Chapter -2

Isolation and characterization of acidophilic heterotrophs from the culture of *Acidithiobacillus ferrooxidans* strains of Garubathan origin

3.1 INTRODUCTION

Acid mine drainage is rich in various metal ions of heavy metals such as iron, Zn, Cu, Pb, Cd etc. with low pH because of the acid production by sulfur or reduced sulfur-oxidizing organisms such as *Acidithiobacillus ferrooxidans* present as a dominant microflora (Banks *et al.*, 1997; Lopez *et al.*, 2001). The environment is therefore thought to be limited in the types of substrates available to the microorganism and the diversity of microflora (Okabayaski *et al.*, 2005). However cultivation-based studies have revealed that the environment is rich in the diversity of microorganisms in AMD (Johnson, 1998; Hallberg and Johnson, 2001). This suggests that the minerals of mining environment themselves remain associated with organic compounds that may come as the metabolic waste product released by acidophilic autotroph such as *Acidithiobacillus ferrooxidans* (Paiment *et al.*, 2001). These organic materials can then be utilized as the source of nutrients by the acidophilic heterotrophs such as *Acidiphilium* species; the process by which the inhibitory effect of organic compounds toward *A. ferrooxidans* is alleviated (Harrison, 1984). The AMD therefore has been found not only to be dominated by the autotrophic bacteria like *A. ferrooxidans, A, thiooxidans, A. caldus, Leptospirillum ferrooxidans* etc but also by the acidophilic heterotroph particularly belonging to the genus *Acidiphilium* (Harrison, 1984; Rawlings, 1997; Goebel and Stackebrandt, 1994; Lopez *et al.*, 2001).

The study of the acidophilic heterotroph of AMD has become very important as this is a common inhabitant of mineral sulfide 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 oxidizing organisms in the environments and have been suggested to have beneficial effects. Pronk *et al.*, (1990) have suggested that these heterotrophs enhance the bioleaching by scavenging or removing the toxic organic compounds from the environment. Beside that these heterotrophs have been found to be capable of reducing ferric iron (both amorphous and crystalline) to soluble ferrous iron which is very important in iron cycling for the mixed culture of *A. ferrooxidans, L. ferrooxidans* and acidophilic heterotrophs in ferrous-iron-glucose medium (Johnson and McGuinness, 1991). It is therefore important to characterize these heterotrophs and establish the relationship with *A. ferrooxidans.*

A. ferrooxidans has been isolated from various natural sources which are geographically different and extremely diverse in terms of their physico-chemical characteristics, such as presence of sulfide minerals, pH, temperature and the presence of organic compounds (Mishra *et al.*, 1983; Karavaiko *et al.*, 2003). Earlier workers reported the ability of *A. ferrooxidans* to grow heterotrophically on ferrous iron and other sulfide minerals (Shafia and Wilkinson, 1969; Tabita and Lundgren, 1971a). Some workers have also reported the mixotrophic nature of *A. ferrooxidans* on glucose and ferrous iron (Barros *et al.*, 1984; Sugio *et al.*, 1982). It was also demonstrated that after prolonged growth in glucose containing medium, *A. ferrooxidans* lose the ability to oxidize ferrous iron and once it loses this ability it is very difficult to grow them autotrophically in ferrous iron medium (Sugio *et al.*, 1982; Barros *et al.*, 1984). It was presumed that after prolonged growth in glucose medium *A. ferrooxidans* culture gives rise to the new heterotrophic culture. These heterotrophs have the ability to utilize more different organic compounds as energy sources (Tabita and Lundgren, 1971b). However, later questions were raised against the purity of *A. ferrooxidans* culture when the *A. ferrooxidans* culture growing on ferrous iron and that growing on glucose medium were found to be serologically different (Shafia *et al.*, 1972).

Guay and Silver (1975) found that the G+C content of *A. ferrooxidans* culture differed when grown in different sulfide minerals and concluded that *A. ferrooxidans* culture may not comprise a distinct species. Further investigation led them to the isolation of an acidophilic heterotroph from *A. ferrooxidans* strains confirming the presence of these heterotrophs as the contaminant in *A. ferrooxidans* culture, which was enriched in glucose medium diluting the population of *A. ferrooxidans*. The absence of the growth on ferrous iron as well as the ability of utilization of different organic compounds by these isolates indicated that the isolates were not *A. ferrooxidans* (Lobos *et al.*, 1986). The heterotroph isolated by Guay and Silver (1975) had the ability to grow in elemental sulfur medium in addition to its ability to exhibit heterotrophic growth on a variety of organic compounds and they named the organism *Thiobacillus acidophilus*. Later on the basis of 16S rRNA sequence analysis this organism was reassigned to a new genus and species *Acidiphilium acidophilum* (Hiraishi *et al.*, 1998).

These findings then led to the investigation of the presence of heterotroph from ostensibly pure culture of *Acidithiobacillus ferrooxidans*. Mackintosh (1978) found that *A. ferrooxidans* culture whether isolated in the liquid culture or received from different

culture collection center were contaminated by heterotrophs and concluded that *A. ferrooxidans* cultures remain as a mixed culture. Harrison *et al.* (1980) with the help of DNA homology study found that *A. ferrooxidans* cultures collected from different collection center as well as those maintained in their own laboratory were contaminated by at least five different heterotrophs other than *Acidiphilium acidophilum*. These isolates were found unable to utilize sulfur or iron as the source of energy. Beside that some of them also contained yellow pigments. Harrison (1981) isolated another acidophilic heterotrophic bacterium directly from acidic mineral environment which was found to be very similar to the one that had been isolated earlier from the culture of *Acidithiobacillus ferrooxidans* but unable to oxidize elemental sulfur. The organism was obligate acidophile whose growth was found to be inhibited in the rich medium. The bacterium was described as *Acidiphilium cryptum* (Harrison, 1981). There after, several other species of *Acidiphilium* (*A. angustum, A. facilis, A. rubrum, A. organovorum etc.*) have been reported and described from several acidic mine drainage or from the culture of *Acidithiobacillus ferrooxidans* (Wichlacz *et al.,* 1986; Lobos *et al.,* 1986).

This chapter deals with the enrichment, isolation and characterization of acidophilic heterotroph isolated from tentatively pure culture(s) of *Acidithiobacillus ferrooxidans* strains of Garubathan.

3.2 MATERIALS AND METHODS

3.2.1 Media

Modified DSMZ 269 was used as the enrichment medium for the acidophilic heterotrophs (http:// www.dsmz . de / microorganisms / html / media / medium 000269.html). Medium contained $(NH_4)_2SO_4$ 2.0g; K_2HPO_4 0.5g; $MgSO_4.7H_2O$ 0.5g; KCl 0.25g; glucose 1.0g, yeast extract 0.1g 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 10% and 1% solution, and the required volume was added to the basal salt solution.

Solid medium for the heterotrophs was prepared by mixing two solutions. 180 ml solution A contained DSMZ 269 basal salt solution and 800 ml solution B contained 12 g agar. The pH of solution A was adjusted to 3.0 with 1 (N) H_2SO_4. Both the solutions were

separately sterilized at 15 psi for 15 minutes. Sterilized solutions were allowed to cool down to about 50°C and 10 ml each of the sterile 10% glucose and 1% yeast extract solutions was 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 by pouring 3ml of the molten medium in culture tubes and allowing them to solidify in slanting position.

Ferric medium was prepared by mixing the two solutions. Solution A was prepared by dissolving 30 g of $FeSO_4.7H_2O$ in 200 ml distilled water. The solution was boiled for 30 min. to convert it into ferric form and allowed cool down to room temperature. The sediments (ferric hydroxide) were separated by passing the solution through Whatman filter paper No.1. Solution B contained all the ingredients of 9K medium except $FeSO_4.7H_2O$ in 800 ml distilled water. The pH of the medium was adjusted to below 3.0 and was sterilized at 15 psi for 15 min. After sterilization under aseptic condition 10 ml of 10% glucose and 10 ml of 1% yeast extract solution were added. The resultant medium was referred to as Ferric Medium.

3.2.2 Enrichment and Isolation of acidophilic heterotrophs

Acidophilic heterotrophs were enriched and isolated from the typical 9K medium culture of acidophilic autotroph. 1.5 ml of the enrichment broth cultures was centrifuged at 10000 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 suspension was then seeded in the modified DSMZ 269 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 Petroff-Hausser counting chamber) was used for plating in the DSMZ 269 plates.

In another method of isolation of 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 directly on the 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 dilution streaking method. The purified colonies were then maintained in the slants with every fortnight transfer on to the fresh DSMZ 269 agar slants.

3.2.3 Characterization of acidophilic heterotrophs from the AMD of Garubathan

3.2.3.1 Micrometry

i. Gram reaction

Gram reaction was carried out following the standard protocol of differential staining using crystal violet and saffranin. Cell size and shape were determined.

ii. Cellular motility

Motility of the cells was determined by following the hanging-drop method as described in Chpater 1.

iii. Slide culture technique

A thin coat of molten DSMZ 269 medium was laid over the sterile cover-slip (18 mm^2). With the help of sterile toothpick acidophilic heterotrophic bacteria was centrally inoculated. Under aseptic condition the inoculated cover-slip was inverted and placed over a sterile slide. The boundary of the cover-slip was immediately sealed with paraffin and the slide was incubated at 28°C ± 2°C. Every day the microscopic observation was carried out.

3.2.3.2 pH tolerance

DSMZ 269 medium was adjusted to different levels of pH range. 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 turbidity of the inoculated and un-inoculated blank culture.

3.2.3.3 Utilization of different organic carbon source

For testing the ability of utilizing different organic carbon sources for growth, glucose in modified DSZM 269 medium was replaced with 0.1% final concentration of L-arabinose, gluconate, malonate asparagine, glycerol, lactose, D-xylose, maltose, mannitol, raffinose, ribose, D-galactose, D-fructose, sucrose, glutamate, lactate, or citrate. The

medium containing glucose and the acidophilic heterotroph served as the positive control and the medium without any inoculum served as the negative control. Growth of the organisms in different organic sources was determined by comparing the turbidity with the positive and negative control after incubating for 96 h at 28°C ± 2°C.

3.2.3.4 Catalase test

Catalase test was performed to observe the activity of catalase enzyme in the heterotrophs. Presence of catalase enzyme was determined by flooding the slants with 1 ml of 3% H_2O_2. As the enzyme is capable of breaking hydrogen peroxide to water and oxygen, the production of effervescence gives the positive test for catalase test. *Escherichia coli* was taken as positive control while *Bacillus subtilis* was taken as negative control

3.2.3.5 Oxidase test

Oxidase test was performed to observe the activity of cytochrome oxidase. The ability of bacteria to produce cytochrome oxidase was determined by the addition of two or three drops of *p*- aminodimethylaniline oxalate on to the colonies grown in the agar plate. The light pink reagent serves as an artificial substrate donating electrons and thereby becoming oxidized to blackish compound in presence of the oxidase. Development of the black coloration on the surface of the colonies is indicative of positive test. *Escherichia coli* colonies were taken as the negative control while *Pseudomonas* colonies were taken as positive control.

3.2.3.6 Antibiotic sensitivity test

Antibiotic sensitivity test was performed by antibiotic sensitivity discs method. 15 ml of sterile DSMZ269 agar medium (pH 5.5) was spread plated with 0.1 ml of concentrated cell suspension of acidophilic heterotrophic bacteria. Three antibiotic discs Penicillin-G 10µg, Streptomycin 10µg and chloramphenicol 30µg) were placed on top of the agar surface of the inoculated plates at equal distance from one another. The plates were incubated at 28°C ± 2°C for 96 hours and the production of the zone of inhibition was observed.

3.2.3.7 Growth in nutrient rich and diluted media

Nutrient rich media such as Nutrient Broth (NB) or Luria Bertani Broth (LB) was adjusted to pH of 3.0. Different concentrations (undiluted, ten and hundred times dilutions) of nutrient rich media were inoculated with the different strains of acidophilic heterotrophic bacterial strains and incubated at 28°C ± 2°C. The medium without an inoculum served as the control. After 96 hrs of incubation, growth was observed by comparing with the control.

3.2.3.8 Growth in agar extract medium

15g of agar was boiled in 100 ml of acidulated water (pH 3.0) for 10 minutes. The resultant solution was filtered through the Whatman filter paper no.1 to remove the solid agar particles. The filtrate so obtained was considered as agar extract medium and was sterilized at 15 psi for 15 minutes. 5 ml of the agar extract solution was taken in sterile culture tubes and heterotrophic strains were inoculated and incubated at 28°C ± 2°C. Uninoculated medium was considered as the control. Growth in the test tubes after an incubation of 96 hrs was compared with the control.

3.2.3.9 Growth in elemental sulfur, reduced sulfur and ferrous sulfur media with or without the supplementation of glucose and yeast extract

The ability of the heterotrophic strains to grow in different autotrophic media in presence or absence of glucose was tested. 1.5 ml of the bacterial culture was taken from log phase and centrifuged at 10000 rpm for 5 minutes. The cell pellets so obtained were washed with sterile 0.01 (N) H_2SO_4 thrice to remove any traces of organic substances from the culture. The pellet was then re-suspended in sterile 0.01 (N) H_2SO_4 and an equal volume of the cell suspension was inoculated in 15 ml of different media. In one set of experiment, the media used were supplemented with glucose (0.1%) while other set was devoid of glucose. The normal DSMZ 269 medium with the heterotrophic bacterial inoculum served as the positive control while the medium without inoculum served as negative control. Increase in the turbidity which indicated the growth of the bacteria, increase or decrease in pH, dissolution of sulfur particles as well as oxidation of the ferrous iron to ferric iron were observed carefully.

3.2.3.10Reduction of ferric iron

To test the ability to reduce ferric iron exponentially growing culture of acidophilic heterotroph was inoculated in 100 ml Erlenmeyer flasks containing 15 ml of ferric medium in duplicates. At a regular time interval the amount of ferrous iron concentration in the medium was measured following the method described in chapter 1. The medium without the inoculum served as the control.

3.2.3.11Tolerance to heavy metal

Experiment to evaluate heavy metal tolerance was conducted in the test tubes containing 5 ml modified DSMZ 269 medium. A series of test tubes having different concentrations of heavy metals was prepared to which identical volume of inoculum from exponential phase of phase was added. The growth was determined by observing the increase in the turbidity. The test tube without any heavy metals was kept as the positive control and that without any inoculum was kept as the negative control. Growth of the cultures in different concentrations of heavy metals were compared with these controls and '+' sign was given to those strains having comparable growth while '-' sign was given to those which did not show any visible growth. The range of concentration of different heavy metals is given in Table 2.1.

3.2.3.12Kinetics of glucose utilization

Modified DSMZ 269 medium was inoculated with known number of cells. The rate of utilization of glucose was determined by the Dinitrosalicyclic acid method by determining the amount of glucose present in the medium at specific time intervals.

Reagents:

a. Dinitrosalicyclic acid (DNS) reagent:

Dinitrosalicyclic acid:	1.0g
Crystalline Phenol:	200mg
Sodium sulphite	50mg
1% NaOH	100ml

(NaOH is given lastly as it deteriorates the reagent)

b. Rochelle's salt solution (40% Potassium sodium tartarate solution)

Procedure:

i. 1 ml of the culture (~2.07×10^5/ml) was inoculated in several flasks containing 15 ml of modified DSMZ medium.

ii. 0.3-1.5 ml of the medium was taken and the volume was made to 3 ml with distilled water.

iii. 3 ml of DNS reagent was added and placed in the boiling water bath for 5 minutes.

iv. With the solution still warm, 1 ml of Rochelle's salt solution was added.

v. The solution was allowed to cool and the absorbance was measured at 540 nm filter.

vi. Steps 2-5 were repeated every after 24 hrs of incubation.

vii. The amount of glucose was calculated from a standard curve using known concentration of glucose.

Table 2.1: Concentration of heavy metal solutions used in heavy metal tolerance experiment

Heavy metals	Range of concentration (mM)
Cu	3.0, 5.0, 10, 13, 15,
Zn	10, 30, 50, 70, 90, 100, 110
Co	2.0, , 5.0, 10, 15, 17, 20
Ni	10, 30, 50, 70, 90, 100, 110
Cd	1.0, 2.0, 3.0, 4.0, 5.0
Cr	0.1, 0.5, 1.0, 1.5, 2.0

3.3 RESULTS:

3.3.1 Isolation of acidophilic heterotrophs from the enriched *A. ferrooxidans* culture and from *A. ferrooxidans* colonies

Heterotrophic colonies were obtained from the enriched medium as well as from the colonies of *A. ferrooxidans*. The colonies that were smooth, cream-white in color (Plate 2.1) were phylogenetically related to *Acidiphilium cryptum* (by 16S rRNA analysis which is dealt in chapter 3). DKAP1.1, DKAP 1.2, DKAP 1.3, DKAP 1.4, GBAP VI.1 and GBAP VI.3 strains were selected for further studies.

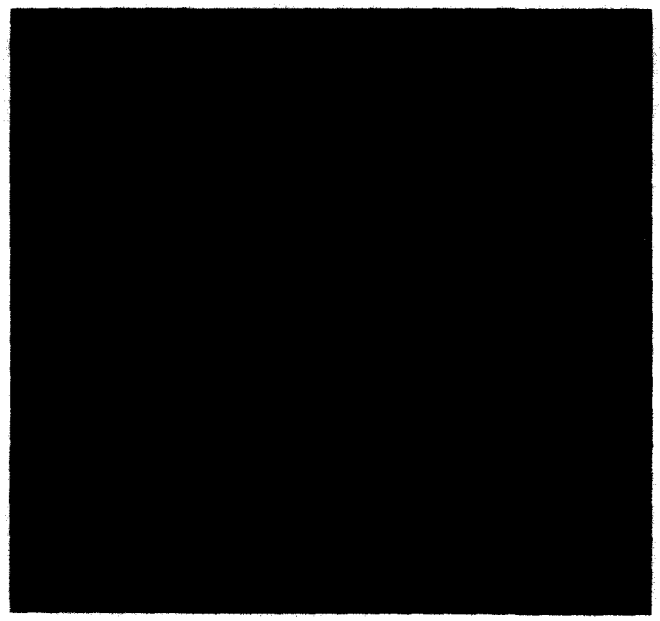

Plate 2.1: Colonies of *Acidiphilium cryptum* developed in DSMZ269 agar plate.

3.3.2 Characterization of acidophilic heterotrophs

3.3.2.1 Morphological Characteristics

Colonies of acidophilic heterotrophic strains were gram negative, smooth in appearance, initially white but turned into cream color on maturity. Cells were motile, small, rod-shaped and ranged in sizes between 0.5-0.8×1.0-1.3μm.

3.3.2.2 Physiological characteristics

Physiological properties of six strains of gram negative acidophilic heterotrophs (DKAP 1.1, DKAP 1.2, DKAP 1.3, DKAP 1.4, GBAP VI.1, and GBAP VI.3) are given in Table 2.2. There was very little difference in their physiological properties among the strains tested, e.g. only DKAP 1.4 was resistant to the antibiotic chloramphenicol, and only GBAP VI.3 was sensitive to streptomycin; strains DKAP 1.2 and GB VI.3 failed to grow in the medium containing lactate and glutamate as sole source of organic carbon respectively. All of tested strains were found to be catalase and oxidase positive and were unable to utilize D-galactose as sole organic carbon source. Beside these all the strains failed to grow in citrate in presence of acetate. These strains were not capable of growing in the nutrient rich media like NB or LB, however the same medium was found to support the growth when it was diluted hundred times. This indicated that the cells prefer very low concentration of nutrients. This fact of oligotrophy exhibited by the strains was further substantiated when cells showed visible growth as tested by the increase in the turbidity in agar extract medium where no glucose or yeast extract was supplemented. The phenotypic features of these strains showed a high degree of similarity (Similarity coefficient of 91.3%) with published characteristics of *A. cryptum* (Harrison 1981).

3.3.2.3 Growth in different autotrophic media with or without the supplementation of glucose

The growth of the heterotrophs (DKAP1.1, DKAP1.3, DKAP1.4, GBAP VI.1, and GBAP VI.3) in ferrous iron and sulfur was found to be positive only in presence of glucose and/or yeast extract, but there was no decrease in pH of sulfur medium and no change in the coloration of ferrous medium to rusty red indicating that the heterotrophs were unable to utilize sulfur or ferrous iron (Table 2.3). However no growth was found in thiosulfate medium containing glucose. In fact cells taken from the thiosulfate medium was found to be non-motile when microscopic observation was carried out. On the other hand no growth was observed in strict autotrophic media (which were not supplemented with glucose). The results indicated that the strains of heterotrophs were obligate organotrophs and presence of high concentration of inorganic cations (Fe^{2+}) did not affect the growth of the heterotrophs.

Table 2.2: Physiological properties of different strains of gram negative heterotroph *Acidiphilium cryptum.*

Properties	**Strains**					
	DKAP 1.1	**DKAP 1.2**	**DKAP 1.3**	**DKAP 1.4**	**GBAP VI.1**	**GBAP VI.3**
a. pH range	1.8.0 - 6.0	1.8.0 - 6.0	2.0 - 6.0	2.0 – 6.0	1.8-6.0	1.6-6.1
b. Temperature	30 – 34°C	30 - 34°C	30 - 34°C	30 - 34°C	30 - 34°C	30 - 34°C
c. Aerobic	+	+	+	+	+	+
d. Catalase	+	+	±	+	+	+
e. Oxidase	+	+	+	±	±	+
f. Organic Carbon Utilization						
L arabinose	+	+	+	+	+	+
glycerol	+	+	+	+	+	+
lactose	±	+	+	+	+	±
D xylose	+	+	+	+	+	+
maltose	+	+	+	+	+	+
mannitol	+	±	+	±	+	+
raffinose	+	+	+	+	+	+
ribose	±	±	+	+	+	+
D fructose	+	+	+	+	±	+
sucrose	+	±	±	±	±	+
glutamate,	+	+	+	+	+	-
lactate	+	-	+	+	+	+
citrate	+	+	+	+	+	+
D galactose	-	-	-	-	-	-
asparagine	-	-	-	±	±	+
Gluconate	+	+	+	±	+	+
g. Growth in LB (dilution)						
10^0	-	-	-	-	-	-
10^{-1}	-	-	-	±	-	-
10^{-2}	+	+	+	+	+	+
h. Growth in NB (dilution)						
10^0	-	-	-	-	-	-
10^{-1}	-	-	-	±	-	-
10^{-2}	+	+	+	+	+	+
i. Growth in citrate in presence of acetate						
	-	-	-	-	-	-
j. Antibiotic Resistance						
Streptomycin	+	+	+	+	+	-
Penicillin	+	+	+	+	+	+
Chloramphenicol	-	-	-	+	-	-

'+', positive growth; '±', moderate growth; '-', absence of growth. NB, Nutrient broth; LB, Luria-Bertani

Table 2.3: Growth of acidophilic heterotrophic strains in different autotrophic media with/without the supplementation of glucose.

Media	Strains					
	DKAP 1.1	DKAP 1.2	DKAP 1.3	DKAP 1.4	GBAP VI.1	GBAP VI.3
1. S^0-medium						
Growth	-	-	-	-	-	-
Initial / Final pH	na	na	na	na	na	na
2. $S_2O_3^{2-}$ medium						
Growth	-	-	-	-	-	-
Initial / Final pH	na	na	na	na	na	na
3. Fe^{2+}-medium						
Growth	-	-	-	-	-	-
Oxidation of Fe^{2+} to Fe^{3+}	na	na	na	na	na	na
4. S^0-medium + glucose (0.1%)						
Growth	+	+	+	+	+	+
Initial / Final pH	same	same	same	same	same	same
5. $S_2O_3^{2}$-medium+ glucose (0.1%)						
Growth	-	-	-	-	-	-
Initial / Final pH	na	na	na	na	na	na
6. Fe^{2+}-medium+ glucose (0.1%)						
Growth	+	+	+	+	+	+
Oxidation of Fe^{2+} to Fe^{3+}	No	No	No	No	No	No

'+', positive growth; '±', moderate growth; '-', absence of growth

3.3.2.4 Reduction of ferric iron

DKAP1.1 and GBAP VI.1 were found to be able to reduce ferric iron. Precipitation of large amount of iron occurred during the preparation of the medium and thus only a small amount of soluble ferric iron remained available in the medium for the reduction process. In this experiment the initial concentration of soluble ferrous iron was found to be near about 1.0 mg/ml. It was observed that there was a steady increment in the soluble ferrous iron from day 1 to day 10 (Fig. 2.1) while there was no increment in ferrous iron content in control, rather ferrous iron content was found to decrease in the later stages of incubation. This ability of ferric iron reduction by the acidophilic heterotrophs could certainly help in the recycling of ferrous in the environment while growing with *A. ferrooxidans*.

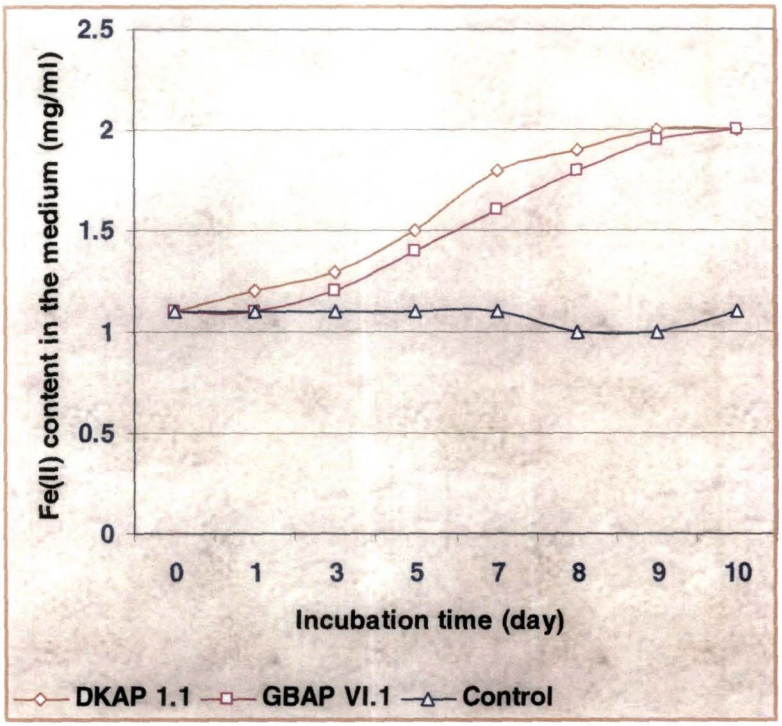

Fig 2.1: Rate of conversion of ferric iron to ferrous iron by *A. cryptum* DKAP1.1 and GBAP VI.1.

3.3.2.5 Rate of glucose utilization

Rate of utilization of glucose was analyzed with five strains of gram negative acidophilic heterotrophs (DKAP1.1, DKAP1.3, DKAP1.4, GBAP VI.1, and GBAP VI.3). All the strains displayed approximately the same rate of glucose utilization except DKAP 1.3 whose rate of utilization was almost two fold more than that of the other strains tested (Fig 2.3). The amount of glucose utilized was determined from the standard curve prepared with known glucose concentration (Fig 2.2).

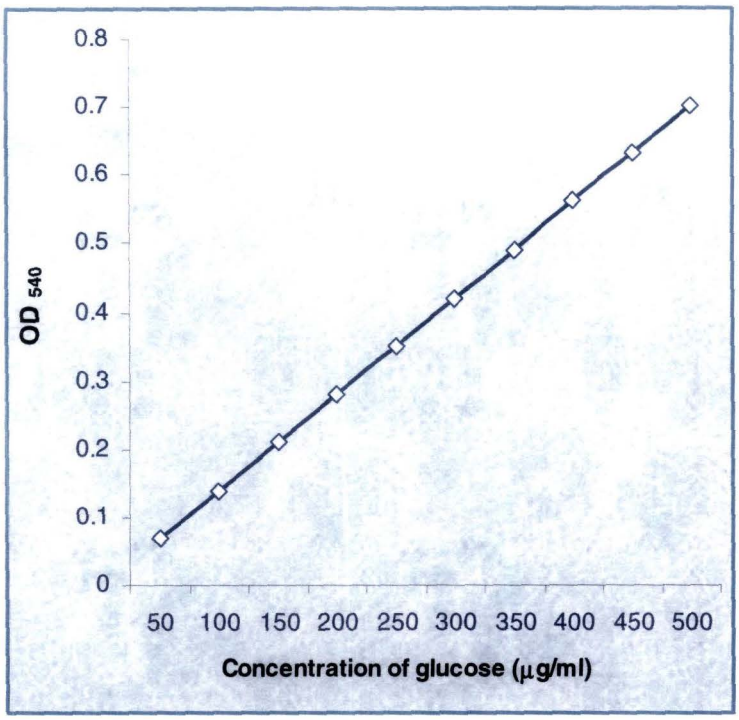

Fig 2.2: Standard curve for glucose

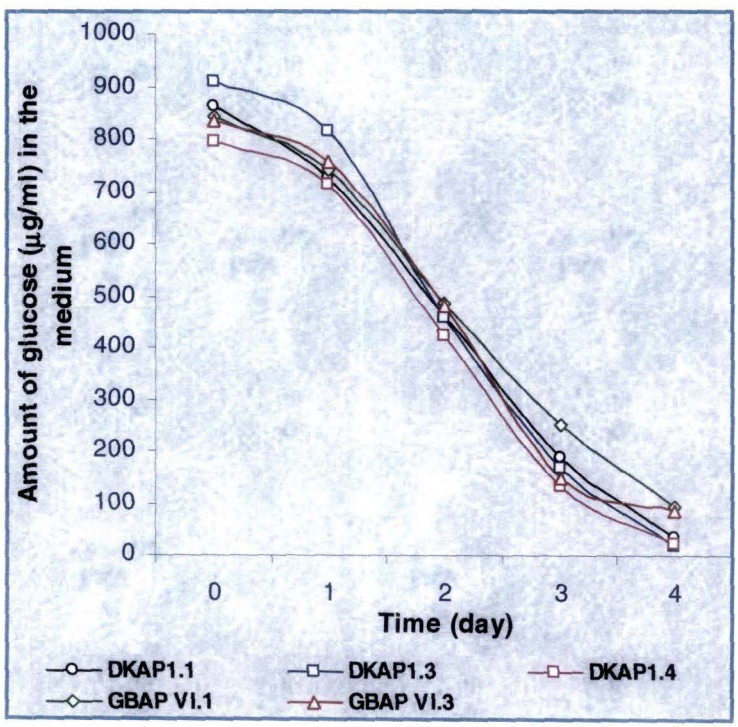

Fig 2.3: Rate of glucose utilization by different strains of acidophilic heterotrophs

3.3.2.6 Tolerance to heavy metal

Most of the strains were able to tolerate heavy metal to a considerably good extent as exhibited by their tolerance to Cu, 15.0 mM; Zn, 100 mM; Ni, 110 mM and Co, 17 mM. Tolerance level to chromium and cadmium was negligible for all the strains tested (Table 2.4).

Table 2.4: Heavy metal tolerance profile of the gram negative acidophilic heterotrophic strains of *A. cryptum*.

Heavy metals		Bacterial Strains				
Name	**Concentration (mM)**	**DKAP1.1**	**DKAP 1.2**	**DKAP 1.3**	**GBAP VI.1**	**GBAP VI .3**
Cu	10	+	+	+	+	+
	13	+	+	±	+	-
	15	±	±	-	±	-
Zn	90	+	+	+	+	+
	100	-	±	+	±	-
	110	-	-	-	-	-
Ni	90	+	+	+	+	+
	100	-	-	±	+	-
	110	-	-	-	±	-
Co	15	+	+	+	+	±
	17	±	±	±	±	-
	20	-	-	-	-	-
Cd	1.0	±	-	±	+	+
	2.0	-	-	-	-	-
Cr	0.5	±	±	±	±	±
	1.0	-	-	-	-	

'+', positive growth; '±', moderate growth; '-', absence of growth

3.4 DISCUSSION

This chapter has described the process of isolation of heterotrophs from the cultures of *Acidithiobacillus ferrooxidans* and presented their atypical physiology of oligotrophism. These gram negative acidophilic heterotrophic strains morphologically and physiologically resembled with *Acidiphilium cryptum* in having the features of creamish-white colony, lacking the ability to grow mixotrophically utilizing elemental sulfur, and being unable to oxidize ferrous iron but capable of utilizing different kinds of organic

molecules (Table 2.2 and Table 2.3). 16S rRNA gene analysis (discussed in chapter 3) and other physiological properties led these heterotrophic isolates to be assigned to the species of *Acidiphilium cryptum.* The cells were highly motile, short rod in shape, having a pH range of 1.6-6.0 indicating that they are acidophilic in nature. The cells were incapable of growing in undiluted nutrient broth or Luria Bertani medium at the concentration used normally for the other heterotrophic organisms but the same medium when diluted hundred times was found to support the growth of these heterotrophs under acidic condition. This showed the oligotrophic nature of the organisms. This was further substantiated when their growth was observed in nutrient poor agar extract medium without any supplementation of glucose and/or yeast extract. They showed the ability to tolerate copper and cobalt up to the level of 15 mM and 17 mM respectively (Table 2.4) which was much lower compared to that of the *A. ferrooxidans;* however they were able to grow in a very high concentration of Zn and Ni (100mM and 110mM respectively). This suggested that the organisms might have been selected and or possessed required genetic system to grow in the environments which are rich in the heavy metal concentrations.

Acidophilic heterotrophs have been found to remain associated with *A. ferrooxidans* strains isolated from a wide variety of sources (Johnson and Kelso, 1983; Mishra *et al.,* 1983). The conditions (e.g. acidic, mesophilic, aerobic, ferrous iron etc.) that are suitable for the growth of *A.* ferrooxidans are also suitable for the perpetuation of some heterotrophic associates. 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). Further the present study also showed that *Acidithiobacillus ferrooxidans* cultures exhibit mixed culture with *Acidiphilium cryptum.* The *A. ferrooxidans* culture was always found to be mixed with heterotrophs like *A. cryptum* even after several rounds of purification processes. This observation has prompted to study the role of *A. ferrooxidans* in the sustenance of the heterotrophic partner.

3.5 CONCLUSION:

1. Acidophilic heterotrophs could be isolated from the culture of *Acidithiobacillus ferrooxidans.* Several strains of these heterotrophs have been characterized and have been assigned to the species *Acidiphilium cryptum.*

2. These heterotrophs were found unable to survive in nutrient rich media but were able to survive in low nutrient medium such as agar extract medium, diluted Nutrient broth or Luria Bertani Broth medium, indicating the oligotrophic nature of the bacteria. In addition to that they were found incapable of oxidizing elemental sulfur or reduced sulfur compounds as well as ferrous iron in presence or absence of glucose, suggesting that they are neither autotrophic or mixotrophic but obligate heterotrophic in nature. In fact the cells were found immotile when they were exposed to thiosulfate possibly suggesting that thiosulfate may be toxic to the organism.

3.6 REFERENCES

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