Chapter -3

Role of *Acidithiobacillus ferrooxidans* in alleviating the inhibitory effect of thiosulfate on the growth of acidophilic *Acidiphilium* species isolated from Garubathan AMD samples

4.1 INTRODUCTION

Acidithiobacillus ferrooxidans, an acidophilic obligate chemolithoautotrophic bacterium, plays an important role in the mining and metallurgical applications. Growth of different strains of *A. ferrooxidans* is inhibited by naturally occurring compounds such as pyruvic acid, citric acid, oxaloacetic acid and glucose (Borichewski, 1967; Harrison, 1984; Frattini *et al.*, 2000). Presence of such inhibitory substances has been shown to decrease the efficiency of bioleaching (Harrison, 1984; Johnson, 1995). This inhibition could be alleviated in presence of heterotrophic acidophiles such as *Acidiphilium* sp. resulting in the enhancement of iron (pyrite) oxidation (Paiment *et al.*, 2001). Cultivation of *A. ferrooxidans* on acidic ferrous iron agar has always been difficult because of the presence of agar hydrolyzed products. Areas circumscribing point inoculations of acidophilic heterotrophs into ferrous iron agar were observed to be colonized by *A. ferrooxidans* (Harrison, 1984). Earlier authors have described a plate count procedure where acidophilic heterotrophic bacterium was used in agar medium to scavenge organic material for colony formation of *A. ferrooxidans* (Butler and Kempton, 1987). Acidophilic heterotrophs like *A. cryptum* required a very lean organic medium (oligotrophic) containing low concentrations of glucose (0.1%) and yeast extract (0.01%). Higher concentration of glucose or complex organic supplements, which are commonly used in most heterotrophic media (eutrophic), completely inhibited growth (Harrison, 1981). On the other hand, acidophilic autotrophs of the genus *Acidithiobacillus* have shown self-inhibition towards their own metabolic by-products released in the environment (Borichewski, 1967). Therefore, growth of *Acidithiobacillus* may require a relationship with acidophilic heterotrophic members of the genus *Acidiphilium* (Peccia *et al.*, 2000).

Geomicrobiological studies have shown the presence of acidophilic heterotrophic bacteria along with ferrous and/or sulfur oxidizing chemolithoautotrophs (Harrison, 1984). Few obligate acidophilic heterotrophic bacteria representing the genus *Acidiphilium,* such as *A. cryptum* (Harrison, 1981), *A. angustum, A. facilis and A. rubrum* (Lazaroff *et al.*, 1982, Jones *et al.*, 1984), *A. organovorum* (Lobos *et al.*,. 1986), and *A. acidophilum* (Guay and Silver, 1975; Harrison, 1983) have been characterized. The acid-laden, sulfidic mineral-rich environments where they are characteristically found are invariably rich in ferrous and ferric ion as well as sulfur and reduced sulfur compounds.

Thiosulfate release from pyrite surface has been suggested by several authors (Luther, 1987; Rimstidt and Vaughan, 2003; Descostes *et al.*, 2004). It has been observed that thiosulfate and polythionates are the significant products of pyrite oxidation in near neutral solution (Schippers and Sand, 1999), while only low amounts of intermediate sulfur oxyanion species have been observed in acidic solution (Druschel and Borda, 2006). In an earlier study it was shown that the glucose-limited chemostat cultures of *Thiobacillus acidophilus* (re-named as *Acidiphilium acidophilum,* Hiraishi *et al.*, 1998) could oxidize thiosulfate (Pronk *et al.*, 1990). Though sulfur di-oxygenase activity has been reported in *A. acidophilum* and *A. cryptum* DSM 2389 (Rohwerdert and Sand, 2003), chemolithotrophic growth of *A. cryptum* on thiosulfate could not be demonstrated. However, there are reports on oxidation of elemental sulfur by *A. cryptum* in the presence of organic substrates (Harrison, 1983; Hallberg *et al.*, 2001).

In this study, while examining the physiological/ growth characteristics of the *Acidiphilium* sp. DKAP1.1 isolated from enrichment culture of *A. ferrooxidans* (from acid mine drainage of Garubathan, West Bengal, India), it was noticed that the *Acidiphilium* strain failed to grow in its routine culture medium in presence of thiosulfate. This observation led us to investigate as to whether growth inhibition by thiosulfate was irreversible in pure culture and the role of the chemolithoautotrophic partner, *A. ferrooxidans,* in alleviating thiosulfate mediated inhibition in mixed culture.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial strains

One acidophilic heterotrophic strain DKAP1.1 and one acidophilic autotrophic strain DK6.1 were used for this study.

4.2.2 Characterization of DK6.1 and DKAP1.1

4.2.2.1 Diagnostic tests for DK6.1

Gram staining, motility, growth in different pH and temperature, autotrophic growth in different energy substrates (ferrous sulfate or elemental sulfur or thiosulfate or tetrathionate) were done according to the procedures described earlier (Chapter 1.)

4.2.2.2 Diagnostic tests for DKAP1.1

Diagnostic tests for DKAP1.1 were done as described in chapter 2.

4.2.2.3 DNA isolation

For the isolation of DNA from DK6.1 and DKAP1.1, pure culture was grown in elemental sulfur medium for two weeks and modified DSMZ 269 medium for 5-6 days respectively. Cells were harvested by centrifuging at 10,000 rpm for 10 min and frozen at -20°C. Genomic DNA was isolated and purified by the procedure described earlier (Chakraborty *et al..*, 1997), and stored in 0.1 X TE buffer or sterile double distilled water (reviewed in detail in chapter 5).

4.2.2.4 Amplification, cloning, sequencing and analysis of 16S rDNA

16S rDNA gene fragments were amplified by PCR with Taq DNA polymerase and a pair set of eubacterial universal primers 27f and 1492 (Lane, 1991). PCR products were treated with a chloroform/isoamyl alcohol mixture and cloned in pGEM-T easy vectors following the manufacturer's instruction and then transformed into *Escherichia coli* XL1 Blue. Nucleotide sequencing was performed with the ABI PRISM^{TM} Dye Terminator Cycle Sequencing Ready Reaction kit and the reactions were analyzed on an ABI PRISM 377 DNA sequencer using SP6 and T7 promoter primers. Nucleotide sequence analysis was performed using BLAST search programs [National Center for Biotechnology Information (NCBI)] (the procedure is reviewed in detail chapter 5).

4.2.2.5 Growth of DKAP1.1 in Elemental sulfur spent medium of autotrophic DK6.1 pure culture

To test the oligotrophic nature, DKAP1.1 was grown in elemental sulfur spent medium of autotrophic DK6.1 pure culture. Three weeks old elemental sulfur culture of 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 sulphide 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 at all present in the spent medium, would be as low as 5µg/l (Roy and Trudinger, 1970; Steudal *et al.*, 1987). DKAP1.1 cells were grown in elemental sulfur spent medium with and without the supplementation of glucose (0.1%) and yeast extract (0.01%). Growth of the culture in different conditions was monitored by counting the viable cells in Petroff-Hausser bacterial counting chamber.

4.2.2.6 Effect of submerged layering of heterotrophic DKAP1.1 cells on the manifestation of chemoautotrophic DK6.1 colonies over modified 9K agar surface

Effect of acidophilic heterotrophs in the growth of acidophilic chemoautotroph was assessed by growing them in single and double layered modified 9K agar plates. The conventionally prepared modified 9K agar plates used for spread-plating of acidophilic chemoautotrophic cells were considered as single layer plates. To prepare double layered plates, 10 ml of molten modified 9K agar medium (45-50°C) was mixed with washed and concentrated log phase cells of acidophilic heterotroph cells (~2 x 10^6 cells) and poured in a Petri- plate to solidify. After solidification of the first, a second layer was made by overlaying it with 10 ml sterile molten modified 9K agar solution and allowed to solidify. Direct spread-plating of diluted *A. ferrooxidans* culture was carried out in both single and double layer plates and were incubated at 28°C. The emergence and increase in diameter of colonies with respect to the period of incubation of plates were recorded.

4.2.2.7 Growth of heterotrophic DKAP1.1 in pure and co-culture with autotrophic DK6.1 in presence of thiosulfate under laboratory condition

20 ml of modified DSMZ 269 medium containing 0.1% glucose and 0.01% yeast extract in each of the 250 ml Erlenmeyer flasks was supplemented with different concentrations of thiosulfate. 100 mM thiosulfate stock solution was prepared in distilled water and sterilized separately. The required quantity of sterile 100 mM thiosulfate solution was added to the medium to obtain the desired concentration (0.5, 0.7, 1.0, 1.5, and 2.0 mM) of thiosulfate. In the first experimental set, pre-grown DKAP1.1 cells at an initial concentration of approximately 2.07-3.3 x 10^5 cells/ ml was added to the thiosulfate containing DSMZ 269 medium. Viability and growth of DKAP1.1 in presence of different concentration of thiosulfate was evaluated by dilution plating on DSMZ 269 agar for

determining the minimum inhibitory concentration. In another set of experiment, DKAP1.1 cells (2.01-2.6 x 10^5 cells/ml) mixed with DK6.1 cells (8.8×10^6 cells/ml) were inoculated in modified DSMZ 269 broth having different concentration of thiosulfate. Survivability of DKAP1.1 cells (in presence of DK6.1) in the thiosulfate supplemented medium was assessed through dilution plating of the mixed culture aliquot at different time intervals on fresh DSMZ 269 agar. On the other hand, viability of the acidophilic chemoautotroph DK6.1 in the thiosulfate supplemented DSMZ broth was determined by periodical inoculation of 50 ml of fresh modified 9K broth with 100 µl of the mixed culture and observation for the oxidation of ferrous iron.

4.3 RESULTS

4.3.1 Phenotypic and 16S rRNA gene characterization of the test strains

Results of Phenotypic characterization of DK6.1 and DKAP1.1 have already been presented in chapter 1 and chapter 2.

BLASTN analysis of 16S rRNA gene sequence of the isolate DK6.1 (Genbank accession no. FJ602383) revealed 99% similarity with partial 16S rRNA gene sequence of *A. ferrooxidans* LMT 4 (Fig 3.1 and Fig 3.2). Distance tree constructed using Fast minimum Evolution of BLASTN 2.2.19+ program (Zhang *et al.*, 2000) presented a robust cluster of DK6.1 sequence with partial 16S rRNA gene of *A. ferrooxidans* LMT4 (AM502931) and uncultured bacterium clone ANG Pin f8 16S rRNA gene (EU 370304).

BLASTN analysis of 16S rRNA gene sequence of the isolate DKAP1.1 (GENBANK accession no. FJ602384) produced maximum similarity (99%) with 16S rRNA gene sequence of *A. cryptum* JF-5 [being the constituent of its complete genome sequence (CP000697)] (Fig 3.3 and Fig 3.4). In the Distance tree constructed using Fast minimum Evolution of BLASTN 2.2.19+ program (Zhang *et al.*, 2000), DKAP1.1 sequence clustered strongly with 16S rRNA gene of *A. cryptum* JF-5 and 16S rRNA gene sequence of *Acidiphilium* sp. SX-F (FJ194544).

4.3.2 Growth of *A. cryptum* DKAP1.1 in Elemental sulfur spent medium of *A. ferrooxidans* DK6.1 pure culture

DKAP1.1 cells were able to grow in three weeks old elemental sulfur spent medium of *A. ferrooxidans* DK6.1, with or without supplementation of glucose and yeast

extract. However growth was not detected in fresh sterile basal salt solution of elemental sulfur medium devoid of glucose and yeast extract (Fig. 3.5). The ability of *A. cryptum* DKAP1.1 to grow in low nutrient medium such as elemental sulfur spent medium explains the oligotrophic nature of the strain.

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tcgcagctac catgcagtcg acggtacagg tcttcggatg ctgacgagtg gcggacgggt gagtaatgcg
taggaatctg tcttttagtg ggggacaacc cagggaaact tgggctaata ccgcatgagc cctgaggggg
aaagcggggg atcttcggac ctcgcgctaa gagaggagcc tacgtccgat tagctagttg gcgggtaaag
gcccaccaag gcgacgatcg gtagctggtc tgagaggacg accagccaca ctgggactga gacacggccc
agactcctac gggaggcagc agtggggaat ttttcgcaat gggggcaacc ctgacgaagc aatgccgcgt
ggatgaagaa ggccttcggg ttgtaaagtc ctttcgtgga ggacgaaaag gcgggttcta atacaatctg
ctgttgacgt gaatccaaga agaagcaccg gctaactccg tgccagcagc cgcggtaata cgggggggtgc
aagcgttaat cggaatcact gggcgtaaag ggtgcgtagc ggtacgttag tctgtcgtga atccccgggc
tcaacctggg aatggcggtg gaaaccggcg cactagagta tgggagaggg tggtggaatt ccaggtgtag
cggtgaaatg cgtagagatc tggaggaaca tcagtggcga aggcggccac ctggcccaat actgacgctg
aggcacgaaa gcgtggggag caaacaggat tagataccct ggtagtccac gccctaaacg atgaatacta
gatgtttggt gcctagcgta ctgagtgtcg tagctaacgc gataagtatt ccgcctggga agtacggccg
caaggttaaa actcaaagga attgacgggg gcccgcacaa gcggtggagc atgtggttta attcgatgca
acgcgaagaa ccttacctgg gcttgacatg tccggaattc tgcagagatg cggaagtgcc cttcggggaa
tcggaacaca ggtgctgcat ggctgtcgtc agctcgtgtc gtgagatgtt gggttaagtc ccgcaacgag
cgcaaccctt gtccttagtt gccagcggtt cggccgggca ctctagggag actgccggtg acaaaccgga
ggaaggtggg gatgacgtca agtcctcatg gcctttatgt ccagggctac acacgtgcta caatggcgcg
tacagaggga agccaagccg cgaggtggag cagaccccag aaagcgcgtc gtagttcgga ttgcagtctg
caactcgact gcatgaagtc ggaatcgcta gtaatcgcgg atcagcatgc cgcggtgaat acgttcccgg
gccttgtaca caccgcccgt cacaccatgg gagtggattg taccagaagc agctagccta acctcgggag
ggcgtaccac ggtacgtc
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Fig. 3.1: Partial 16S rRNA gene sequence of acidophilic autotrophic strain DK6.1

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DK6.1   11    CATGC-AGTCG-ACGGT-ACAGGTCTTCGGATGCTGACGAGTGGCGGACGGGTGAGTAAT  67
              ||||| ||||| ||||| ||||||||||||||||||||||||||||||||||||||||||
Aferr   27    CATGCAAGTCGAACGGTAACAGGTCTTCGGATGCTGACGAGTGGCGGACGGGTGAGTAAT  86
DK6.1   68    GCGTAGGAATCTGTCTTTTAGTGGGGGACAACCCAGGGAAACTTGGGCTAATACCGCATG  127
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Aferr   87    GCGTAGGAATCTGTCTTTTAGTGGGGGACAACCCAGGGAAACTTGGGCTAATACCGCATG  146
DK6.1   128   AGCCCTGAGGGGGAAAGCGGGGGATCTTCGGACCTCGCGCTAAGAGAGGAGCCTACGTCC  187
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Aferr   147   AGCCCTGAGGGGGAAAGCGGGGGATCTTCGGACCTCGCGCTAAGAGAGGAGCCTACGTCC  206
DK6.1   188   GATTAGCTAGTTGGC-GGGTAAAGGCCCACCAAGGCGACGATCGGTAGCTGGTCTGAGAG  246
              |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Aferr   207   GATTAGCTAGTTGGCGGGGTAAAGGCCCACCAAGGCGACGATCGGTAGCTGGTCTGAGAG  266
DK6.1   247   GACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGG  306
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Aferr   267   GACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGG  326
DK6.1   307   GAATTTTTCGCAATGGGGGCAACCCTGACGAAGCAATGCCGCGTGGATGAAGAAGGCCTT  366
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Aferr   327   GAATTTTTCGCAATGGGGGCAACCCTGACGAAGCAATGCCGCGTGGATGAAGAAGGCCTT  386
DK6.1   367   CGGGTTGTAAAGTCCTTTCGTGGAGGACGAAAAGGCGGGTTCTAATACAATCTGCTGTTG  426
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Aferr   387   CGGGTTGTAAAGTCCTTTCGTGGAGGACGAAAAGGCGGGTTCTAATACAATCTGCTGTTG  446
DK6.1   427   ACGTGAATCCAAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGGGG  486
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Aferr   447   ACGTGAATCCAAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGGGG  506
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DK6.1  487   GTGCAAGCGTTAATCGGAATCACTGGGCGTAAAGGGTGCGTA-GCGGTACGTTA-GTCTG  544
             ||||||||||||||||||||||||||||||||||||||||| |||||||||||| |||||
Aferr  507   GTGCAAGCGTTAATCGGAATCACTGGGCGTAAAGGGTGCGTAGGCGGTACGTTAGGTCTG  566
DK6.1  545   TCGTG-AATCCCCGGGCTCAACCTGGGAATGGCGGTGGAAACCGGCGCACTAGAGTATGG  603
             ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Aferr  567   TCGTGAAATCCCCGGGCTCAACCTGGGAATGGCGGTGGAAACCGGCGCACTAGAGTATGG  626
DK6.1  604   GAGAGGGTGGTGGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAACATCA  663
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Aferr  627   GAGAGGGTGGTGGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAACATCA  686
DK6.1  664   GTGGCGAAGGCGGCCACCTGGCCCAATACTGACGCTGAGGCACGAAAGCGTGGGGAGCAA  723
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Aferr  687   GTGGCGAAGGCGGCCACCTGGCCCAATACTGACGCTGAGGCACGAAAGCGTGGGGAGCAA  746
DK6.1  724   ACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGAATACTAGATGTTTGGTGCC  783
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Aferr  747   ACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGAATACTAGATGTTTGGTGCC  806
DK6.1  784   TAGCGTACTGAGTGTCGTAGCTAACGCGATAAGTATTCCGCCTGGGAAGTACGGCCGCAA  843
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Aferr  807   TAGCGTACTGAGTGTCGTAGCTAACGCGATAAGTATTCCGCCTGGGAAGTACGGCCGCAA  866
DK6.1  844   GGTTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATT  903
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Aferr  867   GGTTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATT  926
DK6.1  904   CGATGCAACGCGAAGAACCTTACCTGGGCTTGACATGTCCGGAATTCTGCAGAGATGCGG  963
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Aferr  927   CGATGCAACGCGAAGAACCTTACCTGGGCTTGACATGTCCGGAATTCTGCAGAGATGCGG  986
DK6.1  964   AAGTGCCCTTCGGGGAATCGGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTG  1023
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Aferr  987   AAGTGCCCTTCGGGGAATCGGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTG  1046
DK6.1  1024  AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTTAGTTGCCAGCGGTTCGG  1083
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Aferr  1047  AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTTAGTTGCCAGCGGTTCGG  1106
DK6.1  1084  CCGGGCACTCTAGGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGT  1143
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Aferr  1107  CCGGGCACTCTAGGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGT  1166
DK6.1  1144  CCTCATGGCCTTTATGTCCAGGGCTACACACGTGCTACAATGGCGCGTACAGAGGGAAGC  1203
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Aferr  1167  CCTCATGGCCTTTATGTCCAGGGCTACACACGTGCTACAATGGCGCGTACAGAGGGAAGC  1226
DK6.1  1204  CAAGCCGCGAGGTGGAGCAGACCCCAGAAAGCGCGTCGTAGTTCGGATTGCAGTCTGCAA  1263
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Aferr  1227  CAAGCCGCGAGGTGGAGCAGACCCCAGAAAGCGCGTCGTAGTTCGGATTGCAGTCTGCAA  1286
DK6.1  1264  CTCGACTGCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACG  1323
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Aferr  1287  CTCGACTGCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACG  1346
DK6.1  1324  TTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGATTGTACCAGAAGCAGC  1383
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Aferr  1347  TTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGATTGTACCAGAAGCAGC  1406
DK6.1  1384  TAGCCTAACCT-CGGGAGGGCG-T-ACCACGGTA  1414
             |||||||||| |||||||||| | |||||||||
Aferr  1407  TAGCCTAACCTTCGGGAGGGCGGTTACCACGGTA  1440
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Fig 3.2: Blastn result of 16SrRNA sequence of DK6.1 showing 99% homology with partial 16SrRNA gene sequence of *Acidithiobacillus ferrooxidans* LMT4. Vertical lines show the positions of identical bases. Aferr: *A. ferrooxidans* LMT4

4.3.3 Effect of submerged layering of *A. cryptum* DKAP1.1 cells on the manifestation of *A. ferrooxidans* DK6.1 colonies on Iron-agar

A. ferrooxidans DK6.1 cells grown in ferrous iron medium when dilution plated separately on single and double layered plates, showed marked variation with respect to

the number, emergence and robustness of colonies with time (Table 3.1). A positive effect on growth of *A. ferrooxidans* DK6.1 was observed when it was co-cultured with *A. cryptum* DKAP1.1 in double layered plates. Incorporation of heterotrophic *A. cryptum* DKAP1.1 in an underlay of ferrous-agar medium promoted growth of *A. ferrooxidans* DK6.1 colonies through enhanced ferrous iron oxidation.

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tcgcagctta ccatgcagtc gcacgggcag ggcaacctgt cagtggcgga cgggtgagta acgcgtagga
atctatcctt gggtggggga caaccgtggg aaactacggc taataccgca tgatccctga ggggcaaagg
cgaaagtcgc ctgaggagga gcctgcgtct gattaggtag ttggtggggt aaaggcctac caagcctgcg
atcagtagct ggtctgagag gatgatcagc cacactggga ctgagacacg gcccagactc ctacgggagg
cagcagtggg gaatattgga caatgggcga aagcctgatc cagcaatgcc gcgtggatga agaaggtctt
cggattgtaa agtccttttg gcggggacga tgatgacggt acccgcagaa taagctccgg ctaacttcgt
gccagcagcc gcggtaatac gaaggggggct agcgttgctc ggaatgactg ggcgtaaagg gcgcgtaggc
ggacggcaca gtcaggcgtg aaattcctgg gctcaacctg ggggactgcg tctgagacgt gttgtcttga
gtatggaaga gggttgtgga atttccagtg tagaggtgaa attcgtagat attggaaaga acaccggtgg
cgaaggcggc aacctggtcc attactgacg ctgaggcgcg aaagcgtggg gagcaaacag gattagatac
cctggtagtc cacgctgtaa acgatgtgtg ctggatgttg gggtgcttag cacttcagtg tcgtagctaa
cgcggtaagc acaccgcctg gggagtacgg ccgcaaggtt gaaactcaaa ggaattgacg ggggcccgca
caagcggtgg agcatgtggt ttaattcgaa gcaacgcgca gaaccttacc aggatttgac atggggagta
ccggtccaga gatggacttt cccgcaaggg gctctcgcac aggtgctgca tggctgtcgt cagctcgtgt
cgtgagatgt tgggttaagt cccgcaacga gcgcaaccct cgccttcagt tgccagcatg tttgggtggg
cactctgaag gaactgccgg tgacaagccg gaggaaggtg gggatgacgt caagtcctca tggcccttat
gtcctgggct acacacgtgc tacaatggcg gtgacagtgg gaagccaggt ggtgacaccg agctgatctc
aaaaagccgt ctcagttcgg attgcactct gcaactcgag tgcatgaagg tggaatcgct agtaatcgcg
gatcagcatg ccgcggtgaa tacgttcccg ggccttgtac acaccgcccg tcacaccatg ggatttggtt
tgaccttaag ttggtgcgct aacccgcaag gaggcagcca
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Fig: 3.3: Partial 16SrRNA gene sequence of the acidophilic heterotroph DKAP1.1.

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DKAP1.1   3         GCA-GCTT-AC-CATGC-AGTCGCACGGGCAGGGCAACCTGTCAGTGGCGGACGGGTGAG  58
                    ||| |||| || ||||| ||||||||||||||||||||||||||||||||||||||||||
AcrJF-5   2012865   GCATGCTTAACACATGCAAGTCGCACGGGCAGGGCAACCTGTCAGTGGCGGACGGGTGAG  2012806
DKAP1.1   59        TAACGCGTAGGAATCTATCCTTGGGTGGGGGACAACCGTGGGAAACTACGGCTAATACCG  118
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AcrJF-5   2012805   TAACGCGTAGGAATCTATCCTTGGGTGGGGGACAACCGTGGGAAACTACGGCTAATACCG  2012746
DKAP1.1   119       CATGATCCCTGAGGGGCAAAGGCGAAAGTCGCCTGAGGAGGAGCCTGCGTCTGATTAGGT  178
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AcrJF-5   2012745   CATGATCCCTGAGGGGCAAAGGCGAAAGTCGCCTGAGGAGGAGCCTGCGTCTGATTAGGT  2012686
DKAP1.1   179       AGTTGGTGGGGTAAAGGCCTACCAAGCCTGCGATCAGTAGCTGGTCTGAGAGGATGATCA  238
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AcrJF-5   2012685   AGTTGGTGGGGTAAAGGCCTACCAAGCCTGCGATCAGTAGCTGGTCTGAGAGGATGATCA  2012626
DKAP1.1   239       GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTG  298
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AcrJF-5   2012625   GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTG  2012566
DKAP1.1   299       GACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGGATGAAGAAGGTCTTCGGATTGT  358
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AcrJF-5   2012565   GACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGGATGAAGAAGGTCTTCGGATTGT  2012506
DKAP1.1   359       AAAGTCCTTTTGGCGGGGACGATGATGACGGTACCCGCAGAATAAGCTCCGGCTAACTTC  418
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AcrJF-5   2012505   AAAGTCCTTTTGGCGGGGACGATGATGACGGTACCCGCAGAATAAGCTCCGGCTAACTTC  2012446
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DKAP1.1  419      GTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATGACTGGGCGTAAA  478
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AcrJF-5  2012445  GTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATGACTGGGCGTAAA  2012386
DKAP1.1  479      GGGCGCGTAGGCGGACGGCACAGTCAGGCGTGAAATTCCTGGGCTCAACCTGGGGGACTG  538
                  ||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
AcrJF-5  2012385  GGGCGCGTAGGCGGACGGCACAGTCAGGCGTGAAATTCCTGGGCTCAACCT-GGGGACTG  2012327
DKAP1.1  539      CGTCTGAGACGTGTTGTCTTGAGTATGGAAGAGGGTTGTGGAATTTCCAGTGTAGAGGTG  598
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AcrJF-5  2012326  CGTCTGAGACGTGTTGTCTTGAGTATGGAAGAGGGTTGTGGAATTTCCAGTGTAGAGGTG  2012267
DKAP1.1  599      AAATTCGTAGATATTGGAAAGAACACCGGTGGCGAAGGCGGCAACCTGGTCCATTACTGA  658
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AcrJF-5  2012266  AAATTCGTAGATATTGGAAAGAACACCGGTGGCGAAGGCGGCAACCTGGTCCATTACTGA  2012207
DKAP1.1  659      CGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGT  718
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AcrJF-5  2012206  CGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGT  2012147
DKAP1.1  719      AAACGATGTGTGCTGGATGTTGGGGTGCTTAGCACTTCAGTGTCGTAGCTAACGCGGTAA  778
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AcrJF-5  2012146  AAACGATGTGTGCTGGATGTTGGGGTGCTTAGCACTTCAGTGTCGTAGCTAACGCGGTAA  2012087
DKAP1.1  779      GCACACCGCCTGGGGAGTACGGCCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCG  838
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AcrJF-5  2012086  GCACACCGCCTGGGGAGTACGGCCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCG  2012027
DKAP1.1  839      CACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGGATTTG  898
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AcrJF-5  2012026  CACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGGATTTG  2011967
DKAP1.1  899      ACATGGGGAGTACCGGTCCAGAGATGGACTTTCCCGCAAGGGGCTCTCGCACAGGTGCTG  958
                  |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
AcrJF-5  2011966  ACATGGGGAGTACCGGTCCAGAGATGGACTTTCCCGCAAGGGGCTCCCGCACAGGTGCTG  2011907
DKAP1.1  959      CATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACC  1018
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AcrJF-5  2011906  CATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACC  2011847
DKAP1.1  1019     CTCGCCTTCAGTTGCCAGCATGTTTGGGTGGGCACTCTGAAGGAACTGCCGGTGACAAGC  1078
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AcrJF-5  2011846  CTCGCCTTCAGTTGCCAGCATGTTTGGGTGGGCACTCTGAAGGAACTGCCGGTGACAAGC  2011787
DKAP1.1  1079     CGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGTCCTGGGCTACACACGT  1138
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AcrJF-5  2011786  CGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGTCCTGGGCTACACACGT  2011727
DKAP1.1  1139     GCTACAATGGCGGTGACAGTGGGAAGCCAGGTGGTGACACCGAGCTGATCTCAAAAAGCC  1198
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AcrJF-5  2011726  GCTACAATGGCGGTGACAGTGGGAAGCCAGGTGGTGACACCGAGCTGATCTCAAAAAGCC  2011667
DKAP1.1  1199     GTCTCAGTTCGGATTGCACTCTGCAACTCGAGTGCATGAAGGTGGAATCGCTAGTAATCG  1258
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AcrJF-5  2011666  GTCTCAGTTCGGATTGCACTCTGCAACTCGAGTGCATGAAGGTGGAATCGCTAGTAATCG  2011607
DKAP1.1  1259     CGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCA  1318
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AcrJF-5  2011606  CGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCA  2011547
DKAP1.1  1319     TGGGATTTGGTTTGACCTTAAGTTGGTGCGCTAACCCGCAAGG-AGGCAGCCA  1370
                  |||||||||||||||||||||||||||||||||||||||||||| |||||||||
AcrJF-5  2011546  TGGGATTTGGTTTGACCTTAAGTTGGTGCGCTAACCCGCAAGGGAGGCAGCCA  2011494
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Fig 3.4: Blast result of 16S rRNA sequence of DKAP1.1 showing 99% homology with the partial 16SrRNA sequence of *Acidiphilium cryptum* JF-5 (AcrJF-5). Vertical lines show the position of identical bases.

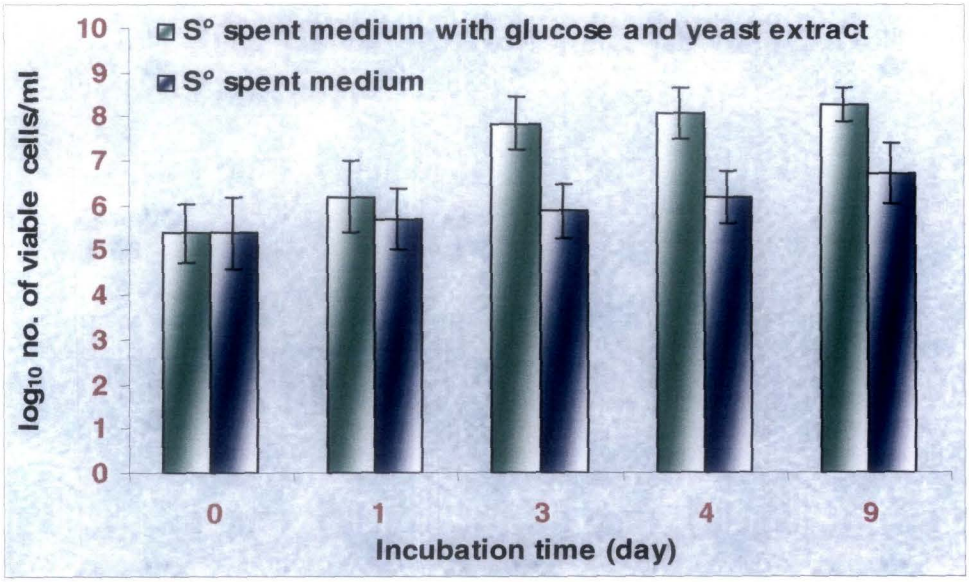

Fig 3.5: Growth of acidophilic heterotroph DKAP1.1 in spent elemental sulfur medium with or without the supplementation of glucose. Error bars represent the standard deviation in replicate samples (n =3).

Table 3.1: Comparison of the rate of emergence and increase in the diameter of *A. ferrooxidans* GBVI colonies in Fe (II) iron agar in single and double layered plates.

Single layered Fe(II)-agar plate				**Double layered Fe(II)-agar plate**			
Dilution of the stock	No. of colonies emerged	No. of days of incubation	Average diameter (mm)	Dilution of the stock	No. of colonies emerged	No. of days of incubation	Average diameter (mm)
10^{-7}	Nil	5	na	10^{-7}	0	5	nd
10^{-7}	-do-	8	na	10^{-7}	14	8	2.0
10^{-7}	-do-	9	na	10^{-7}	14	9	2.6
10^{-7}	-do-	10	na	10^{-7}	14	10	3.2
10^{-7}	10	18	nd	10^{-7}	14	12	3.4
10^{-7}	10	23	0.5	10^{-7}	14	13	3.6

na, not applicable

4.3.4 Inhibitory effect of thiosulfate on growth of *A. cryptum* DKAP1.1 and alleviation of inhibition in co-culture with *A. ferrooxidans* DK6.1

A decrease in viability of *A. cryptum* DKAP1.1 cells in thiosulfate supplemented modified DSMZ 269 medium was observed with the increase in the concentration of thiosulfate (Fig. 3.6). It was found that *A. cryptum* DKAP1.1 could tolerate concentrations of thiosulfate up to 0.7 mM. It was observed that the number of viable cells were higher in 48 h treated cells for a given concentration of thiosulfate as compared to 24 h treatment at the same concentration up to 0.7 mM. However at concentration above 1.0 mM, the effect of thiosulfate on the viability was much more pronounced with respect to both the concentration and time of treatment. At 1.0 mM concentration viable cells could be obtained from 24 h treated culture but no viable cells were obtained after 48 h treatment. On further increase of concentration no viable cells could be obtained from either 24 or 48 h treated cells (Fig. 3.6). On the other hand, when pure culture of *A. ferrooxidans* DK6.1 was incubated in modified DSMZ 269 medium for ≥ 24 h with or without thiosulfate, the cells lost their ability to oxidize ferrous iron when transferred to fresh 9K medium.

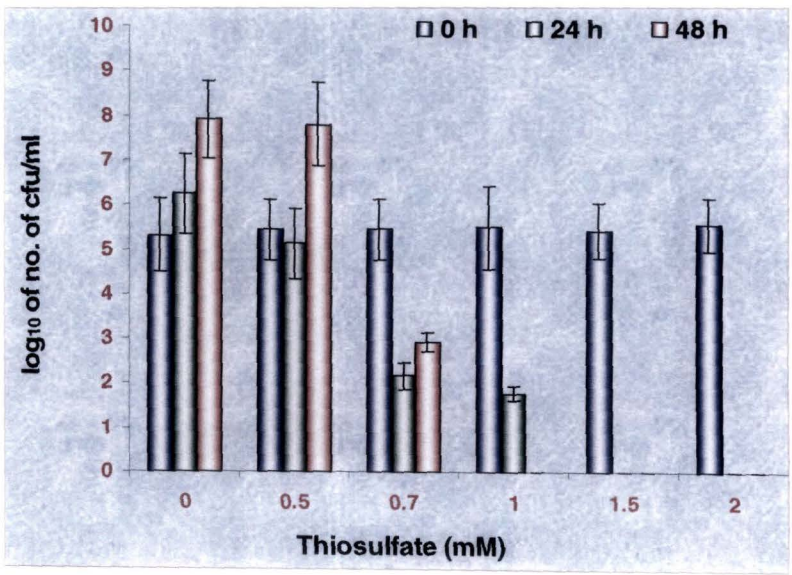

Fig 3.6: Viable cell number of pure culture of *A. cryptum* DKAP1.1 cells in DSMZ 269 medium containing different thiosulfate concentrations.

The death of *A. cryptum* DKAP1.1 cells due to presence of thiosulfate in DSMZ 269 medium was prevented in co-culture with *A. ferrooxidans* DK6.1. Survival of the co-cultured DKAP1.1 cells even in presence 2.0 mM thiosulfate was noted (Fig. 3.7).

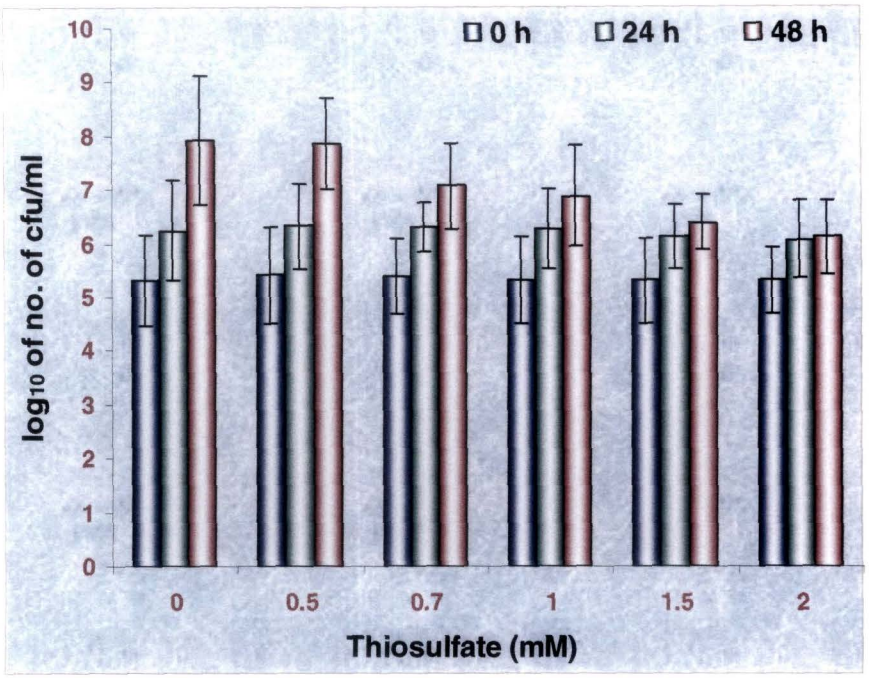

Fig 3.7: Viable cell number of *A. cryptum* DKAP 1.1 in co-culture (with *A. ferrooxidans* DK6.1) cells at different thiosulfate concentrations. Error bars represent the standard deviation on replicate

A. ferrooxidans DK6.1 cells in co-culture remained viable (qualitatively tested by their ability to oxidize ferrous iron in 9K medium) throughout the expanse of the experiment. The lethal effect of thiosulfate on *A. cryptum* DKAP1.1 in pure culture was found directly related to the concentration of thiosulfate in the medium and the lethality could be alleviated in mixed culture with *A. ferrooxidans* DK6.1.

4.4 DISCUSSION

A. ferrooxidans, unique among the acidophilic chemolithotrophic bacteria in its ability to oxidize ferrous iron and sulfidic minerals, was first isolated from acidic mine drainage (AMD) water (Temple and Colmer, 1951) and has since been isolated from a variety of acidic environments. Also, there are numerous reports of the isolation of acidophilic heterotrophs from reportedly pure cultures of *A. ferrooxidans* (Guay and

Silver, 1975; Harrison *et al.*, 1980). These heterotrophs survive during prolonged cultivation of *A. ferrooxidans* in inorganic media, probably by scavenging low concentration of organic compounds excreted by *A. ferrooxidans.* Few of the acidophilic heterotrophic bacteria like *A. cryptum* and *A. acidophilum*, have the ability to reduce ferric ion to ferrous state. In this respect it has been suggested that the removal of (toxic) organic compounds and regeneration of ferrous ions by the heterotrophs may also be advantageous to *A. ferrooxidans* (Harrison, 1984; Marchand and Silverstein, 2003).

The present work was carried out to gain a better understanding of the interactions among acidophilic chemolithoautotrophs and heterotrophs in AMD environments enriched with low amount of organic carbon substrates. In the microbial ecology of acid mine environments, members of the genus *Acidiphilium* may have considerable relations with sulfur- and iron-oxidizing bacteria. Certain conditions like acidic, mesophilic, aerobic, ferrous iron or other mineral sulfide substrate environment, which support growth of sulfur- and iron-oxidizing bacteria, are appropriate for the maintenance of some heterotrophic partners. Pure culture studies on *Acidithiobacilus thiooxidans* have shown that this bacterium excrete pyruvic and oxalo-acetic acids that are self inhibitory at 2×10^{-5} to 7×10^{-5} M (Borichewski, 1967), and therefore, growth of *A. thiooxidans* may necessitate a partnership with an acidophilic heterotroph. It has also been shown that *A. ferrooxidans* cells excrete organic compounds, such as pyruvate, glutamate, aspartate, serine, glycine, and other amino acids, which the heterotrophs can use as substrates for growth (Arkesteyn and deBont, 1980; Schnaltman and Lundgren, 1965). On the other hand, lysed cells of *A. ferrooxidans* may provide an additional source of organic matter. In each case, the quantity of obtainable organic material in autotrophically grown *A. ferrooxidans* cultures is very small. Consequently, the acidophilic heterotrophs have to be competent forager of these organic compounds. Since some of these compounds have been shown to be inhibitory to *A. ferrooxidans* (Harrison, 1984; Tabita and Lundgren, 1971), it is probable that the association is mutualistic in nature. Members of the genus *Acidiphilium* are capable of iron reduction. This biological reduction of Fe^{3+} to Fe^{2+} helps to compensate ferric iron production and thus attenuates acid production in acid mine drainage (Pronk and Johnson, 1992). The aim of this work was to determine the role of the autotroph in such an extreme habitat which was of advantage for the survival of the heterotrophs. The chemolithoautotrophic strain, *A. ferrooxidans* DK6.1, used in this study was isolated and purified from the AMD sample of Garubathan, West Bengal, India. The acidophilic

heterotrophic strain used in this study was isolated from the AMD enrichment medium (typically used for isolation of *A. ferrooxidans* strains). It was characterized as *A. cryptum* DKAP1.1. We wanted to explore the degree of obligate oligotrophy in *A. cryptum* DKAP1.1 as we have observed that it failed to grow in nutrient rich medium (eutrophic medium). The elemental sulfur spent medium of three weeks old *A. ferrooxidans* DK6.1 (which basically contained the residual basal salts and leachates/ excreta of the pure culture of the autotroph as total dissolved organic carbon) culture could support the growth of *A. cryptum* DKAP1.1. (Fig. 3.5). This growth in elemental sulfur spent medium was found to be enhanced by a factor of more than 10 in presence of glucose and yeast extract.

It is well accepted that thiosulfate is central in much of the thinking concerning pyrite oxidation pathways (Druschel and Borda, 2006). Thiosulfate was found to be an important product during short term (< 24 h) experiment of pyrite oxidation at pH 5- 9.5 (Goldhaber and Rey, 1977; Goldhaber, 1983). The rate of tetrathionate formation in pyrite-surface-catalyzed oxidation of thiosulfate was found to be of first order with respect to the pyrite surface concentration and fractional order with respect to thiosulfate concentration (Yong and Schoonen, 1995). Tetrathionate accumulation to the extent of 1.3% (after 24 h of incubation) was observed in chemical pyrite oxidation with 10 mM $FeCl_3$ at pH 1.9 at 28°C (Schippers and Sand, 1999). Conversely, at neutral pH, the accumulation of thiosulfate (approximately 0.5 mM) was evident in pyrite oxidation (Schippers *et al.*, 1996). However, pyrite oxidation attending the formation of sedimentary roll-type uranium deposits does not occur in very low pH range (Goldhaber and Rey, 1977) and intermediate sulfur compounds like sulfite, thiosulfate, and tetrathionate offer suitable substrates for moderately acidophilic thiobacilli. The role of thiosulfate as an initial soluble intermediate in the pathway of pyrite oxidation has been postulated by different authors (Luther, 1987; Rimstidt and Vaughan, 2003). Earlier authors have suggested that pyrite oxidation generates thiosulfate, which reacts to tetrathionate in acidic solutions containing ferric iron. The acid-insoluble metal sulfides FeS_2, MoS_2, and WS_2 are chemically attacked by iron (III) hexahydrate ions, generating thiosulfate, which is oxidized via tetrathionate, disulfane-monosulfonic acid, and trithionate to sulfuric acid (Schippers and Sand, 1999). The predominant metal sulfide dissolving microorganisms are extremely acidophilic bacteria that are able to oxidize either inorganic sulfur compounds and/or Fe^{2+} ions. Although autotrophic Fe^{2+} oxidizing microorganisms are believed to play a more important role in acidic mineral rich environments, heterotrophic microbes have routinely been

cultured and detected in these extremely substrate limited environmental niches (Peccia *et al.*, 2000). *A. ferrooxidans* thrives on the oxidation of reduced sulfur compounds and in addition, is able to oxidize H_2, HCOOH, Fe^{2+} ions and other metal ions. *Leptospirillum ferrooxidans* and *L. ferriphilum* can grow only by aerobically oxidizing Fe^{2+} ions. Bacteria of the genus *Acidiphilum* display most diverse metabolic flexibility. Addition of thiosulfate profoundly affected the growth of cultures of acidophilic bacteria typical of acid mine drainage (AMD) sites: the iron and sulfur oxidizing autotrophic bacteria *A. ferrooxidans*, and a common heterotrophic strain *A. cryptum*. Growth of *A. cryptum* DKAP1.1 on DSMZ 269 medium was significantly inhibited in the presence of 1.0 mM thiosulfate in spite of favorable cultural conditions. The actual mechanism of this inhibition is not known. However, the lethal effect of the dual presence of tetrathionate and thiosulfate on *Escherichia coli* has been reported (Palumbo and Alford, 1970). The maximum lethal effect on *E. coli* occurred at a 3 to 1 molar ratio of thiosulfate-tetrathionate in growth medium. Tetrathionate is known to react with free sulfhydryl groups of enzyme and to cause their inactivation (Parker and Allison, 1969). Thiosulfate can also react with sulfhydryl groups (Postgate, 1963). It was therefore suggested that thiosulfate-tetrathionate mixture in growth medium of *E. coli* interfered with the synthesis, the activity, or both, of sulfur-containing enzymes or cell wall and membrane components (Palumbo and Alford, 1970). Thiosulfate is unstable in acidic solutions. Inhibition of growth of heterotrophic *A. cryptum* DKAP1.1 in presence of thiosulfate in acidic growth medium could be due to similar effect as was suggested for *E. coli*.

When *A. ferrooxidans* DK6.1 cells that were inoculated at the 1.0 g/l glucose condition in DSMZ 269 medium (with or without thiosulfate) were transferred to a glucose-free 9K ferrous medium after 24 h of incubation, no iron oxidation was observed (even after a month incubation) suggesting that incubation in presence of dissolved organic carbon including glucose had an acute, and perhaps irreversibly toxic, effect on the autotroph. Interestingly, in co-culture both the autotroph and the heterotroph survived in thiosulfate containing DSMZ 269 medium. In the mixed-culture flask that experienced viability of *A. cryptum* DKAP1.1, it is likely that consumption of reduced sulfur species by autotrophic bacteria relieved the inhibitory stress of thiosulfate or its oxidized products in acidic DSMZ 269 medium, thereby allowing *A. cryptum* DK6.1 to grow. On the other hand, following *A. cryptum* DKAP1.1 growth due to heterotrophic metabolism, it was likely that a significant fraction of the total organic carbon including glucose was not

present at inhibiting high concentration to affect the viability of *A. ferrooxidans* DK6.1. Inhibition of growth of *A. ferrooxidans* in the presence of 1.0 g/l glucose and alleviation of the glucose toxicity by an acidophilic heterotroph in co-culture was demonstrated by earlier authors (Marchand and Silverstein, 2003). In this chapter we have also demonstrated a positive growth effect for *A. ferrooxidans* DK6.1 caused by co-culturing in ferrous iron agar medium in the presence of *A. cryptum* DKAP1.1. It was shown by earlier authors that heterotrophic cells can enhance iron oxidation by *A. ferrooxidans* in aerobic condition, even if the heterotrophs are not actively metabolizing organic carbon (Marchand and Silverstein, 2003). The presence of heterotrophic *A. cryptum* DKAP1.1 culture in the agar plate was probably responsible for lowering the bio-available total organic carbon (which resulted from acid hydrolysis of agar) to a level that permitted enhancement of iron oxidation by *A. ferrooxidans* DK6.1. Acidophilic heterotrophic bacterium has been used by earlier authors to scavenge organic material from normal agar in order to facilitate colony formation of *A. ferrooxidans* (Butler and Kempton, 1987). This work has provided additional evidences in support of the synergism between obligately autotrophic *A. ferrooxidans* and obligately oligotrophic heterotroph *A. cryptum* perceived to occur in natural ecosystem.

4.5 CONCLUSION

1. Increase in the robustness of colony formation of *A. ferrooxidans* in ferrous iron agar plate in deliberate presence of *Acidiphilium cryptum* was observed. This suggested that the heterotroph plays an important role in the manifestation of colonies in ferrous iron agar plate.

2. The ability of *A. cryptum* to grow in elemental sulfur spent medium showed the oligotrophic nature of the organism and also provided the best possible explanation for the co-existence of *A. cryptum* with *A. ferrooxidans* culture.

3. Thiosulfate at the level of ≥ 0.7 mM was found to be toxic to the strains of *Acidiphilium cryptum*. However, this toxicity could be alleviated in presence of *A. ferrooxidans* which provides the additional support to the synergism that exists between *A. ferrooxidans* and *A. cryptum*.

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