CHAPTER 2

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

2.1 Introduction

2.1.1 Facultative sulfur lithoautotrophy

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

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

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

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

2.1.2 Thiosulfate oxidation

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

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

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

2.1.3 Sulfite oxidation

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

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

2.1.4 Organotrophy

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

2.2 Materials and methods

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

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

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

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

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

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

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

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

2.2.4 Consumption of thiosulfate by acid-tolerant chemolithotrophic isolates

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

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

2.2.5 Thiosulfate and sulfite dehydrogenase assay of acid-tolerant isolates

2.2.5.1 Preparation of cell-free extract

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

2.2.5.2 Thiosulfate dehydrogenase (EC 1.8.2.2) assay

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

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

2.2.5.3 Sulfite dehydrogenase (EC 1.8.2.1) assay

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

2.2.6 Demonstration of organotrophic growth by the acid-tolerant isolates

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

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

2.2.6.2 Growth of acid-tolerant isolates in R2A medium

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

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

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

2.2.7 Biochemical tests

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

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

The calculation of association coefficients for two organisms

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

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

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

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

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

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

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

2.2.7.1 Indole test

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

2.2.7.2 Methyl Red test

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

2.2.7.3 Voges-Proskauer test

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

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

2.2.7.4 Catalase test

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

2.2.7.5 Oxidase test

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

2.2.7.6 Citrate utilization test

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

2.2.7.7 Urease test

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

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

2.2.7.8 Nitrate reduction test

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

2.2.7.9 Gelatin hydrolysis

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

2.2.7.10 Starch hydrolysis

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

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

2.2.7.11 Casein hydrolysis

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

2.2.7.12 Lipase test

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

2.2.7.13 Hydrogen Sulfide production test

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

2.2.7.14 Fermentation of sugars

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

2.2.7.15 Utilization of organic acid salts

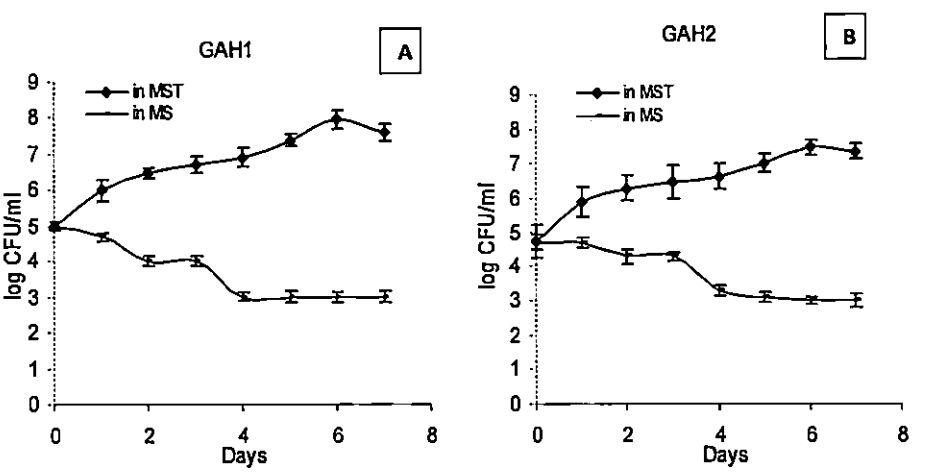
The test for the utilization of organic acid salts was carried out using HiBio-ID/HiCarbo system according to manufacturer's instruction (HiMedia, Mumbai, India). 50 µl of growing cultures were added to the media and growth was observed after 2-3days after incubation at 30 ^{0}C.

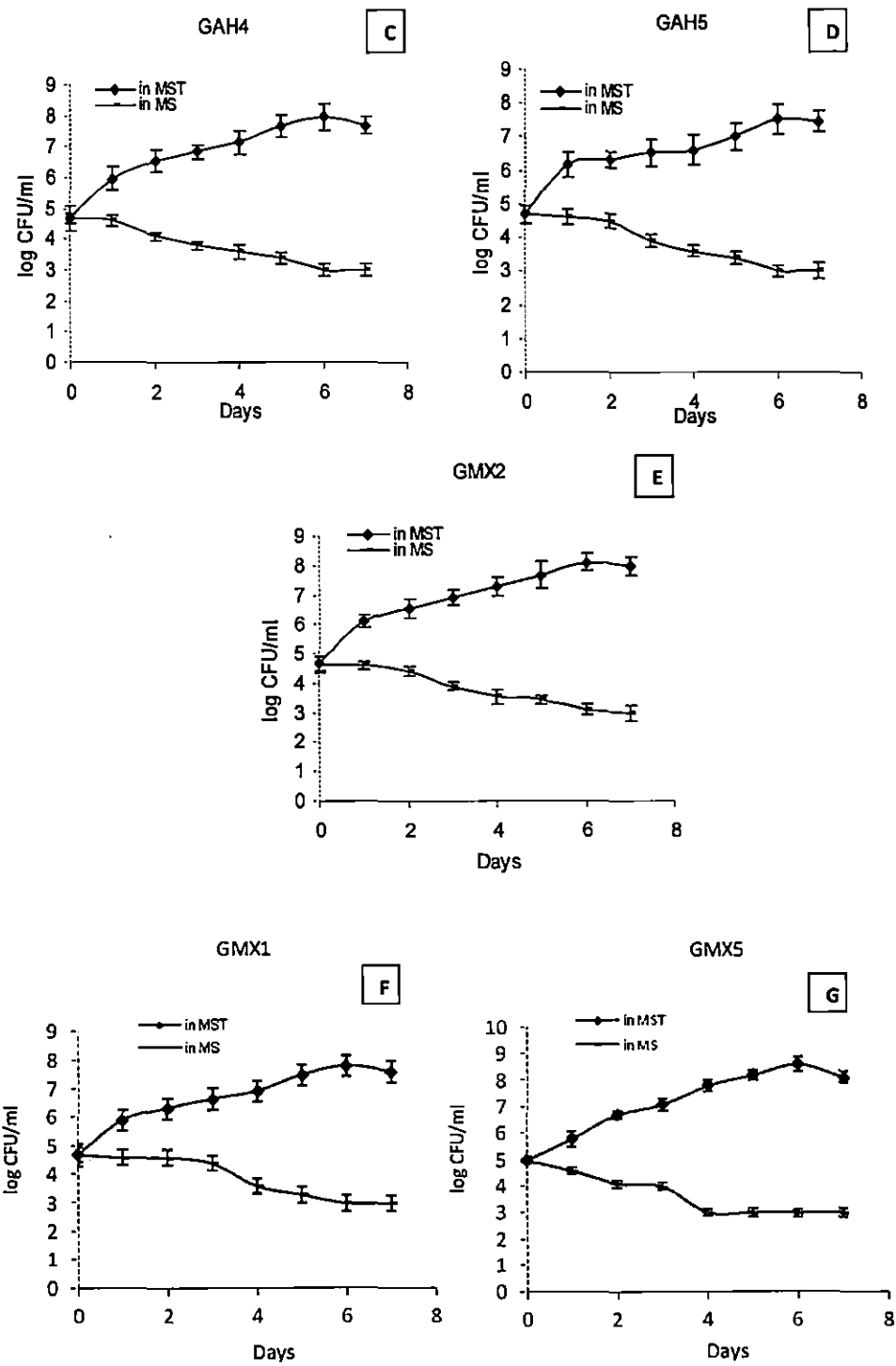
2.3 Results

2.3.1 Demonstration of chemolithotrophic growth by the acid-tolerant isolates

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

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

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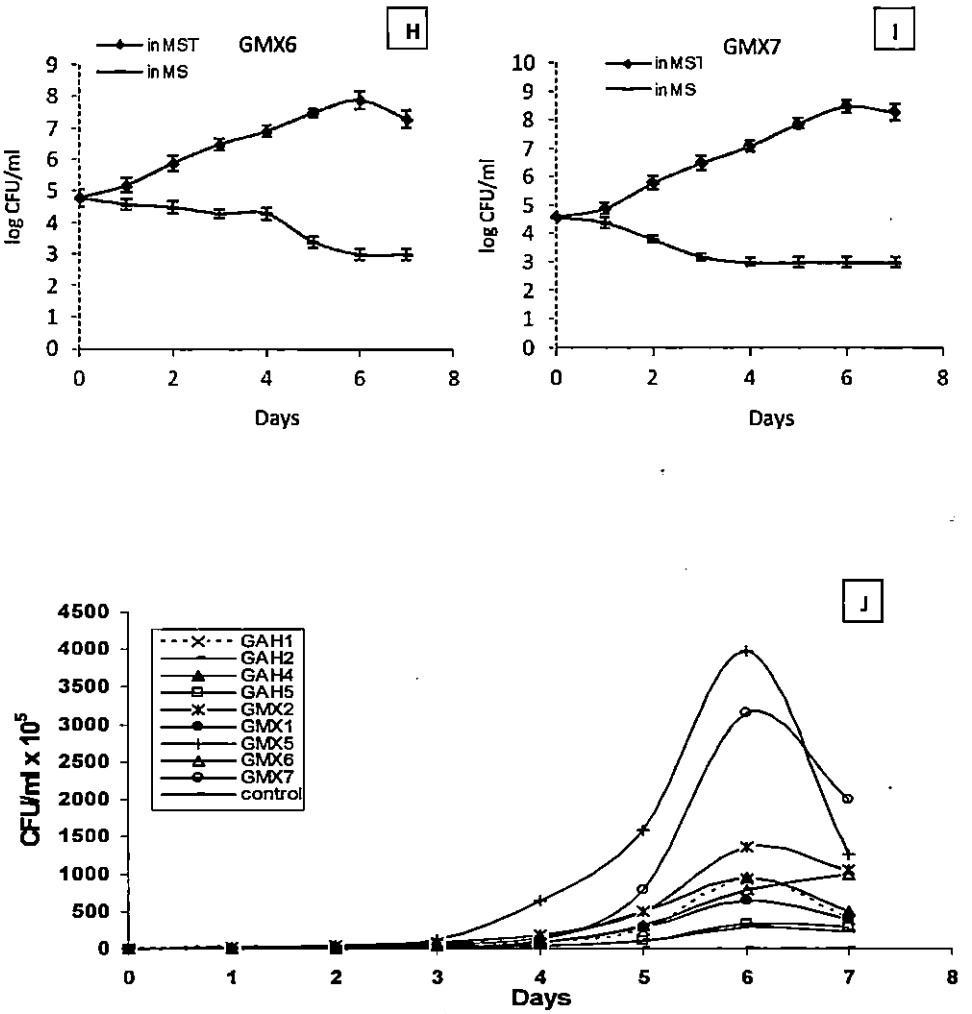

Fig. 2.1: Chemolithotrophic growth of acid-tolerant isolates on thiosulfate (10 gl^{-1}) in MST medium. (A) GAH1, (B) GAH2, (C) GAH4, (D) GAH5, (E) GMX2, (F) GMX1, (G) GMX5, (H) GMX6, (I) GMX7, and (J) results of all test isolates represented together. MS medium was used as control in all the experiments.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

2.3.2 Mixotrophic growth by the acid-tolerant isolates

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

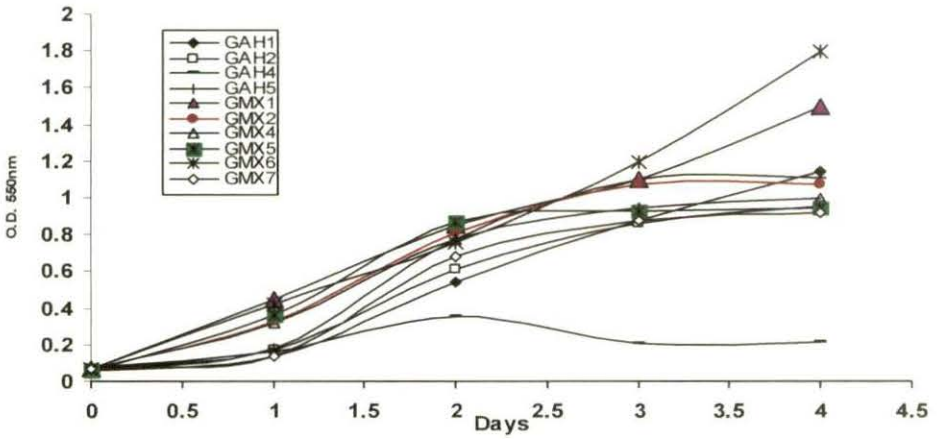

Fig. 2.2: Mixotrophic growth of acid-tolerant isolates on thiosulfate (10 gl^{-1}), and succinate (5 gl^{-1}) in MSTSY medium at $O.D._{550nm}$. Results of all test isolates have been represented together. MSTSY medium devoid of inoculum was used as negative control.

2.3.3 Consumption of thiosulfate by acid-tolerant mixotrophic isolates

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

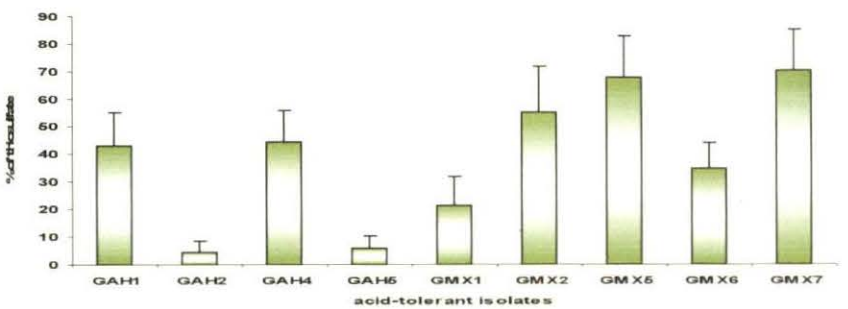

Fig 2.3: Thiosulfate consumption (percent consumption) by acid-tolerant heterotrophic isolates in 72 h. Error bar represent the standard error for 3 replicates.

2.3.4 Thiosulfate and sulfite dehydrogenase assay of acid-tolerant isolates

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

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

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

2.3.5 Organotrophic growth by the acid-tolerant isolates

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

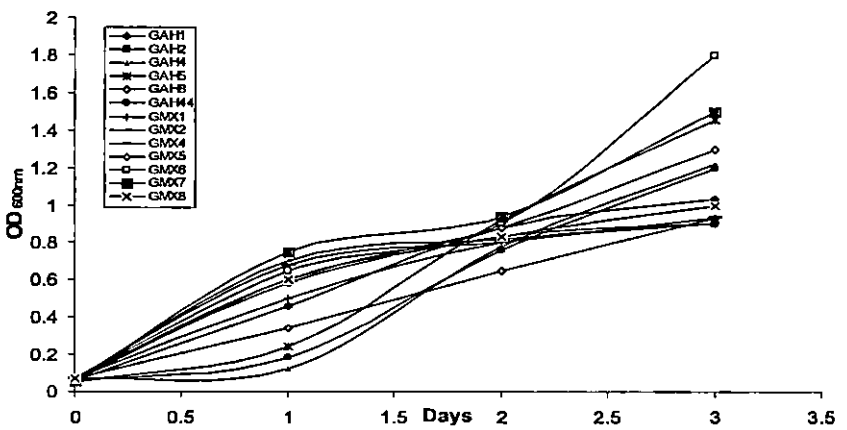

Fig. 2.4: Organotrophic growth of acid-tolerant isolates on succinate (5 gl^{-1}) in MSSY medium at $O.D._{600nm}$. Results of all test isolates represented together. MSSY medium devoid of inoculum was used as control.

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

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

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

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

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

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

+, positive; -, negative

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

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

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

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

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

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

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

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

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

2.4 Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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