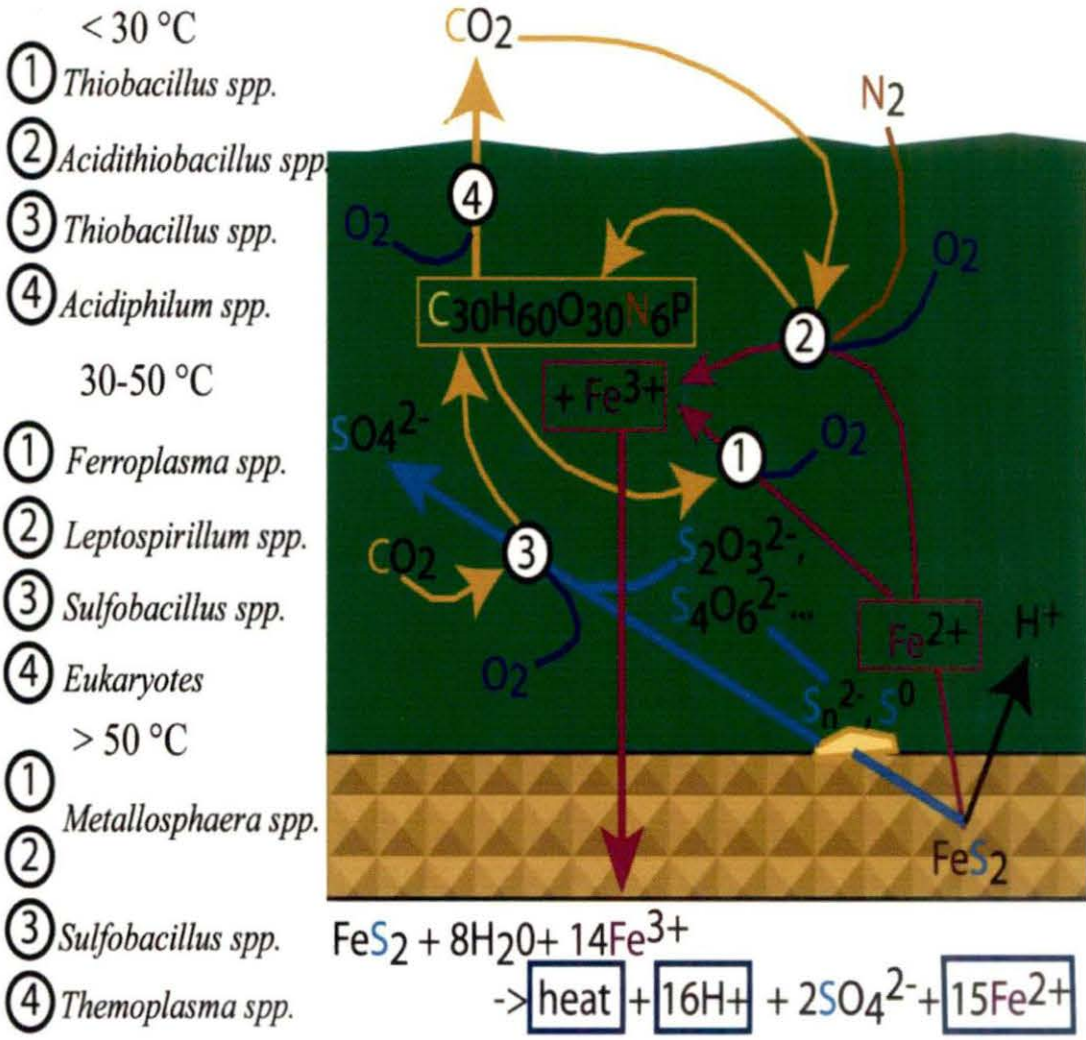
CHAPTER 1

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

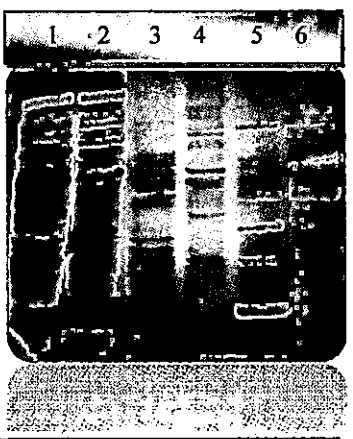
Heterotrophic colonies were obtained from the enrichment culture medium as well as from the colonies of *A. ferrooxidans*. On an average twenty five bacterial colonies were obtained at 10^{-3} dilution from modified DSMZ 269 media and mixotrophic media (MSTSY), viz., GMX1, GMX2, GMX3, GMX4, GMX5, GMX6, 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.

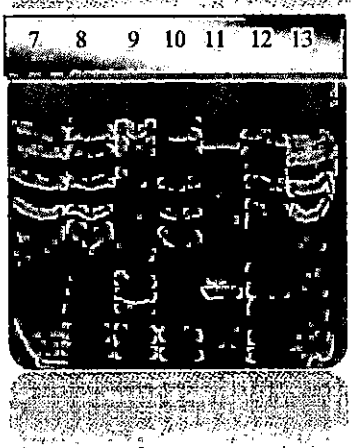
1.3.3 Preliminary selection of acid-tolerant heterotrophic isolates based on total protein profile

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.



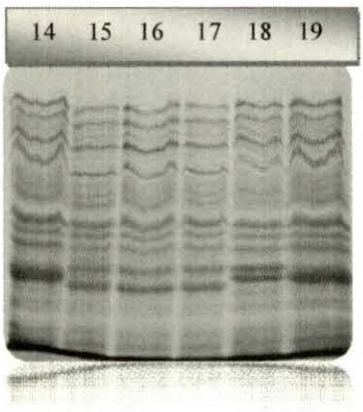
(Fig. 1.2a)

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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