5.2.

5.2.14 Identification of bacterial colonies that contain recombinant plasmids by α-complementation method

To a pre-made Luria–Bartani agar plate containing 50 $\mu g ml^{-1}$ ampicillin, 0.5 mM isopropylthiogalactosidase (IPTG) and X-gal (80 $\mu g \mu l^{-1}$) transformed competent cells were plated as described earlier. It was possible to recognize colonies that carry

putative recombinant plasmids by blue-white screening of the colonies. Insertion of foreign DNA into the polycloning site of plasmid pBluescript KS(+) leads to the incapability of complementation. Bacteria carrying recombinant colonies are therefore white colonies. Recircularized vectors containing bacteria could utilize chromogenic substrate X-Gal and thus form blue colonies.

5.2.15 Checking of clones for metal tolerance

E. coli cells containing recombinant plasmids were selected and purified. LB plates supplemented with metal salts [Zn(II), 10 mM; As(III), 0.25 mM; Ni(II), 25 mM; Co(II), 2 mM; and Cu(II), 0.3 mM] were prepared. Clones of *E. coli* cells were streaked on respective metal containing LB plates. Clones showing positive growth on these plates were selected and plasmid isolation was done by alkaline lysis (mini prep.).

5.2.16 Sequencing of the clones

The recombinant plasmids were directly used for sequencing of the inserts using primers for T7 and T3 promoters. Nucleotide sequencing was performed with the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kits (Perkin-Elmer) using specific primers and the reaction was analyzed in an 'AB1 PRISM 377 DNA Sequencer'.

5.2.17 Analysis of the sequences

Tools and techniques used for the analysis of the sequences is described in chapter 4 under section 4.2.7

5.3 Results

5.3.1 Metal resistance profile of *Acidiphilium* sp. DK2AH2

Strain	Co(II)	Ni(II)	Cu(II)	Zn(II)	As(III)
	mM	mM	mM	mM	mM
DK2AH2	5.0	450.0	0.3	30.0	1.0

5.3.2 Detection of Plasmids in *Acidiphilium* sp. DK2AH2 and restriction analysis of the plasmid pDK2AH2

Plasmid was detected in only one bacterial isolate viz., *Acidiphilium* sp. DK2AH2 among the fifteen bacterial acid-tolerant heterotrophic isolates tested [subject to experimental limitation in following only one method of plasmid extraction for all test strains (by alkaline lysis method, both maxi and mini preparation)]. The obligate acidophilic, metal resistant bacteria *Acidiphilium* sp. DK2AH2 contained multiple (five) plasmids. The molecular sizes of different plasmids were determined by using size markers of standard plasmids of known molecular sizes (Macrina *et al.*, 1982). The size of the largest plasmid detected and isolated was named pDK2AH2; the size of which was determined to be 55 kb. In descending order, the sizes of the plasmids obtained were approximately 55 kb, 23kb, 4 kb, 2 kb, and 1 kb (Fig. 5.1). The biggest plasmid (approx. 55 kb) was sliced off from low melting point agarose gel (after separation of plasmids), purified, checked, and used as stock plasmid DNA (pDK2AH2) for further analysis. The restriction patterns of the **plasmid,** pDK2AH2, by using different restriction enzymes were studied. *Eco*RI restricted fragments were cloned in pBS (described later in the section).

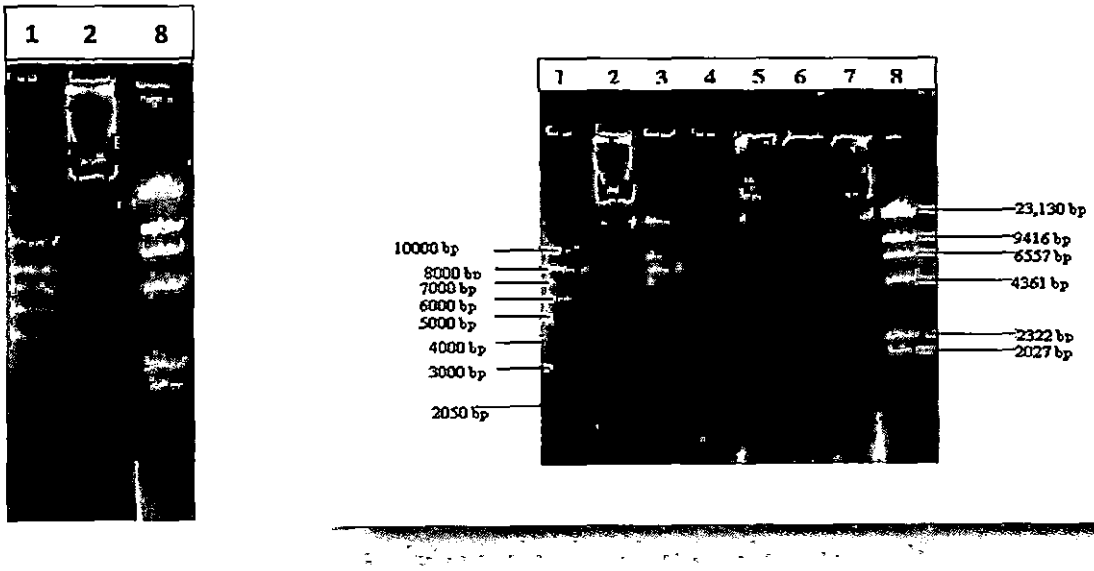


Fig. 5.1a: size determination and restriction digestion patterns of DK2AH2 plasmids.

Lane 1: super coiled plasmid DNA ladder

Lane 2: plasmid profile of DK2AH2

Lane 3: λDNA *Eco*RI digest (marker)

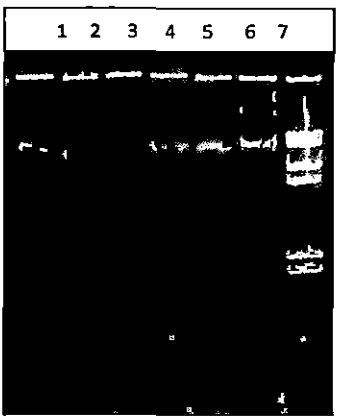
Lane 4: blank

Lane 5: *Pst*I digestion pattern of DK2AH2 plasmids

Lane 6: *Bam*HI digestion pattern of DK2AH2 plasmids

Lane 7: *Pvu*I digestion pattern of DK2AH2 plasmids

Lane 8: λDNA *Hind*III digest (marker)



Lane 1- *Not* I digested pattern of DK2AH2 plasmids

Lane 2- *Xho* I digested pattern of DK2AH2 plasmids

Lane 3- *Nco* I digested pattern of DK2AH2 plasmids

Lane 4- *Sma* I digested pattern of DK2AH2 plasmids

Lane 5- *Spe* I digested pattern of DK2AH2 plasmids

Lane 6- DK2AH2 plasmids

Lane 7- λ *Hind* III digest (marker)

Fig. 5.1b: size determination and restriction digestion patterns of DK2AH2 plasmids.

Nine different restriction enzymes were used to digest the pDK2AH2. The detailed calculations for the fragments generated by the restriction enzymes are given in Table 5.1. Six restriction enzymes viz., *Pst* I, *Bam* HI, *Pvu* I, *Sma* I, *Spe* I, and *Not* I could not digest the pDK2AH2. Three restriction enzymes *Eco* RI, *Xho* I, and *Nco* I could generate fragments of different sizes (Table 5.1).

Table. 5.1: Size and number of fragments generated after digestion of plasmid DNAs with different restriction endonucleases

Restriction enzyme	*Number of fragments*	*Number of sites*	*Size of fragments (kb)*	*Total length (kb)*
Eco RI	**6**	**6**	**17,15,9,6,5,3**	**55**
Pst I	---	---	---	---
Bam HI	---	---	---	---
Pvu I	---	---	---	---
Xho I	**6**	**6**	**14,12,10,8,6,3**	**55**
NCO I	**5**	**5**	**15,12,10,8,6**	**51**
Sma I	---	---	---	---
Spe I	---	---	---	---
Not I	---	---	---	---

5.3.3 Curing of plasmids in *Acidiphilium* sp. DK2AH2 [using Acridine Orange (AcOr)]

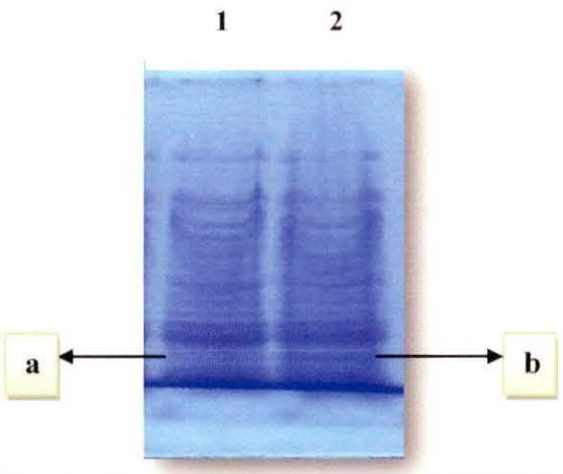
The culture of *Acidiphilium* sp. DK2AH2 was subjected to curing of the plasmids from the strain DK2AH2 using AcOr (100 μgml^{-1}). The plasmid-less isolate was obtained and was named as DK2C. The absence of plasmid DNA in the cured isolate, DK2C was verified by alkaline lysis method. Interestingly, losses of all five plasmids were observed. This indicates a curing frequency of almost >80% for plasmids in DK2AH2 strain using this method, which is comparatively higher than the frequencies normally obtained using traditional curing techniques.

Two of the metal resistant properties of the strain DK2C were found to be lost. The metals, Cu(II) and Zn(II) supplemented modified DSMZ 269 agar plates showed no growth of the DK2C strain. Besides the loss of Cu(II) and Zn(II) resistance phenotype, DK2C was also found incapable of biofilm formation (result has been described in 5.3.4)

Lane 1 : pDK2AH2

Lane 2 : AcOr treated pDK2AH2

Fig. 5.2: Plasmid profile of cured DK2C and uncured DK2AH2 strains of *Acidiphilium* sp.



Lane 1: strain DK2C

Lane 2: strain DK2AH2

a- absent, b- present

Fig. 5.3: Protein profile of DK2C and DK2AH2 strains

Whole cell protein profile of the two strains, DK2AH2 and DK2C was found to be identical, except the absence of one band in DK2C. It confirmed the purity of the strain (i.e DK2C is directly derived from DK2AH2 by dispensing the plasmids only); presence of identical protein bands except absence of one single band may signify loss of expression of certain plasmid encoded proteins.

5.3.4 Biofilm formation phenotype of *Acidiphilium* sp. DK2AH2 and DK2C

Biofilm formation character was found to be associated with plasmid genes. This finding was confirmed by checking the biofilm formation in the plasmid bearing DK2AH2 and plasmidless DK2C culture and the percentage of formation of biofilm in DK2C was nearly negligible.

Fig. 5.4: Production of biofilm by DK2AH2

Fig. 5.5: No biofilm Production by DK2C

5.3.5 Cloning of the pDK2-5 and pDK2-9 fragments of pDK2AH2

The restriction fragments of pDK2AH2 generated by *Eco* RI were subjected to shotgun cloning purpose (Sambrook *et al.*, 1989), using the vector pBS KS^{+}. The recombinant molecule (vector+insert) was transformed into *E.coli* XL1blue. Three positive clones viz., pDK2-3, pDK2-5, and pDK2-9 were found and were subjected to restriction digestion by *Eco* RI. The purified clones were sequenced and analysed.

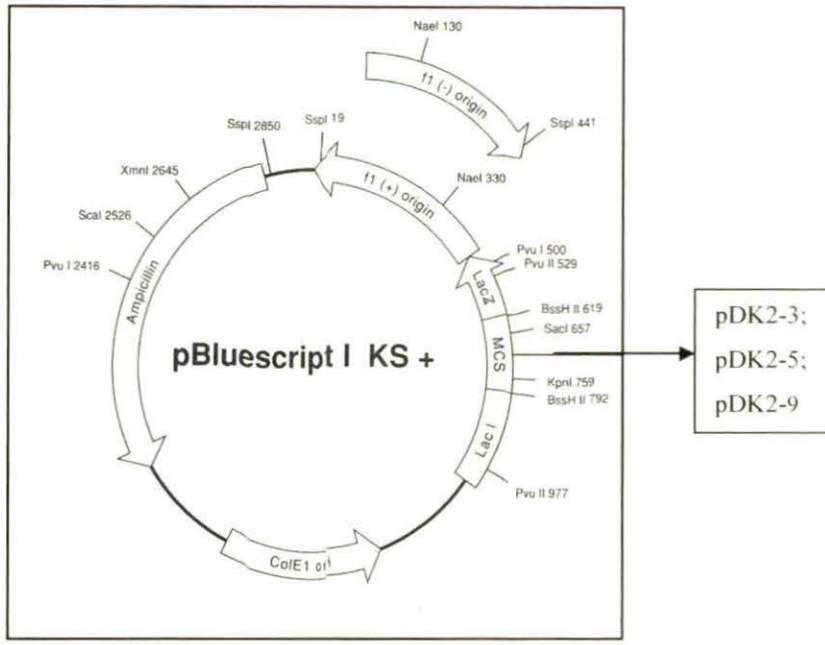


Fig. 5.6: Diagrammatic representation of pBS KS+ vector containing 5 kb and 9 kb insert DNA of pDK2-3, pDK2-5, and pDK2-9

pBluescript II KS(-), pBluescript II KS(+)

M13/pUC sequencing primer (-20), 17-mer (#S0100) — T7 promoter — T7 transcription start

```
5′ G TAA AAC GAC GGC CAG TGA GCG CGC GTA ATA CGA CTC ACT ATA GGG CGA ATT
3′ C ATT TTG CTG CCG GTC ACT CGC GCG CAT TAT GCT GAG TGA TAT CCC GCT TAA
LacZ ←— Val Val Ala Leu Ser Ala Arg Tyr Tyr Ser Glu Ser Tyr Pro Ser Asn
```

653 — Ecl136II, SacI, BstXI, BtgI, Cfr42I, OliI, Eco52I, NotI, XbaI, BcuI, BamHI, Cfr9I, SmaI, PstI, EcoRI

```
GGA GCT CCA CCG CGG TGG CGG CCG CTC TAG AAC TAG TGG ATC CCC CGG GCT GCA GGA ATT C
CCT CGA GGT GGC GCC ACC GCC GGC GAG ATC TTG ATC ACC TAG GGG GCC CGA CGT CCT TAA G
Ser Ser Trp Arg Pro Pro Pro Arg Glu Leu Val Leu Pro Asp Gly Pro Ser Cys Ser Asn
```

Eco32I, HindIII, Bsu15I, HincII, SalI, XmiI, PspXI, XhoI, Eco0109I, ApaI, Bsp120I, Acc65I, KpnI — 760

```
GA TAT CAA GCT TAT CGA TAC CGT CGA CCT CGA GGG GGG GCC CGG TAC CCA
CT ATA GTT CGA ATA GCT ATG GCA GCT GGA GCT CCC CCC CGG GCC ATG GGT
Ser Ile Leu Ser Ile Ser Val Thr Ser Arg Ser Pro Pro Gly Pro Val Trp
```

```
GCT TTT GTT CCC TTT AGT GAG GGT TAA TTG CGC GCT TGG CGT AAT CAT GGT CAT AGC TGT TTC CTG 3′
CGA AAA CAA GGG AAA TCA CTC CCA ATT AAC GCG CGA ACC GCA TTA GTA CCA GTA TCG ACA AAG GAC 5′
```

T3 transcription start — T3 promoter — M13/pUC reverse sequencing primer (-26), 17-mer

```
Ser Lys Asn Gly Lys Thr Leu Thr Leu Glu Ser Ser Pro Thr Ile Met Thr Met
```

5.3.6 Analyses of pDK2-3, pDK2-5, and pDK2-9 sequence

BlastN was performed using NCBI tool which revealed that the sequence of pDK2-5(Fig. 5.9 and 5.11) and pDK2-9 (Fig. 5.13 and 5.15) are >80% homologous to the hypothetical protein of *Acidiphilium multivorum* AIU301 plasmid and hypothetical protein of *A. cryptum* JF5 (Fig. 5.10a,b and 5.14a,b). Similarly, further sequence analysis of pDK2-5 and pDK2-9 revealed that -2 frame of the sequence contained an open reading frame (ORF) (164 and 101 amino acid long respectively), showing 100% and 81% homology respectively with hypothetical protein APM_0575 of *Acidiphilium* sp. PM.

Partial nucleotide sequence of pDK2-3 was done with T3 primer (Fig. 5.7) which revealed that +2 frame of the sequence contained an ORF showing 74% homology with hypothetical protein of uncultured Sphingobacteria and glycosyl transferase protein of uncultured Flavobacteria (Fig. 5.8a); and -1 frame showing 71% homology with conserved domain protein of *E. coli* MS 69-1 (Fig. 5.8b).

```
CATTACCCTGCACTGGCCGACTGTTTACCCCGACCGGACTGGCAAAACCCTGCCGTTACCCGGCTT
AATCGCCTTGAACCACATCCCCCTTTCGCCAGCTGGAGAAATACCGAAGAGGCCCGCACCGATCCC
CCTTCCCAACATTTGCGCCCCCTGAATGGCGAATGGGACGCGCCCTGTAGATCCGCATTAAGCGCG
GCGGGCGGGGTGCTTACACGCAGAGTGACCGCTACATTTTGGAGCGCCGTACCGCCTGCTACTTTA
TATTTCTTACCTTCGTTTGTCGCCCCGTTCGCCAGCTTTTCGGTCACCTCTAAATACTGCCCTCCC
TTTACGATGACGATTGTGGCTTCACGGCCCTGCGACCCACACAACTTGATTATCTGATGGTTGCGT
ATGATATCATCGGCTGATACCCGTATCTCCACGTTTGATTGTGGGACACGTCTGCCAGGGCGGATC
GTGTGCGATTGCGCAAACTCAGCTTATGACGTCTATTATTTGATTCATCAGGTATTTGCCGATGCA
GCCGATTAGGTTGAGATGATCTGATTCAGC (558)
```

Fig. 5.7: Partial nucleotide sequence of pDK2-3; sequence done with T3 primer.

```
  2 attaccctgcactggccgactgtttaccccgaccggactggcaaa
      I  T  L  H  W  P  T  V  Y  P  D  R  T  G  K
 47 accctgccgttacccggcttaatcgccttgaaccacatccccctt
      T  L  P  L  P  G  L  I  A  L  N  H  I  P  L
 92 tcgccagctggagaaataccgaagaggcccgcaccgatccccctt
      S  P  A  G  E  I  P  K  R  P  A  P  I  P  L
137 cccaacatttgcgccccctga 157
      P  N  I  C  A  P  *                    (a)
```

Fig. 5.8a: ORF obtained from pDK2-3 insert. **a**, 51 amino acid long at +2 frame showing 74% homology with hypothetical protein of uncultured Sphingobacteria (1) and glycosyl transferase protein of uncultured Flavobacteria (2).

1. hypothetical protein S18_1049_0001 [uncultured Sphingobacteria]

```
pDK2-3    1  ITLHWPTVYPDRTGKTLPLPGLIALNHIPLSPAGEIPKRPAPIPLPNICA  50
             IT+HWP+ Y   TGKTL LP LIAL HIPLSPAG I KRPAPI LPN CA
hypothet 18 ITIHWPSFYNVVTGKTLALPNLIALQHIPLSPAGVIAKRPAPIALPNSCA  67
```

2. glycosyl transferase protein,family 1 [uncultured Flavobacteria]

```
pDK2-3 1    ITLHWPTVYPDRTGKTLPLPGLIALNHIPLSPAGEIPKRPAPIPLPNICA  50
            IT+HWP+ Y   TGKTL LP LIAL HIPLSPAG I KRPAPI LPN CA
gly tra 212 ITIHWPSFYNVVTGKTLALPNLIALQHIPLSPAGVIAKRPAPIALPNSCA  261
```

```
144 atgttgggaaggggatcggtgcgggcctcttcggtatttctcca
    M  L  G  R  G  I  G  A  G  L  F  G  I  S  P
 99 gctggcgaaaggggatgtggttcaaggcgattaagccgggtaac
    A  G  E  R  G  M  W  F  K  A  I  K  P  G  N
 54 ggcagggttttgccagtccggtcggggtaa 25
       G  R  V  L  P  V  R  S  G  *        (b)
```

Fig. 5.8b: ORF obtained from pDK2-3 insert. **b**, 39 amino acid long at -1 frame showing 71% homology with conserved domain protein of *E. coli* MS 69-1.

conserved domain protein [Escherichia coli MS 69-1]

```
pDK2-3    1   MLGRGIGAGLFGISPAGERGMWFKAIKPGNGRVLPVRS  38
              +LGR IGAGLF I+PAGERGM  KAIK GN RV PV +
cons dm   30  LLGRAIGAGLFAITPAGERGMCRKAIKLGNARVFPVTT  67
```

```
GCGATGGGAGACCATAGAGACAGAGGAAAGAAAAAAAAAAACACACACGAACAACACGCCAAACAA
TGACACCGGGGGAGTACGAAAAAGAGAGAGAAGAGGAAGAAGAGAAAAAAAAACGGGCTGACATTC
TTATCCTCCTTCTCAGCTGAAATGTGATACCCTCCTGATCGAGGCAGGGGCGCGGGCGAAGGCGGT
TTGATGCGCACGAACTGTGACAGTATGACTTGCTCCCTTCCTCGTGCCATGCGCGCCGGCGATTTC
AAGCCGATGAACATGCCGAATAACCGACACCGCGATCGAGACAGAAAATCACGCGCCGCCTTCGGC
CACGAGCCGTCCATCTCGGCGAGGTCTTGCCCCGCCTCAACAGAATACTCCACTCTTTGCTATTGT
CTCGAAACCCGGATGGCGGTCCTCCCGCGGATCGAGGTGTGGGTGACTGATCTGGAGACTCTGCAC
ACTCGCCACCGCGCCGGACTCCCCCTCCCTGCCTCCGTCCCGAAAAATCC    (512)
```

Fig. 5.9: Partial nucleotide sequence of pDK2-5; sequence done with T7 primer.

```
pDK2-5     155     TGTGATACCCTCCTGATCGAGGCAGGGGCG-CGGGC-GA-AGGCGGTTTGATGCG-CACG  210
                   ||||||||||| | || | | |||||||||| ||||| || |||||||||||||| ||||
Ac mult 205021     TGTGATACCCAC-TG-TAG-GGCAGGGGCGTCGGGCCGAGAGGCGGTTTGATGCGGCACG
205077

pDK2-5     211     AACTGTGACAGTATGACTTGCTCC--CTTCCTCGTGCCATGCGCGCCGGCGATTTC-AAG  267
                   ||   |||| || ||||| |||||  ||||||||||||||||||||||||||||||| |||
Ac mult 205078     AA---TGACGGT-TGACTAGCTCCAGCTTCCTCGTGCCATGCGCGCCGGCGATTTCAAAG
205133

pDK2-5     268     CC-GATGAAC-ATGCC-GAATAACCGAC---ACCGCGATCGAGACA--GAAAATCA--CG  317
                   || ||||||| | ||| ||||||| |||    ||||||||||||||   |||||||   |
Ac mult 205134     CCAGATGAACAACGCCAGAATAACGGACCAGGCCGCGATCGAGACAAGAAAAATCAACAG
205193

pDK2-5     318     CGCCGCCTTCGGCCA--CGAGCCGTCCA  343
                   ||||||||||||||||  |||||||| ||
Ac mult 205194     CGCCGCCTTCGGCCAGGCGAGCCGTTCA  205221                    (a)
```

```
pDK2-5     225       GACTTGCTCC-CTTCCTCGTGCCATGCGCGCC----GGCGATTTCAAGCC--GATGAACA  277
                   |||| ||||  | |||||| ||| ||||||||    || ||  |||  ||  ||||| ||
Gly tra    2065010   GACTGGCTCGGCCTCCTCGCGCCGTGCGCGCCAAACGGAGACCTCAGACCCAGATGATCA  2065069

pDK2-5     278       TGCCGA--ATAACCGACA---CCGCGATCGAGAC-AGAAAA-TCAC--GCGCCGCCTTCG  328
                   |||| || || | |    |||||| |||||| |||||| |||   ||||||||||||
Gly tra    2065070   ATCCGAGAATGACGGCCCAGGCCGCGACCGAGACGAGAAAAATCAGCAGCGCCGCCTTCG  2065129

pDK2-5     329       GCCA--CGAGCCGTCCA  343
                     ||||  |||||||| ||
Gly tra    2065130   GCCAGGCGAGCCGTTCA  2065146                              (b)
```

Fig. 5.10: BlastN result of pDK2-5 nucleotide sequence **(a)** showing 83% homology with + strand of *Acidiphilium multivorum* AIU301 plasmid pACMV1, Features flanking this part of subject sequence: 63 bp at 5' side: hypothetical protein; 484 bp at 3' side: hypothetical protein and **(b)** showing 72% homology with + strand of *Acidiphilium multivorum* AIU301, Features flanking this part of subject sequence: putative glycosyltransferase and hypothetical protein.

```
TGGATTCCGGAACTGAGCCCCAGGAAGGTCGCCGTACCCAACTGGGTGGCCATTCCGTTCAGCGCA
TTGCTCGCGTCGTAGTAAGTGGTCTCGGTTACACTCGATAGCGCGCCCGAGGGCTGGTTCAGCGTG
AACCCGCTCAGGAAGGACTGGATGCCGGTCGGCGACGTGATTCCGGTTTCCGACGCGTAGAGATAC
AGCGTGCCACCCGCAGACGTGGAGGTATTGATGCTGTTGGTGCTAAAGTTGTCTGCAGGCAGAAGA
TCGCCGCTGCTACCGATCTTGTTGATGGTGAACGTACCGAAAGAGCCATTGTAGTAGGGTCCGCCC
GGGGCCGGAGACTGCGTGACATAATTCGCGTAACCGGCCTCCTGCAGGGTGATGGTGATCACAGGC
GTCGCATGGGCGACCGGCGTTCCGACCAGAGCAATGGCGCCGCATATCGCTGCGCCGAGTAATGTC
GATTTCAGTGATTTACGTTTCATTGTCCTCATTTCCTAGTCGCCTTGGCCACGAAGTTGCTGCCGT
TTGCCGTAAAATTTGATGATGCGCGTTTACCGCTAAATTTTGAAAGCAAAAACAATGCCATTTTTA
TTAGCTTTTGTTTTCAATACCTTATCATTACGTTTCGGCACG     (636)
```

Fig. 5.11: Partial nucleotide sequence of pDK2-5; sequence done with T3 primer.

```
494 atgaggacaatgaaacgtaaatcactgaaatcgacattactcggc
    M  R  T  M  K  R  K  S  L  K  S  T  L  L  G
449 gcagcgatatgcggcgccattgctctggtcggaacgccggtcgcc
    A  A  I  C  G  A  I  A  L  V  G  T  P  V  A
404 catgcgacgcctgtgatcaccatcaccctgcaggaggccggttac
    H  A  T  P  V  I  T  I  T  L  Q  E  A  G  Y
359 gcgaattatgtcacgcagtctccggccccgggcggaccctactac
    A  N  Y  V  T  Q  S  P  A  P  G  G  P  Y  Y
314 aatggctctttcggtacgttcaccatcaacaagatcggtagcagc
    N  G  S  F  G  T  F  T  I  N  K  I  G  S  S
269 ggcgatcttctgcctgcagacaactttagcaccaacagcatcaat
    G  D  L  L  P  A  D  N  F  S  T  N  S  I  N
224 acctccacgtctgcgggtggcacgctgtatctctacgcgtcggaa
    T  S  T  S  A  G  G  T  L  Y  L  Y  A  S  E
179 accggaatcacgtcgccgaccggcatccagtccttcctgagcggg
    T  G  I  T  S  P  T  G  I  Q  S  F  L  S  G
134 ttcacgctgaaccagccctcgggcgcgctatcgagtgtaaccgag
    F  T  L  N  Q  P  S  G  A  L  S  S  V  T  E
 89 accacttactacgacgcgagcaatgcgctgaacggaatggccacc
    T  T  Y  Y  D  A  S  N  A  L  N  G  M  A  T
 44 cagttgggtacggcgaccttcctggggctcagttccggaatcca 1
    Q  L  G  T  A  T  F  L  G  L  S  S  G  I
```

Fig. 5.12: ORF obtained from pDK2-5 insert. 164 amino acid long at -2 frame showing 100% homology with hypothetical protein APM_0575 of *Acidiphilium* sp. PM.

```
                hypothetical protein APM_0575 [Acidiphilium sp. PM]

 pDK2-5 4    MKRKSLKSTLLGAAICGAIALVGTPVAHATPVITITLQEAGYANYVTQSPAPGGPYYNGS  63
             MKRKSLKSTLLGAAICGAIALVGTPVAHATPVITITLQEAGYANYVTQSPAPGGPYYNGS
 hypoth 1    MKRKSLKSTLLGAAICGAIALVGTPVAHATPVITITLQEAGYANYVTQSPAPGGPYYNGS  60

 pDK2-5 64   FGTFTINKIGSSGDLLPADNFSTNSINTSTSAGGTLYLYASETGITSPTGIQSFLSGFTL  123
             FGTFTINKIGSSGDLLPADNFSTNSINTSTSAGGTLYLYASETGITSPTGIQSFLSGFTL
 hypoth 61   FGTFTINKIGSSGDLLPADNFSTNSINTSTSAGGTLYLYASETGITSPTGIQSFLSGFTL  120

pDK2-5  124  NQPSGALSSVTETTYYDASNALNGMATQLGTATFLGLSSGI  164
             NQPSGALSSVTETTYYDASNALNGMATQLGTATFLGLSSGI
hypoth  121  NQPSGALSSVTETTYYDASNALNGMATQLGTATFLGLSSGI  161
```

```
ATCAGGCTGGTTGTGATACCCACTGTAGGGCAAGGGGCGTCGGGCCGAGAGGCGGTTTGATGCGGC
ACGAATGACGGTTGACTTGCTCCAGCTACTCGGTGCCATGCGCGCCGGCGATTTCAAAGCCAGATG
AACAATGCCAGAATAACCGACCAGGCCGCGATCGAGACAAGAAAAAATCAACAGCGGCGGTTTTCG
GCAAGGCAAGCTTTTCAGCACTCCTCGTGGCGTAATGTATTCTGGCCCCCGGCACTCAGAAGCGAA
ATAACTTCCCACAACCAATTTGCATCAATTGTATCCATACGGCGAAGAACTCCGCGTGATTGGGGG
GCGGTGTTCAACATCTCCCGGGTCCGTTGATCAGGTGCCTGGGCGGCTGTACTTGATTCTTGCTTG
AGGATCAGTCATGCCATCAGTCCCCGCCGATCCGCCTGCCTGAAGAACTTTTGAACCGCCCGGGCC
TCTCTTTGCCGAAGTCAATCTCGTCCATTGCCCCATGGGGGAAGGGCGAAACTTAAACCTCCTAAT
TTCGCCCAAGTTTTGTGAAGTTGGGAACAAACTATAAAGGGCGGGTTGGTCTCAAAATTCGGGAAA
AGGGCCTCCTTTTTTGGGAAGAGGCC    (620)
```

Fig. 5.13: Partial nucleotide sequence of pDK2-9; sequence done with T7 primer.

```
pDK2-9   1       ATCAGGCTGGTTGTGATACCCACTGTAGGGCAAGGGGCGTCGGGCCGAGAGGCGGTTTGA  60
A mul    205010  ATCAGGCTGGTTGTGATACCCACTGTAGGGCA-GGGGCGTCGGGCCGAGAGGCGGTTTGA  205068

pDK2-9   61      TGCGGCACGAATGACGGTTGACTTGCTCCAGCTACTCGGTGCCATGCGCGCCGGCGATTT  120
A mul    205069  TGCGGCACGAATGACGGTTGACTAGCTCCAGCTTCCTCGTGCCATGCGCGCCGGCGATTT  205128

pDK2-9   121     CAAAGCCAGATGAACAATGCCAGAATAACCGACCAGGCCGCGATCGAGACAAGAAAAAAT  180
A mul    205129  CAAAGCCAGATGAACAACGCCAGAATAACGGACCAGGCCGCGATCGAGACAAG-AAAAAT  205187

pDK2-9   181     CAACAGCGGCGGTTTTCGGCAAGGCAAGCTTTTCAGCACTCCTCGTGGCGTAATGTATTC  240
A mul    205188  CAACAGC-GCCGCCTTCGGCCAGGCGAGCCGTTCAGCA-TCCTCGTGGCGT-ATGTATCT  205244

pDK2-9   241     TGGC----------------------CCCCGGCACTCAGAAGCGAAATAACTTCCCACAAC  279
A mul    205245  TGCCGCGGCACTCAGATGTATCTTGCCGCGGCACTCAGACGCGAAATAAC-TCCGAC-AT  205302

pDK2-9   280     CAATTTGCATCAATTGTATCCATACGGCGAAGAACTCCGCGTGATTGGGGGGCGGTG-TT  338
A mul    205303  CAATTTGCATCGATTGTATCCATATGGCGAAGACCTCGGCGTGATTGGGGGGCGGTGTTT  205362

pDK2-9   339     CAACATCTCCCGGGTCCGTTGATCAGG-TGCCTGGGCGGCTGTACTTGATTCTTGCTTGA  397
A mul    205363  CAGCATCCCCTGGGTCCGTTGATCAGGCTGCCTGGGCGGCTGTACTTGGATCTTGC-TGA  205421

pDK2-9   398     G-GAT-CAGT--CATGC-CATCA  415
A mul    205422  GCGATCCAGTTCCATGCGCATCA  205444
```

(a)

```
pDK2-9 1         ATCAGGCTGGTTGTGATACCCAC---TGTAGGGCAAGGGGCGTCGGGCCGAGAGGCGGTT  57
Acryp  174018    ATCAGGCTGGTTGTGATACACACGTTTGTAGGGCAAGGGGCGTCGGGCCGAGAGGCGGTT  173959

pDK2-9  58       TGATGCGGCACGAATGACGGTTGACTTGCTCCAGCTA------CTCGGTGCCATGCGCGC  111
Acryp  173958    TGATGCGGCGCGAATGATGGTTGACTAGCTGTAGCTTAGCTTCCTCG-TGCCAATCGCGC  173900

pDK2-9 112       CGGCGATTTCAAAGCCAGATGAACAATGCCAGAATAACCGACCAGGCCGCGATCGAGACA  171
Acryp  173899    CGGCGAATTCAAAACCAGATGAACAACGCCAGAATAACGGCCCAGGCCGCGATCGAGACA  173840

pDK2-9  172      AGAAAAAATCAACAGCGGCGGTTTTCGGCAAGGCAAGCTTTTCAGCACTCCTCGTGGCGT  231
Acryp  173839    AGA-AAAATCAACAGC-GCCGCCTTCGGCCAGGCGAGCCGTTCGGCA-TCCTCGTGGCGT  173783

pDK2-9  232      AATGTATTCTGGCCCCCGGCACTCAGAAGCGAAATAACTTCCCACAACCAATTTGCATCA  291
Acryp  173782    -ATGTATCTTG--CCGCGGCACTCAGACGCGAAATAAC-TCCGAC-ATCAATTTGCATCA  173728

pDK2-9  292      ATTGTATCCATACGGCGAAGAACTCCGCGTGATT-GGGGGGCGGTG-TTCAACATCTCCC  349
Acryp  173727    ATTGTATCCATACGGCGAAGACCTCGGCGTGGTTGGGGGGGCGGTGTTTCAGCATCCCCT  173668

pDK2-9  350      GGGTCCGTTGATCAGG-TGCCTGGGCGGCTGTACTTGATTCTTGCTTGAG-GAT-CAGT-  405
Acryp  173667    GGGTCCGTTGATCAGGCTGCCTGGGCGGCTCTACCTGGATCTTGC-TGAGCGATCCAGTT  173609

pDK2-9  406      -CATGC-CATCA  415
Acryp  173608    CCATGCGCATCA  173597
```

(b)

Fig. 5.14: BlastN result of pDK2-9 nucleotide sequence **(a)** showing 85% homology with + strand of *Acidiphilium multivorum* AIU301 plasmid pACMV1, Features flanking this part of subject sequence: 52 bp at 5' side: hypothetical protein; 261 bp at 3' side: hypothetical protein and **(b)** showing 85% homology with - strand of *Acidiphilium cryptum* JF-5 plasmid pACRY01, Features flanking this part of subject sequence: hypothetical protein.

```
TGGATTCCGGAACTGAGCCCCAGGAAGGTCGCCGTACCCAACTGGGTGGCCATTCCGTTCAGCGCA
TTGCTCGCGTCGTAGTAAGTGGTCTCGGTTACACTCGATAGCGCGCCCGAGGGCTGGTTCAGCGTG
AACCCGCTCAGGAAGGACTGGATGCCGGTCGGCGACGTGATTCGGGTTTCCGACGCGTAGAAGATA
CAGCGTGCCACCCGCAGACGTGGAGGTATTGATGCTGTTGGGTGCTAAAGTTGTCTGCGAGCAGAA
GATCGCCGCTGCTACCGATCTCTGTGATGGTGGACGTACCGAAAGAGCCACTGTAGTAAGGTCCGC
CCAGGGGCCGGAGACTGCGTGACATATTCGCCGTAACCCGCCTCCTGCAAGGGTGATTGTGAATAC
CAGGCGCCCCAGGGCGAACCGCGTCCCACCAAAACAATGGCGCCCCCATCCCTGCCCGAGAAAGGC
GAATCCCGGGATAACGTTCATTGGCTCATTCAATCGCTGGCCCAAGTGCGGTCGTTGCCGTAAAAT
TGGTAAAGCGCGTTACGCCTATTTTGAAGCAAACATGCATTTTACCCTGGTTTTAAAACTATAATA
ATTCGCCCACCAGGACATGAAGTTTAACCTTC  (626)
```

Fig. 5.15: Partial nucleotide sequence of pDK2-9; sequence done with T3 primer.

```
445 atgggggcgccattgttttggtgggacgcggttcgccctggggcg
    M  G  A  P  L  F  W  W  D  A  V  R  P  G  A
400 cctggtattcacaatcacccttgcaggaggcgggttacggcgaat
    P  G  I  H  N  H  P  C  R  R  R  V  T  A  N
355 atgtcacgcagtctccggcccctgggcggaccttactacagtggc
    M  S  R  S  L  R  P  L  G  G  P  Y  Y  S  G
310 tctttcggtacgtccaccatcacagagatcggtagcagcggcgat
    S  F  G  T  S  T  I  T  E  I  G  S  S  G  D
265 cttctgctcgcagacaactttagcacccaacagcatcaatacctc
    L  L  L  A  D  N  F  S  T  Q  Q  H  Q  Y  L
220 cacgtctgcgggtggcacgctgtatcttctacgcgtcggaaaccc
    H  V  C  G  W  H  A  V  S  S  T  R  R  K  P
175 gaatcacgtcgccgaccggcatccagtccttcctga 140
    E  S  R  R  R  P  A  S  S  P  S  *
```

Fig. 5.16: ORF obtained from pDK2-9 insert. 101 amino acid long at -2 frame showing 81% homology with hypothetical protein APM_0575 of *Acidiphilium* sp. PM.

hypothetical protein APM_0575 [Acidiphilium sp. PM]

```
pDK2-9   39  GGPYYSGSFGTSTITEIGSSGDLLLADNFSTQ  70
             GGPYY+GSFGT TI +IGSSGDLL ADNFST
hypoth   53  GGPYYNGSFGTFTINKIGSSGDLLPADNFSTN  84
```

5.4 Discussion

Plasmids of acidophilic heterotrophs of the genera *Acidiphilium* and *Acidocella,* were demonstrated to carry metal resistant genes. While genes conferring arsenic resistance in *Acidiphilium multivorum* are similar to those analyzed from other sources, there is no sequence similarity with the reported Cd- and Zn-resistant genes with cloned plasmid DNA fragments from *Acidiphilium symbioticum* KM2 and *Acidocella* GS19h strains which were found to have link with Cd and Zn resistance phenotype. Such observations have indicated some novel aspects of metal resistance in acidophilic bacteria. *Acidiphilium organovorum* contained at least three distinct plasmids; one of them was > 30 kb, and the other two were < 4.0 kb (Lobos *et al.*, 1986). Another AMD strain *Acidiphilium multivorum* AIU 301 was shown to harbour multiple plasmids of different sizes. One of the plasmids, pKW301A (56 kb), when transferred into *Escherichia coli* JM109 by electroporation, an *E. coli* transformant carrying pKW301 displayed resistance to sodium arsenite, sodium arsenate, and mercuric(II) chloride (Suzuki *et. al.*, 1997). A cryptic plasmid pAM5 (5161bp) bearing approximately copy number of 50-60 of *Acidiphilium multivorum* JCM-8867, which was completely sequenced, revealed five putative open reading frames of which two ORFs, ORF1 and ORF3 showed significant identity with various plasmid encoded mobilization (Mob) and replication initiation (Rep) proteins, respectively. One of the recombinant clones, pSK1 bearing pAM5 mob region was mobilized to *Escherichia coli* in presence of conjugative plasmid pRK2013 (Singh and Banerjee, 2007). Another acidophilic bacterium, *Acidiphilium symbioticum* H8, resistant to high levels of several heavy metals, hydrophobic agents, and organic solvents, was found to host a 9.6 kb plasmid pASH8. One of the clones of the plasmid bank was found to encode a single putative open reading frame (ORF) showing significant homology to several rusticyaninA1 proteins. Another clone, pASH8, encoded for a 43-kDa protein having conserved domain homology with several outer envelope TolC proteins, functionally complemented an *Escherichia coli* tolC mutant strain, making it resistant to several toxic hydrophobic agents, earlier for which it was sensitive. The third clone encoded for a putative 318-aa AcrA (acriflavine resistance protein A) protein and the clone was resistance to plasmid curing dye acriflavine. The clone contained a truncated ORF, showing significant homology to cation-efflux pump AcrB (Singh *et. al.*, 2010). *A. multivorum* AIU301T, on whole genome analysis, was found to contain nine replicons; one circular chromosome (3,749,411 bp, 67.6% G+C,

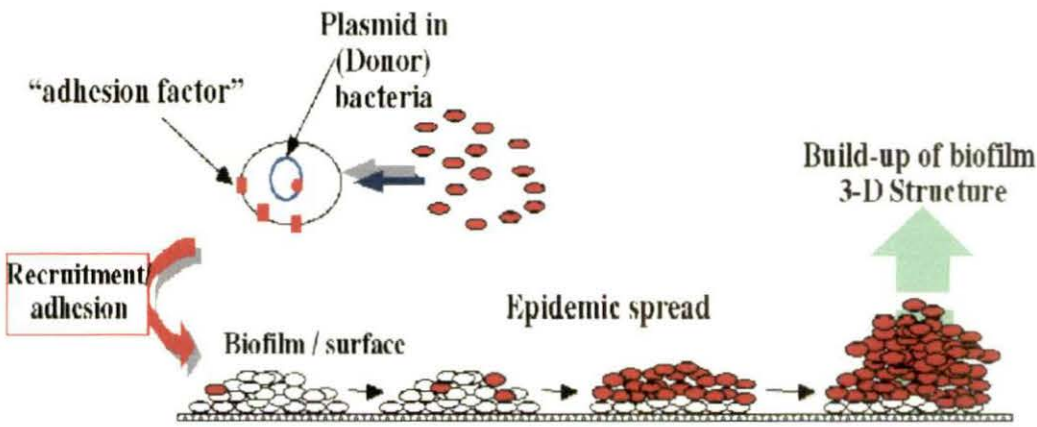
3,449 ORF) and **eight circular plasmids** pACMV1 (271,573 bp, 62.9% G+C, 284 ORF), pACMV2 (65,564 bp, 61.9% G+C, 69 ORF), pACMV3 (54,248 bp, 61.2% G+C, 61 ORF), pACMV4 (40,588 bp, 60.1% G+C, 44 ORF), pACMV5 (14,328 bp, 59.0% G+C, 19 ORF), pACMV6 (12,125 bp, 59.6% G+C, 14 ORF), pACMV7 (5,178 bp, 57.6% G+C, 8 ORF) and pACMV8 (1,728 bp, 60.9% G+C, 1 ORF). Several genes with putative involvement in heavy metal-transport, and three operons (which appeared to contribute to arsenic resistance) were identified from the chromosome and circular plasmids. *A. multivorum* AIU301T chromosome showed overall high conservation of synteny with *A. cryptum* JF-5 chromosome which has been sequenced, excluding a few regions (http://www.nbrc.nite.go.jp/e/index.html). In the present study, five plasmids, sizes approximately 55 kb, 23kb, 4 kb, 2 kb, and 1 kb, were reported from the strain DK2AH2 (Fig. 5.1). The largest plasmid detected and isolated was named pDK2AH2 (55 kb); restriction digested and gene bank was constructed with *EcoR*I fragments of the same. Three gene bank clones, pDK2-3, pDK2-5 and pDK2-9, were partially sequenced. Sequence analyses of pDK2-5 (Fig. 5.9 and 5.11) and pDK2-9 (Fig. 5.13 and 5.15) revealed significant similarities (>80% homologous) to the hypothetical protein of *Acidiphilium multivorum* AIU301 plasmid and hypothetical protein of *A. cryptum* JF-5 (Fig. 5.10a,b and 5.14a,b). Translated sequences of pDK2-5 and pDK2-9 revealed that -2 frame of the sequence contained an open reading frame (ORF) (164 and 101 amino acid long respectively), showing 100% and 81% homology respectively with hypothetical protein APM_0575 of *Acidiphilium* sp. PM. Partial nucleotide sequence of pDK2-3 on in-silico translation revealed that +2 frame of the sequence contained an ORF showing 74% homology with hypothetical protein of uncultured *Sphingobacteria* and glycosyl transferase protein of uncultured *Flavobacteria* (Fig. 5.8a); and -1 frame showing 71% homology with conserved domain protein of *E. coli* MS 69-1 (Fig. 5.8b).

Building up of evidences that Acidiphilium strains do possess the property of biofilm formation: As natural consequence, the *Acidiphilium* strains inhabiting AMD are often subjected to several physico-chemical stresses such as high and low temperatures and exposure to various heavy metals, etc. Stress induced changes in cell morphology has been observed with *Acidiphilium symbioticum* H8 where maximum alterations in size occurred when the bacterium was exposed to sub-inhibitory concentrations of Cu and Cd. Loosely packed coccobacillus-type normal cells formed characteristic chains of coccoidal lenticular shape with constrictions at the junctions between them in the

presence of Cd; Cu induced transformation of cells to become round shaped; Ni caused the cells to aggregate, but Zn showed no effect. Respective metal depositions on the cell surface were confirmed by scanning electron microscopy equipped with energy dispersive X-ray analysis (Chakravarty and Banerjee, 2008). Such changes in cellular morphology are generally observed with biofilm forming strains and biofilm formation is also induced by stress factors. It may be so that the authors (Chakravarty and Banerjee, 2008) have missed the evidences to relate such changes with biofilm formation; and biofilm formation is also related to metal resistance in bacteria. In one of the successive studies on *Acidiphilium* strains, extracellular polymeric substances (EPS) that constitute biofilm was reported from *Acidiphilium* 3.2Sup(5) (Tapia *et al.*, 2009). Electron microscopy (both TEM and SEM) enabled to determine the degree of attachment and the growth of the biofilm overtime on two solid supports: carbon fibre cloth and graphite rods (Tapia *et al.*, 2009). In the present study it was shown that *Acidiphilium* sp. DK2AH2 is also capable of forming biofilm on glass surface. Extracellular polymeric substances (EPS) produced by microorganisms are a complex mixture of biopolymers primarily consisting of polysaccharides, as well as proteins, nucleic acids, lipids and humic substances. EPS make up the intercellular space of microbial aggregates and form the structure and architecture of the biofilm matrix. The key functions of EPS comprise the mediation of the initial attachment of cells to different substrata and protection against environmental stress and dehydration.

Association of plasmid borne genes with formation of biofilm: Many chromosomal genes have now been shown to be involved in different stages of biofilm development. By contrast, the contribution of the extra-chromosomal plasmid gene pool (representing as much as 10-20 % of total bacterial DNA) to biofilm biology is poorly understood. As a consequence, with the exception of biotechnology application and antibiotic resistance spread, the role of plasmids in bacterial ecology has been largely overlooked. Even though conjugation has been studied first and foremost in liquid, most natural bacterial populations are found associated with environmental surfaces in complex multispecies communities called biofilms. Biofilms are ideally suitable for the exchange of genetic material of various origins, and it has been shown that bacterial conjugation occurs within biofilms. It was shown that conjugative plasmids contribute directly to the capacity of the bacterial host to form a biofilm. Natural conjugative

plasmids expressed factors that induced planktonic bacteria to form or enter biofilm communities, which favour the infectious transfer of the plasmid. This general connection between conjugation and biofilms suggested that medically relevant plasmid-bearing strains are more likely to form a biofilm (Ghigo, 2001). Though biofilms have been envisaged to be involved in persistence, the process of biofilm formation remained complex and poorly understood in *E. coli* O157:H7. A mini-Tn5 transposon insertion library was constructed in strain EDL933 and screened for biofilm-negative mutants using a microtiter plate assay in order to comprehend the genetics of this process. Of 11,000 independent insertions 95 of (0.86%) them failed to produce biofilm and transposon insertions were located in 51 distinct genes/intergenic regions that must be involved either directly or indirectly in biofilm formation. All of the 51 biofilm-negative mutants showed reduced biofilm formation on both hydrophilic and hydrophobic surfaces. Thirty-six genes were unique including genes on the virulence plasmid pO157. The type V secreted autotransporter serine protease EspP and the enterohemolysin translocator EhxD were found to be directly involved in biofilm formation (Dudley *et al.*, 2006). In addition, EhxD and EspP were also important for adherence to T84 intestinal epithelial cells, suggesting a role for these genes in tissue interactions *in vivo* (Puttamreddy *et al.*, 2010).



In the present study it was shown that plasmids in *Acidiphilium* sp. DK2AH2 on getting cured the biofilm forming ability is lost to a great extent coupled to the sensitivity towards Cu(II) and Zn(II). Significant advances have been made to reveal new insights

into biofilms and their constituents. The expansion of knowledge in relation to molecular mechanisms involved in bacterial-mineral attachment may be relevant in the enhancement of bioleaching timing and efficiency.

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General Discussion & Summary

General Discussion and Summary

The study of acidophilic heterotrophs of AMD has become very important because they are regular inhabitants of mineral sulphide environments (Berthelot *et al.*, 1997). Several characteristic features of these heterotrophs have been suggested to have beneficial effect on the bioleaching as well as for the sustenance of the iron-oxidising organisms in the environments. Earlier studies have shown that both sulfur- and iron-oxidizing bacteria such as *Acidithiobacillus ferrooxidans*, *A. thiooxidans* and *Leptospirillum ferrooxidans* were present in rather high numbers (Baker and Banfield, 2003). *A. ferrooxidans* and *Ferroplasma* has been considered principally responsible for the extreme conditions of AMD systems (Bond *et al.*, 2000; Okabayashi *et al.*, 2005). The presence of heterotrophs associated with chemolithotrophs like *A. ferrooxidans* has been observed (Wichlacz and Unz, 1981; Bond *et al.*, 2000), but studies of such heterotrophs are largely restricted to obligately acidophilic bacteria like *Acidiphilium* sp. (Johnson and McGinness, 1991; Bruneel *et al.*, 2003; Okabayashi *et al.*, 2005). It was observed that a significant symbiosis exists between heterotrophic and certain autotrophic species. Acidophilic autotrophs produces organic materials as their by product. Heterotrophs makes a way by utilizing/removing organic compounds toxic for the autotrophs (Pronk *et al.*, 1990; Das and Mishra, 1996). Mixotrophic growth was evidenced by certain facultative chemolithotrophs where limited amount of both inorganic and organic substrates are present in AMD (Clark and Norris, 1996). Assuming larger degree of metabolic plasticity of facultative acidophilic heterotrophs over obligately acidophilic heterotrophs, in the present study, cultivation and characterization of acidtolerant heterotrophs from AMD samples from Garubathan ore reserves of West Bengal, India, has been attempted to study their physiology, to understand their role in nature and to identify their potential in biotechnological applications.

The enrichment techniques, isolation, purification and preliminary screening of acid-tolerant heterotrophic isolates from AMD sites of Garubathan have been described in this chapter1. Isolation and identification of acid-tolerant heterotrophs from AMD samples from Garubathan, India, has partially revealed diversity of the strains in terms

of pH tolerance, growth at a wide range of temperature, and chemolithotrophy. Primarily, eighty one acid-tolerant heterotrophic isolates were obtained from the AMD samples. Out of which ten heterotrophic isolates were purified from the autotrophic culture of *Acidithiobacillus ferrooxidans* colonies. Earlier authors (Johnson and Kelso, 1983; Mishra *et al.*, 1983) have also detected acidophilic heterotrophs in *A. ferrooxidans* cultures. It has been found that some organic substrates such as pyruvate, glutamate, aspartate, serine, glycine, and other amino acids excreted by *A. ferrooxidans* can be utilized by the heterotrophs for their growth (Schnaltman and Lundgren, 1965; Arkesteyn *et al.*, 1980; Ingledew, 1982). Several types of acidophilic heterotrophs (*Acidiphilium cryptum*, *A. acidophilum*, *A. organovorum* etc.) that are gram negative in nature were recovered from *A. ferrooxidans* enrichment cultures (Guay and Silver, 1975; Harrisson *et al.*, 1980; Lobos *et al.*, 1986). An important molecular technique has proved to be useful in typing bacterial strains is Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of whole cell bacterial proteins of the isolates, wherein differences seen in protein bands have been successfully used to group bacteria (Krech *et al.* 1988; Huey and Hall, 1989; Maiti *et al.*, 2009). In this study too, SDS-PAGE of total protein isolated from the strains was used to screenout the similar banding patterns and finally eighteen of them showing differential banding patterns of protein was selected for further studies. Twelve of the eighteen acid-tolerant isolates were found to be Gram negative. All the isolates showed growth at diverse range of pH, (from 1 to 12) and temperature (from 4 °C to 42 °C). The isolates showed best growth at pH 3 and temperature (28-30) °C except GMX1, GMX7, and GAH1which showed psychro-tolerant nature growing best at 16 °C. Nine acid-tolerant heterotrophic isolates were tentatively found to be mixotrophic (facultative sulfur-oxidizing) strains (Table1.1). Reduced sulfur species of sulfidic mineral origin in Garubathan AMD sites of Darjeeling Himalayas must have favoured abundant microbial populations of sulfur-oxidizers (both mesophilic and psychro-tolerant), including autotrophic *Acidithiobacillus* species as well as heterotrophic sulfur-oxidizing strains. These strains may provide genetic resources for the development of novel biotechnological processes. Hence, in the following chapter i.e chapter 2 contained the detailed study of facultative sulfur lithoautotrophy, organotrophy and biochemical characterization of these isolates.

Lithoautotrophic sulfur oxidizers are found in environments rich in H_2S, such as AMD. Particularly most sulfur-oxidizing bacteria are both acidophilic and autotrophic (Pronk *et al.*, 1991). Nine of the acid-tolerant isolates could grow chemolithoautotropically on thiosulfate (Fig. 2.1) however four isolates, GAH2, GAH5, GMX1, GMX6 grew relatively poorer than the others; whereas, elemental sulfur medium could not support the growth of any of the acid-tolerant isolates. Concurrent utilization of organic (succinate) and inorganic (thiosulfate) substrates, termed as mixotrophic nutritional ability (Matin, 1978), were shown by ten of the isolates reported in this study (Fig. 2.4). GMX4 which could not sustain in MST medium was able to grow when yeast extract was added to the medium (MSTSY). These mixotrophic behaviours of the isolates were also evident during the study of *Thiobacillus* A2 which showed mixotrophic metabolism when in the natural environment both inorganic sulfur compounds and organic substrates were available simultaneously (Gottschal and Kuenen, 1980). All nine isolates have demonstrated growth by utilizing thiosulfate as the energy source (Fig. 2.3). Rate of depletion of thiosulfate in the medium with GMX7 and GMX5 was highest among all the nine isolates. Chemolithotrophic growth of acid-tolerant isolates on thiosulfate in MST medium (Fig. 2.1J) and the data regarding consumption of thiosulfate (% consumption) by these isolates (Fig. 2.3) supported each other which means cell growth was coupled with the utilization of thiosulfate. The diversity of physiology was evident from the wide range of physicochemical requirements like temperature, pH tolerance etc. Similarly autotrophic growth of different isolates in this study in batch cultures on thiosulfate produced different growth yields. It was inferred from the growth experiment that external physical parameters do not influence the growth yield on thiosulfate for different isolates. Concurrently it also enabled to set up a working hypothesis that there may be difference in the mode of dissimilar sulphur compounds oxidation pathways, electron transport mechanisms and modes of energy conservation. Abundance of reduced sulfur species of sulfidic mineral origin in Garubathan AMD sites of Darjeeling Himalayas could be the reason of the prevailing diversity amongst culturable microbial populations of sulfur-oxidizers (both mesophilic and psychrotolerant), including autotrophic *Acidithiobacillus* species (Gurung and Chakraborty, 2009) as well as heterotrophic sulfur-oxidizing strains. Similar diversity

of thiosulfate-oxidizing bacteria has been reported from marine sediments and hydrothermal vents (Teske *et al.*, 2000). Chemolithoautotrophy on thiosulfate is also known in non-thiobacilli of facultative lithotrophs like *Paracoccus pantotrophus*, hydrogen oxidizing heterotrophs, and heterotrophic marine pseudomonads (Ruby *et al.*, 1981; Kelly, 1989). The final product of chemolithotrophic sulfur compounds oxidation is sulfate. The role and production of polythionates as intermediates in oxidations of sulfur, sulfide and thiosulfate to sulfite had been addressed starting from the initial studies (Kelly, 1982, 1989). Peck (1960) first proposed a separate view of thiosulfate oxidation independent of tetrathionate formation, in which $S_2O_3^{2-}$ was cleaved to S^{2-} and SO_3^{2-} by the enzyme thiosulfate reductase. He showed that S^0 was produced from sulfide (S^{2-}) oxidation, while the SO_3^{2-} was oxidized to sulfate forming APS (adenylyl sulfate) as the intermediate product. Kelly (1989) proposed a 'common pathway", a hypothetical scheme proposed on the basis of the discrete available data generated with the various strain, viz., *T. concretivorus* (strain *T. thiooxidans*), *T. thiooxidans*, *T. thioparus, T. novellus, T. ferrooxidans, T. denitrificans*, and *T. neapolitanus.* All these strains oxidize thiosulfate. The differences on the oxidative metabolism of thiosulfate by different members of sulfur lithotrophs were primarily due to the differences in the mechanism of initial cleavage of S-S bond of thiosulfate. Tabita *et al.*, (1969) proposed the involvement of two different enzymes in the metabolism of thiosulfate- (i) Rhodanese and (ii) thiosulfate oxidizing enzyme. Rhodanese cleaves the S-S bond, thereby producing membrane associated (S^0) and sulfite (SO_3^{2-}). The thiosulfate oxidizing enzyme, thiosulfate cytochrome c oxidoreductase catalyzes union of two molecules of thiosulfate to form one molecule of tetrathionate ($S_4O_6^{2-}$), which could be further oxidized to produce thiosulfate ($S_2O_3^{2-}$) and sulfite (SO_3^{2-}). The oxidation of thiosulfate without the formation and accumulation of polythionates has been best studied with facultative species *T. versutus*. Cytochrome-dependent complete oxidation of thiosulfate to sulfate was demonstrated with enzyme preparations (Kelly, 1989). A multi-enzyme system that performs the sequence of oxidation reactions of thiosulfate was identified in some greater detail (Kelly, 1989). Thiosulfate oxidation pathways operating in sulfur-oxidising bacteria have been tentatively grouped in three categories (Meyer *et al.*, 2007): (a) pathway involving breakdown of thiosulfate to polythionate intermediates by thiosulfate dehydrogenase and tetrathionate hydrolase, which is

common in extremophilic sulfur oxidisers (*Acidithiobacillus*, *Thermothiobacillus*, and *Halothiobacillus*) (Kelly *et al.*, 1997); (b) pathway for direct conversion of thiosulfate to sulfate without sulfur globule formation by multienzyme complex (Sox) system, active in photo- and chemotrophic alphaproteobacteria (Friedrich *et al.*, 2001; Mukhopadhyaya *et al.*, 2000); (c) branched thiosulfate oxidation pathway involving formation of sulfur globules operating in sulfur-storing bacteria (Hensen *et al.*, 2006). Sox enzyme system is present in diverse thiosulfate oxidizing bacteria. Activity of thiosulfate dehydrogenase, which occurs in a wide range of chemolithotrophic bacteria, was observed in the cell-free extracts of nine of the thiosulfate oxidizing strains, with GAH2 showing the least activity; GMX1, GMX5 and GMX6 showing no activity. (Table 2.1). Even if cell free extracts produces tetrathionate, doubts prevail about the nature of the intermediate; as tetrathionate may not always be a normal intermediate in thiosulfate oxidation by normal cells (Dam *et al.*, 2007).

Studies in energy conservation in sulfur oxidation constitute electron transport (ETS) and translocation of proton. Electrons generated from sulfur substrates oxidations may couple the redox reaction at the level of cytochrome *c* or electron enters at cytochrome *b* or more higher status in the electron transport chain for energy generation (Kelly, 1989). The terminal reaction, $SO_3^{2-} + H_2O \rightarrow SO_4^{2-} + 2H^+ + 2e^-$, is common in all the sulfur compound oxidation to produce sulfate from sulfite. In almost all the members of thiobacilli the enzyme responsible for the catalysis of this reaction, sulfite cytochrome *c* oxidoreductase, has been found to be actively present. The electron negative redox couple of SO_3^{2-}/ SO_4^{2-} is adequate to couple to the electron transport system at the level of flavin-quinone-cytochrome *b*. In some species it has been shown that cytochrome *c* is the physiological electron acceptor and the start point where the energy is coupled to the electron transport chain. Alternatively, adenosine phosphosulfate (APS) reductase has been shown to be operative in thiosulfate metabolism in several strains of *Thiobacillus*. It catalyzed the same terminal oxidation reactions of sulfite to sulfate. The electrons enteringthe chain at level of cytochrome *b*, are benefited in two ways- by conserving energy synthesizing ATP at the site of transfer in between cytochrome *b* to cytochrome *c* sector in the ETS and also at the cytochrome *c* / oxidase sector. These differences in major electron transport chain cause

the differences in growth yields e.g., *T. denitrificans* and *T. tepidarius*, produce better yields of growth when compared to the growth of *T. versutus* and *T. neapolitanus* (Kelly, 1989). Sulfite dehydrogenase activity was observed in cell-free extracts of all of the nine test strains (Table 2.1). *soxC* encodes a sulfite dehydrogenase, the requirement for which in thiosulfate-dependent lithotrophic growth, experimentally verified in *Paracoccus denitrificans* (Mukhopadhyaya *et al.*, 2000).

Organotrophic growth for the acid-tolerant isolate was done on MSSY and R2A medium. Thirteen of the acid-tolerant isolates showed good growth on both the media (Fig. 2.4 and Table 2.2). R2A medium was greatly preferred (better growth within 24 h) by them. The isolates also displayed a wide range of substrate utilization as well (Table 2.3). R2A is a low-nutrient containing medium. Oligotrophic bacteria are generally tested for their ability to grow in R2A medium (Bhowal and Chakraborty, 2011). The isolates DK1AH1 and DK2AH2 were excluded from biochemical characterization study as they could not survive in pH more than 5.5. The rest of the acid-tolerant heterotrophic isolates showed varying differential properties from each other biochemically. The results of S_{SM} values scored between the isolates have reflected similar behaviour as obtained from total protein profile. Growth of acid-tolerant isolates in R2A medium has impelled us for the identification of oligotrophic property of these isolates (elaborated in chapter 3).

Acid mine drainage contains relatively low concentration (10 mg/l) of dissolved organic carbon (Kolmert and Johnson, 2001), and, as such, is called an oligotrophic environment. For the purposes of enumeration, oligotrophic aquatic bacteria have been tentatively defined as bacteria that develop on first cultivation on media with a minimal content of organic matter of either 1 mg or 1 to 15 mg of C per liter, usually supplied as complex mixtures of peptone, Trypticase, and other nutrients. Eutrophic bacteria, on the other hand, have been considered to be organisms able to grow on similar nutrients but at levels supplying 2 or more g of C per liter (Akagi *et al.*, 1977). The two types of media, differing widely in nutrient level, have been used to determine the distribution of oligotrophic and eutrophic bacteria in the sea (Akagi *et al.*, 1977) and in lake water

(Ishida *et al.*, 1980). Oligotrophic bacteria were considered to be those organisms which grew at the lower nutrient level but failed to grow when transferred to the higher level. In another study organisms able to grow only at the lower concentration of nutrients were called obligate oligotrophs, whereas those which grew at both low and high concentrations of nutrients were termed facultative oligotrophs (Ishida *et al.*, 1980, 1982). When one detects growth by measuring changes in numbers of viable cells in a liquid medium by using the plate count technique (Van der Kooij *et al.*, 1980; ZoBell and Grant, 1942), the concentrations of nutrients required to produce detectable increases in cell numbers are at least two orders of magnitude smaller than those needed to produce turbidity in a liquid medium or colonies on a solid medium. Thus, using plating techniques, ZoBell and Grant in 1943 were able to show that *Escherichia coli*, *Staphylococcus citreus*, *Bacillus megat erium*, *Proteus vulgaris*, and *Lactobacillus lactis* multiplied in solutions containing 0.1 mg of glucose (supplying 40 µg of C per liter) (ZoBell and Grant, 1942). Lower concentrations were not tested for technical reasons. Even *Pseudomonas aeruginosa* has been shown to grow in tap water at 25 µg of C per liter supplied by any one of a number of compounds (Van der Kooij *et al.*, 1982), and *Aeromonas hydrophila* multiplied when C supplied as glucose was added at 10 µg/liter (Van der Kooij *et al.*, 1980). These organisms are ordinarily not considered to be oligotrophs, yet they more than qualify when the current definition of an oligotroph as an organism which can grow in a medium containing nutrient supplying 1 to 15 mg of C per liter is applied. Oligotrophic bacteria are generally tested for their ability to grow in R2A medium. Very recently, diluted Luria-Bertani broth has been used to detect oligotrophic bacteria from environmental water samples (Kumar *et al.*, 2010; Oh *et al.*, 2009). Sixteen acid-tolerant heterotrophic strains from Garubathan AMD are facultatively oligotrophic capable of growing in R2A and diluted Luria-Bertani as well as in diluted modified DSMZ 269 media (Table 3.1). There are numerous reports of the isolation of acidophilic heterotrophs from extensibly pure culture of *Acidithiobacillus ferrooxidans* (Guay and Silver, 1975; Harrison *et al.*, 1980) and they are the part of the consortium present in the sulfide mineral occurrence sites (Harrison, 1981, Berthelot *et al.*, 1997). Although these environments are very poor in organic materials, the heterotrophs probably survive by scavenging on the low concentration of organic compounds excreted by *A. ferrooxidans* and other autotrophic

chemolithotrophs. Heterotrophic acidophiles are able to utilize organic materials produced by acidophilic autotrophs. The culture filtrate from the autotroph *A. ferrooxidans* contained sufficient organic matter to support heterotrophic growth of *Sulfobacillus thermosulfidooxidans* TH1 (Norris and Kelly, 1980) and *Acidiphilium* sp. DKAP1.1 (Gurung and Chakraborty, 2009). Of the seven strains reported in this study, two strains, which tolerated pH to the extreme of 1.0, GAH1 and GAH4, were able to grow in sterile acid mine water (pH 1.0-1.5) and elemental sulfur spent medium of *A. ferrooxidans* (pH 1.5-2.0) while the others (lesser acid-tolerant; Table 1.1 of chapter 1) have shown growth in the said media only when pH was increased to 3.0 - 4.0. DOC measurements of AMD samples from Garubathan (17-22 mg l^{-1}) and elemental sulfur spent medium of *A. ferrooxidans* (65-71 mg l^{-1}) have confirmed very low carbon content which supported the oligotrophic growth of the acid-tolerant strains. This demonstrated the adaptation of these acid-tolerant heterotrophs to a low nutrient condition that usually prevails in the mineral rich environments. The ability to grow in low nutrient condition, on the other hand is highly beneficial to the autotrophic partner *A. ferrooxidans.*

Microorganisms surviving in AMD environment meet substantial selective pressure to develop resistance mechanism to metal ions, supporting them with a competitive selective advantage. As a result, in shaping the characteristics of microbial communities in acidic environments in terms of both structure and function, the efficacy of diverse heavy metal resistance mechanisms would play a significant role (Dopson *et al.*, 2003). Acidophilic heterotrophic bacteria representing *Acidiphilium* and *Acidocella* genera were found to resist high levels of Cd, Zn, Ni, and Cu (Ghosh *et al.*, 1997; Mahapatra and Banerjee, 1996). Distinct patterns of heavy metal resistance in isolates from coal mining environments of Brazil were evidenced, being the Zn and Ni resistance the most widespread (Castro-Silva *et al.*, 2003). The AMD sites selected in this study is restricted to an area of 0.247 sq.km where zinc ore has been found to occur (Shah *et al.*, 1974-75). Not surprisingly, the strains isolated from Garubathan had been able to resist the heavy metal ions. Acid-tolerant heterotrophic isolates from AMD samples could be classified into nine groups on the basis of distinct patterns of metal resistance (Table 3.2). Isolates of Group III, strains GAH2 and GAH5, tolerated Co(II),

Ni(II), and Zn(II) as high as 35, 30, and 40 mM respectively. Similarly, isolates of Group IX, strains DK1AH1 and DK2AH2 tolerated Ni(II) and Zn(II) at its most 90, 20; and 450, 30 mM. The maximum tolerance of 100 mM Zn (II) was shown by GAH4. They could tolerate cobalt, nickel, copper, zinc, and arsenite concentration up to the level of 35 mM, 450 mM, 5 mM, 100 mM, and 10 mM respectively. However, maximum tolerable concentration of chromium, cadmium, and mercury was found to be only 1.5 mM, 1 mM, and 0.1 mM respectively. The strains have shown multiple metal tolerance as well as higher metal tolerance ability due to the selective pressure as the site from where these strains were obtained was rich in mineral occurrences.

For further characterization, it was important to test the sensitivity of the acid-tolerant isolates towards several antibiotics. The antibiotics tested were selected to represent 7 different classes: Aminoglycosides, Antifolates, Cephalosporins, Penicillin, Quinolones, and Others. The detailed antibiotic tolerance profiles of the acid-tolerant isolates were given in Table 3.3. Four (GAH4, GAH44, DK1AH1, and DK2AH2) out of eighteen strains were found to be sensitive towards antibiotics tested. However five (GAH1, GAH3, GAH8, GAH9, and GAH10) of them were resistant to all the 12 panel of antibiotics tested. The rest of the strains showed resistance towards two or more than two antibiotics. The resistance to a particular heavy metal has been correlated to antibiotics and other heavy metal resistance in a variety of organisms (Austin and Colwell, 1977; Luli *et al.*, 1983; Sabry *et al.*, 1997) and the role of plasmids in confering resistance to both antibiotics and metals has been previously demonstrated (Foster, 1983; Lyon and Skurray, 1987). Hence, research followed a natural tract to uncover phylogenetic affiliations of the AMD isolates (elaborated in Chapter 4).

The outcome of the 16S rRNA sequence comparisons was presented in Chapter 4, indicating phylogenetic diversity among the isolates studied. The 16S phylogeny of the fifteen acid-tolerant, thiosulfate metabolising bacteria isolated from AMD samples of Garubathan, India, fell into the genus *Burkholderia* , *Comamonas*, *Serratia*, *Psychrobacter*, *Acidiphilium* and *Bacillus* (Fig. 4.3-4.17) of the sub-class *Beta-proteobacteria, Gamma-proteobacteria, Alpha-proteobacteria* and *Firmicutes*. Despite extremities, a diverse range of bacteria, including representatives of *Proteobacteria*, *Nitrospirae*, *Firmicutes*, and *Actinobacteria*, inhabit AMD environments. Besides the

most studied group of bacteria under *Gamma-proteobacteria*, bacteria under *Alpha-* and *Beta-proteobacteria* were also identified in the acid mine environments (Baker and Banfield, 2003; Valverde *et al.*, 2006; Xiao *et al.*, 2009). Reduced sulfur species of sulfidic mineral origin in Garubathan AMD sites of Darjeeling Himalayas favor abundant microbial populations of sulfur oxidizers (both mesophilic and psychrotolerant), including autotrophic *Acidithiobacillus* species as well as heterotrophic sulfur-oxidizing strains. Similar diversity of thiosulfate oxidizing bacteria has been reported from marine sediments and hydrothermal vents (Teske *et al.*, 2000).

High sequence similarities of the 16S rRNA gene sequence of the two strains with *Burkholderia* species and clustering of them with *Burkholderia pyrrocinia* in the phylogenetic tree (Fig. 4.18) suggest that the isolates GAH2 and GAH5 can be assigned as novel acid- and metal-tolerant (elaborated in chapter 3) thiosulfate-oxidizing strains of *Burkholderia pyrrocinia*. In the phylogenetic trees, isolates GMX5 and GMX8 clustered with *Bacillus megaterium* with a bootstrap support of 100%. Another Gram positive heterotrophic strain, GMX6, clustered with *B. cereus* with a bootstrap support of 78%.

Amplification of soxB gene homologs and Phylogeny with soxB gene sequences: The *soxB* gene encodes the SoxB component of the periplasmic thiosulfate-oxidizing Sox enzyme complex, which has been proposed to be widespread among the various phylogenetic groups of sulfur-oxidizing bacteria (SOB) that convert thiosulfate to sulfate with and without the formation of sulfur globules as intermediate. Thiosulfate oxidation pathways operating in sulfur-oxidising bacteria have been tentatively grouped in three categories (Meyer *et al.*, 2007): (a) pathway involving breakdown of thiosulfate to polythionate intermediates by thiosulfate dehydrogenase and tetrathionate hydrolase, which is common in extremophilic sulfur oxidisers (*Acidithiobacillus*, *Thermothiobacillus*, and *Halothiobacillus*) (Kelly *et al.*, 1997); (b) pathway for direct conversion of thiosulfate to sulfate without sulfur globule formation by multienzyme complex (Sox) system, active in photo- and chemotrophic alphaproteobacteria (Friedrich *et al.*, 2001; Mukhopadhyaya *et al.*, 2000); (c) branched thiosulfate oxidation pathway involving formation of sulfur globules operating in sulfur-storing

bacteria (Hensen *et al.*, 2006). Sox enzyme system is present in diverse thiosulfate oxidizing bacteria.

Two pairs of degenerate soxB primers (Petri *et al.*, 2001; Meyer *et al.*, 2007) were used to amplify gene sequences. Two of the sequences obtained from the PCR products allowed a first view of the distribution of this gene among sulfur oxidizing *Beta-proteobacteria* and its phylogenetic relationships. Phylogenetic trees constructed with diverse *soxB* gene homologues available in the databases revealed that *Gamma-proteobacteria* and *Alpha-proteobacteria* were not monophyletic and formed at least four and two clusters respectively, while *Beta-proteobacteria* were shown to be monophyletic (Anandham *et al.*, 2008; Meyer *et al.*, 2007; Petri *et al.*, 2001). With introduction of two novel *soxB* gene homologues of *Burkholderia* spp. (strains, GAH2 and GAH4) in the database, the SoxB phylogenetic tree presented in this report has established for the first time that *Beta-proteobacteria* were not monophyletic but formed at least two distinct groups (Fig. 4.37). *Thiobacillus thioparus*, *T. denitrificans*, *Herminimonas arsenicoxydans*, *Methylibium petroleiphilum*, and *Pandorea* spp. formed a distinct cluster (cluster I), congruent to the cluster obtained in an earlier study (Anandham *et al.*, 2008) and the newly described *Burkholderia* strains GAH2 and GAH4 formed a new group among *Beta-proteobacteria* (cluster II). The *soxB* gene homologues of GAH2 and GAH4 were closest to *soxB* homologue of phototrophic sulfur-oxidising *Allochromatium vinosum* ABE01359. A partial *sox* gene cluster constituted of *soxB* and *soxXA*, detected in by PCR assay, was predicted to be essential for reduced sulfur compound oxidation in *A. vinosum* (Friedrich *et al.*, 2001). Inactivation of *fcc*A and *aprBA* genes in *A. vinosum*, which codes for cytochrome subunit of flavocytochrome c-sulfide dehydrogenase and adenosine-5′-phosphosulphate reductase respectively, left hydrogen sulfide and sulfite oxidation unaffected (Dahl, 1996; Reinartz *et al.*, 1998). Event of lateral *soxB* gene transfer is therefore the most reasonable explanation for the inferred close relationship of *soxB* from thiosulfate -metabolising *Burkholderia* spp. (strains GAH2 and GAH4) and *A. vinosum* species that are distantly related on the basis of the 16S rRNA gene phylogeny.

Interestingly, amplicons generated by degenerate soxB primers produced sequence similar to the genes for ABC transport protein (ATP binding) in GAH1,

GAH5 and GMX7 (Fig. 4.29-4.34). Part of gene sequence in GMX7 (Fig. 4.35) was found to possess truncated portion of thiosulfate binding protein. ABC systems constitute probably the largest superfamily of proteins ever detected in living organisms. Most ABC systems are primary transporters that transport unidirectionally molecules through membranes (Schneider and Hunke, 1998). The ABC-type drug transporters also belong to the ABC superfamily, the members of which all contain a highly conserved ATP-binding cassette (Higgins, 1992; Van Veen *et al.*, 2001). They utilize the energy released by ATP-hydrolysis to pump cytotoxic compounds out of the cell. The substrates handled by these transporters are extraordinarily varied, ranging from small molecules (ions, carbohydrates, amino acids, antibiotics) to macromolecules (polysaccharides, proteins). Purified ABC proteins or modules have been shown to bind and hydrolyze ATP (Dayan *et al.*, 1996; Higgins *et al.*, 1985; Ko *et al.*, 1994; Morbach *et al.*, 1993; Muller *et al.*, 1994; Shimabuku *et al.*, 1992; Thiagalingam and Grossman, 1993), making reasonable the hypothesis that they couple the energy of ATP hydrolysis to the movement of substrates by an unknown mechanism.

As the acid-tolerant heterotrophic isolates GAH1, GAH5 and GMX7 isolated from AMD possess facultative thiosulfate chemolithoautotrophic property, the sequence obtained for ATP binding ABC transporters, probably participate in the sulfur metabolism. A number of transport and binding genes appear to play roles in sulfur metabolism of *A. ferrooxidans*. Recently in one of the study it was seen that the the expression level of transport and binding protein encoding genes for ABC transporter was up-regulated when *A. ferrooxidans* culture was grown on sulfur (Xia *et al.*, 2010). Candidate genes potentially encoding uptake of inorganic sulfate were discovered, including a sulfate permease belonging the SulP family of the major facilitator superfamily of transporters (MFS) and a possible sulfate/thiosulfate/molybdenate ATP binding cassette (ABC)-type transporter in *A. ferrooxidans* (Valdés *et al.*, 2003).

In the last and final Chapter 5, the authoress has attempted to relate heavy metal tolerance phenotype with the plasmid (if any) determinants present in any of the acid-tolerant heterotrophic isolates. In the present study, five plasmids, sizes approximately 55 kb, 23kb, 4 kb, 2 kb, and 1 kb, were reported from the *Acidiphilium* strain DK2AH2

(Fig.5.1). Plasmids of acidophilic heterotrophs of the genera *Acidiphilium* and *Acidocella*, were demonstrated by earlier authors to carry metal resistant genes. While genes conferring arsenic resistance in *Acidiphilium multivorum* are similar to those analyzed from other sources, there is no sequence similarity with the reported Cd- and Zn-resistant genes with cloned plasmid DNA fragments from *Acidiphilium symbioticum* KM2 and *Acidocella* GS19h strains which were found to have link with Cd and Zn resistance phenotype. Such observations have indicated some novel aspects of metal resistance in acidophilic bacteria. *Acidiphilium organovorum* contained at least three distinct plasmids; one of them was > 30 kb, and the other two were < 4.0 kb (Lobos *et al.*, 1986). Another AMD strain *Acidiphilium multivorum* AIU 301 was shown to harbour multiple plasmids of different sizes. One of the plasmids, pKW301A (56 kb), when transferred into *Escherichia coli* JM109 by electroporation, an *E. coli* transformant carrying pKW301 displayed resistance to sodium arsenite, sodium arsenate, and mercuric(II) chloride (Suzuki et. al., 1997). Another acidophilic bacterium, *Acidiphilium symbioticum* H8, resistant to high levels of several heavy metals, hydrophobic agents, and organic solvents, was found to host a 9.6 kb plasmid pASH8. One of the clones of the plasmid bank was found to encode a single putative open reading frame (ORF) showing significant homology to several rusticyaninA1 proteins. Another clone, pASH8, encoded for a 43-kDa protein having conserved domain homology with several outer envelope TolC proteins, functionally complemented an *Escherichia coli* tolC mutant strain, making it resistant to several toxic hydrophobic agents, earlier for which it was sensitive. The third clone encoded for a putative 318-aa AcrA (acriflavine resistance protein A) protein and the clone was resistance to plasmid curing dye acriflavine. The clone contained a truncated ORF, showing significant homology to cation-efflux pump AcrB (Singh et. al., 2010). *A. multivorum* AIU301T, on whole genome analysis, was found to contain nine replicons; one circular chromosome (3,749,411 bp, 67.6% G+C, 3,449 ORF) and **eight circular plasmids** pACMV1 (271,573 bp, 62.9% G+C, 284 ORF), pACMV2 (65,564 bp, 61.9% G+C, 69 ORF), pACMV3 (54,248 bp, 61.2% G+C, 61 ORF), pACMV4 (40,588 bp, 60.1% G+C, 44 ORF), pACMV5 (14,328 bp, 59.0% G+C, 19 ORF), pACMV6 (12,125 bp, 59.6% G+C, 14 ORF), pACMV7 (5,178 bp, 57.6% G+C, 8 ORF) and pACMV8 (1,728 bp, 60.9% G+C, 1 ORF). Several genes with putative involvement in heavy

metal-transport, and three operons (which appeared to contribute to arsenic resistance) were identified from the chromosome and circular plasmids. *A. multivorum* AIU301T chromosome showed overall high conservation of synteny with *A. cryptum* JF-5 chromosome which has been sequenced, excluding a few regions (http://www.nbrc.nite.go.jp/e/index.html). The largest plasmid detected and isolated from *Acidiphilum* strain DK2AH2 was named pDK2AH2 (55 kb); restriction digested and gene bank was constructed with *EcoR*I fragments of the same. Three gene bank clones, pDK2-3, pDK2-5 and pDK2-9, were partially sequenced. Sequence analyses of pDK2-5 (Fig. 5.9 and 5.11) and pDK2-9 (Fig. 5.13 and 5.15) revealed significant similarities (>80% homologous) to the hypothetical protein of *Acidiphilium multivorum* AIU301 plasmid and hypothetical protein of *A. cryptum* JF5 (Fig. 5.10a,b and 5.14a,b). Translated sequences of pDK2-5 and pDK2-9 revealed that -2 frame of the sequence contained an open reading frame (ORF) (164 and 101 amino acid long respectively), showing 100% and 81% homology respectively with hypothetical protein APM_0575 of *Acidiphilium* sp. PM. Partial nucleotide sequence of pDK2-3 on in-silico translation revealed that +2 frame of the sequence contained an ORF showing 74% homology with hypothetical protein of uncultured *Sphingobacteria* and glycosyl transferase protein of uncultured *Flavobacteria* (Fig. 5.8a); and -1 frame showing 71% homology with conserved domain protein of *E. coli* MS 69-1 (Fig. 5.8b).

As natural consequence, the *Acidiphilium* strains inhabiting AMD are often subjected to several physico-chemical stresses such as high and low temperatures and exposure to various heavy metals, etc. Stress induced changes in cell morphology has been observed with *Acidiphilium symbioticum* H8 where maximum alterations in size occurred when the bacterium was exposed to sub-inhibitory concentrations of Cu and Cd. (Chakravarty and Banerjee, 2008). Such changes in cellular morphology are generally observed with biofilm forming strains and biofilm formation is also induced by stress factors. It may be so that the authors (Chakravarty and Banerjee, 2008) have missed the evidences to relate such changes with biofilm formation; and biofilm formation is also related to metal resistance in bacteria. In one of the successive studies on *Acidiphilium* strains, extracellular polymeric substances (EPS) that constitute biofilm was reported from *Acidiphilium* 3.2Sup(5) (Tapia *et al.* 2009). Electron

microscopy (both TEM and SEM) enabled to determine the degree of attachment and the growth of the biofilm overtime on two solid supports: carbon fibre cloth and graphite rods (Tapia *et al.* 2009). In the present study it was shown that *Acidiphilium* sp.DK2AH2 is also capable of forming biofilm on glass surface. Extracellular polymeric substances (EPS) produced by microorganisms are a complex mixture of biopolymers primarily consisting of polysaccharides, as well as proteins, nucleic acids, lipids and humic substances. EPS make up the intercellular space of microbial aggregates and form the structure and architecture of the biofilm matrix. The key functions of EPS comprise the mediation of the initial attachment of cells to different substrata and protection against environmental stress and dehydration. Many chromosomal genes have now been shown to be involved in different stages of biofilm development. By contrast, the contribution of the extra-chromosomal plasmid gene pool (representing as much as 10-20 % of total bacterial DNA) to biofilm biology is poorly understood. As a consequence, with the exception of biotechnology application and antibiotic resistance spread, the role of plasmids in bacterial ecology has been largely overlooked. Even though conjugation has been studied first and foremost in liquid, most natural bacterial populations are found associated with environmental surfaces in complex multispecies communities called biofilms. Biofilms are ideally suitable for the exchange of genetic material of various origins, and it has been shown that bacterial conjugation occurs within biofilms. It was shown that conjugative plasmids contribute directly to the capacity of the bacterial host to form a biofilm. Natural conjugative plasmids expressed factors that induced planktonic bacteria to form or enter biofilm communities, which favour the infectious transfer of the plasmid. This general connection between conjugation and biofilms suggested that medically relevant plasmid-bearing strains are more likely to form a biofilm (Ghigo, 2001). Though biofilms have been envisaged to be involved in persistence, the process of biofilm formation remained complex and poorly understood in *E. coli* O157:H7. A mini-Tn5 transposon insertion library was constructed in strain EDL933 and screened for biofilm-negative mutants using a microtiter plate assay in order to comprehend the genetics of this process. Of 11,000 independent insertions 95 of (0.86%) them failed to produce biofilm and transposon insertions were located in 51 distinct genes/intergenic regions that must be involved either directly or indirectly in biofilm formation. All of the 51

biofilm-negative mutants showed reduced biofilm formation on both hydrophilic and hydrophobic surfaces. Thirty-six genes were unique including genes on the virulence plasmid pO157. The type V secreted autotransporter serine protease EspP and the enterohemolysin translocator EhxD were found to be directly involved in biofilm formation. In addition, EhxD and EspP were also important for adherence to T84 intestinal epithelial cells, suggesting a role for these genes in tissue interactions in vivo (Puttamreddy *et al.*, 2010). In the present study it was shown that plasmids in *Acidiphilium* sp. DK2AH2 on getting cured the biofilm forming ability is lost to a great extent coupled to the sensitivity towards Cu(II) andZn(II). Significant advances have been made to reveal new insights into biofilms and their constituents. The expansion of knowledge in relation to molecular mechanisms involved in bacterial-mineral attachment may be relevant in the enhancement of bioleaching timing and efficiency

Finally, it may be concluded in a way that isolation and identification of acid-tolerant bacteria from AMD samples from Garubathan, India, have revealed diversity of the strains in terms of phylogenetic affiliation and thiosulfate chemolithotrophy. These strains may provide genetic resources for the development of novel biotechnological processes.

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Publications

A. List of publications:

a) Papers published:

- **Bhowal, S.,** and R. Chakraborty. **2011.** Five novel acid-tolerant oligotrophic thiosulfate-metabolizing chemolithotrophic acid mine drainage strains affiliated with the genus *Burkholderia* of *Beta-proteobacteria* and identification of two novel *soxB* gene homologues. ***Res. Microbiol.*** **162:** 436-445.

- **SahaBhowal, S.,** and R. Chakraborty. **2011.** Inorganic Sulfur compound oxidation phenotype in cultivable acid- and cold-tolerant mixotrophic bacterium designated as Psychrobacter sp. GMX7, isolated from Acid Mine Drainage of Garubathan, India. ***W. Cong. Biotechnol.*** Omics publishing Group. doi:10.4172/1948-5948.1000001.

b) Manuscript under preparation:

- **Bhowal,** S., A. Kumar, R. Chakraborty, and P. Vandamme. 2011. *Burkholderia garubathanensis* sp. nov., aciduric oligotrophic bacteria isolated from acid mine drainage samples in Garubathan, India. ***International Journal for Systematic and Evolutionary Microbiology.***

B. Sequence Submission in GENBANK / EMBL:

- The accession numbers for the **16S rRNA gene sequences** determined from **Acid-tolerant heterotrophic bacteria** isolated from **Acid Mine Drainage samples** from **Garubathan** of North Bengal are as follows:

  FR686457, FN293172, AM992063, AM998532, AM992537, AM999539, AM998531, AM992536, AM992535, AM992062, AM422128, AM422129, AM412315, AM403733, AM403732

- The accession numbers for the soxB **gene sequences** determined from **thiosulfate-oxidising bacteria** isolated from **Acid Mine Drainage samples** from **Garubathan** of North Bengal are as follows:

  FN995256, FN995257, FR837593, FR837594

C. Bacterial culture deposited in Culture Collections:

- **Bhowal. S.**, and R. Chakraborty. 2010. *Burkholderia garubathanensis* strain GAH44, BCCM™/LMG Bacteria Collection, Gent, Belgium, Acc no. LMG 26026.

- **Bhowal. S.**, and R. Chakraborty. 2010. *Burkholderia garubathanensis* strain GAH44, DSMZ Bacteria Collection. Braunschweig, Germany. Acc no. DSM 23993.

D. Paper presentation at National Symposium/ Seminar/ Conference:

- Poster presentation - **S. Saha Bhowal** and R. Chakraborty. "*Diversity of heterotrophic bacteria in Acid mine drainage samples of Garubathan, India, revealed through culture-dependent techniques*". **4th Congress of European Microbiologists, FEMS 2011** at Geneva, Switzerland. June 26 -30, 2011.

- Poster presentation - **S. Saha Bhowal** and R. Chakraborty. "*Inorganic Sulfur compound oxidation phenotype in cultivable acid- and cold-tolerant mixotrophic bacterium designated as Psychrobacter* sp. GMX7, *isolated from Acid Mine Drainage of Garubathan, India*". World Congress on "**Biotechnology**" by OMICS publishing groups at Hyderabad, Mar 21-23, 2011.

- Oral presentation - **S. Bhowal** and R. Chakraborty. "*Colony morphology variation of a facultative oligotroph Pseudozyma* sp. GAH10, *influenced by cultural conditions*". National Conference on "**Diversity and prospects of microbial resources" MIDICON 2010** by Dept. of Microbiology, North Bengal University, Feb 26-28, 2010.

- Poster presentation - A. Chakraborty, **S. Bhowal** and R. Chakraborty. "*Molecular characterization of cryptic plasmid of Acidiphilium* sp. DK2AH2". National Symposium on **"Diversity and Fuctionality of Plant and Microbes" BOTSYMP 2008** by Dept. of Botany. North Bengal University, Jan 24-25, 2008.

- Oral presentation - **S. Bhowal** and R. Chakraborty. "*Exploring the diversity of acidophilic heterotrophs in ARD of Garubathan of India*". National Symposium on **"Diversity and Fuctionality of Plant and Microbes" BOTSYMP 2008** by Dept. of Botany. North Bengal University, Jan 24-25, 2008.