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.

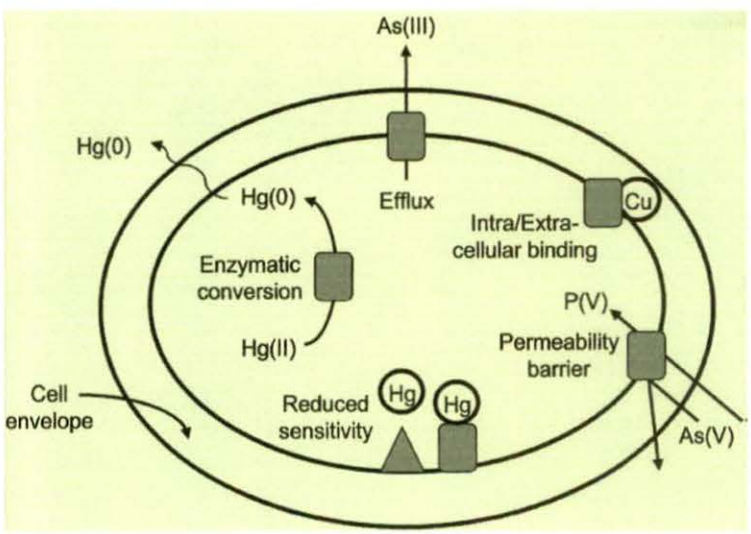

Fig. 3.1 : Schematic diagram of metal resistance mechanisms in acidophilic micro-organisms. The resistance mechanisms (clockwise from the top) include: efflux of the toxic metal out of the cell, e.g. As(III) efflux by the ArsB protein; intra/extracellular binding of the metal reducing

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).

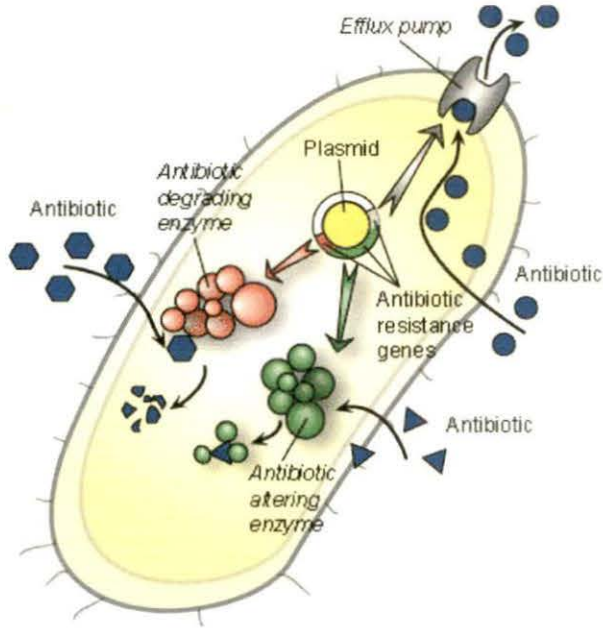

Fig. 3.2: Mechanisms of Antibiotic Resistance (Yim, 2011).

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.

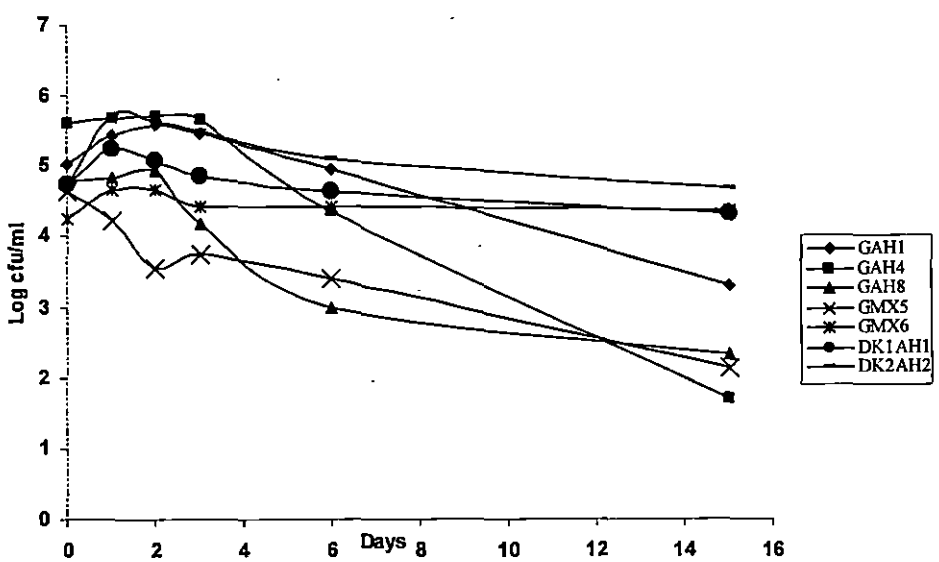

Fig. 3.3: Viability and growth of acid-tolerant heterotrophic isolates, GAH1, GAH4, GAH8, GMX5, GMX6, DK1AH1, and DK2AH2 in sterile acid mine water (pH-1.5).

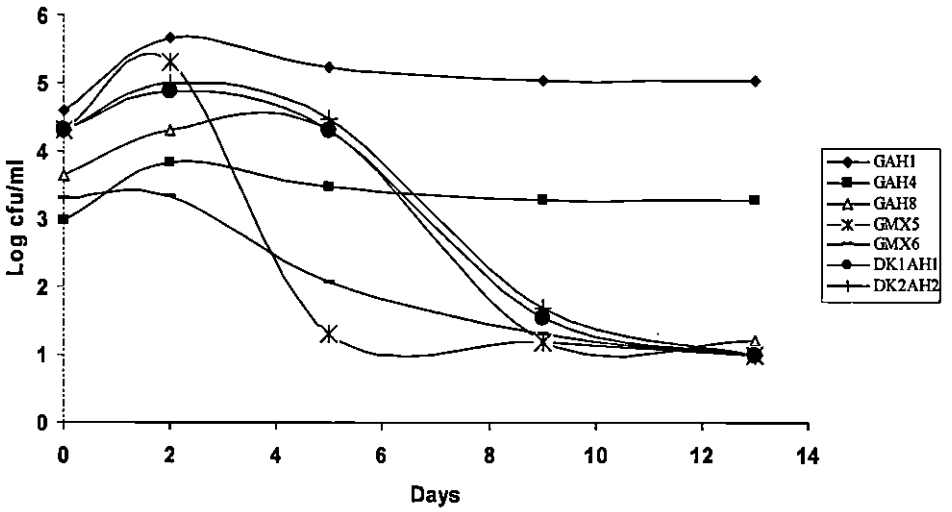

Fig. 3.4: Viability and growth of acid-tolerant heterotrophic isolates, GAH1, GAH4, GAH8, GMX5, GMX6, DK1AH1, and DK2AH2 in elemental sulfur spent medium of *A. ferrooxidans* DK6.1 pure culture (pH- 2.0)

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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