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Biodiversity study of Ambamata multimetal mine, Gujarat: Isolation and characterization of Sulphur and Iron utilizers

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Abstract

After screening of many ores available at department of Microbiology, Gujarat University, for their metal content and gangue material, pollymetallic sulphidic ore from the Ambamata multimetal mine, Ambaji, Gujarat, India was selected for study. The ore contained zinc, copper and lead along with Sulphur and pyrite. Chemical analysis of the ore showed the presence of 7.5% Zn, 6.7% Fe, 3.0% Pb, 0.545 % Cu, 0.064% Cd, and 0.002% Ag. Because of the lower concentration of the minerals, this ore could not be utilized for metal recovery by conventional metal extraction methods and hence heap bioleaching was tested for its feasibility. Three Iron oxidizers DA, DB and DC were isolated and tested for iron oxidation kinetics. Isolate DC was the fastest growing among them, so was used for further study after adaptation. Sulphur oxidizing isolate DS1 decreased the medium pH from 3.5 to 0.4, indicating it as potent acid generator at the site. Thiosulphate oxidizing isolate TH1 showing 78% thiosulphate oxidation within 11 days and decreased the medium pH from 4.5 to less than 3.0, proved to be the other potential acid generator at the site. Eleven strains of heterotrophic bacteria were isolated from various water samples collected from Ambamata multi metal mine site on the basis of their potential to utilize thiosulphate as an energy source under the aerobic conditions. All the isolates were analyzed for their growth pattern, sulphate production, medium pH change and thiosulphate utilization. Isolated organisms showed great diversity in colony morphology. Sequence analysis the 16S rRNA genes of the selected isolates showed their affiliation to genomovars of Pseudomonas stutzeri. The data suggested that thiosulphate utilizing bacteria, in particular those belonging to *Pseudomonas stutzeri*, can play a significant role in bioleaching. The isolates showed different substrate utilization profile when tested on Biolog® plates. A mixed consortium, predominantly *Acidithiobacillus ferrooxidans* isolated from Ambamata multi metal mine water sample was used as inoculum for the experiment. Repeated sub culturing was performed to ensure activation and the adaptation of the strain and reduction in lag phase. Subsequently, the bacterial lag phase was reduced and the log phase initiated immediately after inoculation of the strains in fresh media. After three sets of sub culturing, a steady state of the iron oxidation rate was achieved. This bacterial culture was then inoculated in 9k medium containing 2% pyrite and aerated continuously. Repeated transfer of the consortium in pyrite containing media reduced the time from 104 hours to 48 hours required for >98% pyrite oxidation. The cells were finally transferred to 9k medium having 2% metal sulphide.

Keywords: Iron oxidizers, *Acidithiobacillus ferrooxidans*, *Pseudomonas stutzeri*, metal sulphide, consortium, 16s rRNA, Heterotrophic thiosulphate- oxidizing bacteria, chemolithotrophs, chemolithoheterotrophs.

1. Introduction

Most of the research on the microbiology of acidic mine waters has focused on chemolithotrophic action of the iron- and sulphur-oxidizing thiobacilli. However, it has long been recognized that a variety of heterotrophic bacteria also oxidize reduced, inorganic Sulphur compounds, but thiosulphate is the usual substrate for an incomplete oxidation of inorganic Sulphur (Bernd 2007, Colmer 1947, Jhonson 2008).

The heterotrophic thiosulphate- oxidizing bacteria appear to be a diverse taxonomic group and have been recovered from a wide variety of habitats, including soils, freshwater as well as both inshore and offshore marine environments.

Interest in the microbiology of low pH regions has extended considerably. The present study entailed a search for heterotrophic bacteria with potential for survival and reproduction at the depressed pH values typically encountered in acidic mine sites (Almeida 2009, Colmer 1950, Natrajan 2008, Nooris 2007). Some insight is provided on the species diversity, phylogenetic affiliation, culture characteristics as well as thiosulphate oxidation and pH shifting patterns of mine bacteria were investigated in order to identify possible habitat related common thiosulphate utilizing heterotrophic denominators and recurring differences in physiology and phylogenetic identity of these bacteria.

Our knowledge of Microbial ecology of acidic environments has changed significantly over the past few years (Simons at al 2002, Singer et al 1970). Initially it was thought that such ecosystems were relatively simple biological systems containing mainly of obligate chemolithotrophic bacteria such as *Acidithiobacillus ferroxidans* and *Acidithiobacillus thiooxidans* (Bernd 2007, Gadd 2010, Johnson 2011, Johnson et al 2001, and Nooris 2000).

Today however such environments are known to support a high diversity of acidophilic microorganism including iron oxidizers, Sulphur oxidizers, facultative Sulphur oxidizing heterotrophs and obligate heterotrophs. The acidophilic bacteria in bioleaching environment metabolize organic substrates to support growth in pH range 1.5-6.0. The removal of organic substances that are potentially inhibitory to obligate chemolithotrophs such as *A. ferroxidans* is thought to enhance bioleaching. Indeed, mix Cultures of Autotrophic and heterotrophic acidophiles are more efficient in leaching metals from ore.

Mine waters are generally thought to be biologically sterile; however, they are hardly lifeless. Microorganisms such as bacteria, fungi, yeasts, algae, archaea, and protozoa are common and abundant in mining waters. For example, there are over 1300 different forms of microorganisms identified in the infamous acid waters of the Rio Tinto, Spain (Bernd 2007,).

It is becoming clear that bioleaching of metal sulfide ores involves a much more complex community of microorganisms than initial findings suggested, and that under different environmental conditions; different organisms may dominate the process (Bernd 2007).

Bacteria isolated from mining environments are numerous and include *Acidithiobacillus thiooxidans*, *Acidithiobacillus ferrooxidans*, *Leptospirillum ferrooxidans*, and *Thiobacillus thioparus.* These bacteria function best in an acid, aerobic environment ($pH<4$). The bacteria need minor nitrogen and phosphorous for their metabolism, and they depend on the oxidation of ferrous iron, hydrogen sulfide, thiosulphate, Sulphur, and metal sulfides for energy (Nooris 2011, Rowe et al 2007). They also transform inorganic carbon into cell building material. The inorganic carbon may originate from the atmosphere or from the dissolution of carbonates. The bacterial activity produces metabolic waste (i.e. Sulphuric acid, Fe^{3+}) that accelerates the oxidation of sulfides.

Certain microorganisms survive or even thrive in mining environments because: (a) they tolerate elevated concentrations of dissolved metals and metalloids; and (b) they use the energy from the chemical oxidation reactions for their own growth (Bernd 2007).

Resistance of bacteria to metal cations and oxyanions is plasmid-mediated in many instances. Plasmid-mediated resistance to mercury, tellurium, arsenic, silver, nickel, cobalt, zinc, cadmium, copper, and lead has been reported for heterotrophic bacteria whose pH optima are near neutrality.

It has been reported by Hutchins et al. in 1986 that oxidation of sulfide minerals by chemolithotrophs (e.g. *A. ferrooxidans*) results in some gold solubilization but selected heterotrophs have more effect. However, it is yet to be determined if microbes other than *A. ferrooxidans* and *A. thiooxidans* contribute promisingly to industrial-scale metal leaching (Ehrlich 2009b).

Also recently Kondrat and collogues reported numerous presence of heterotrophic organisms in different kind of mining samples especially of group γ_Proteobacteria (Kondrat'eva et al. 2012). A number of fungus as well as the occurrence of chemolithotrophic bacteria Pseudomonas sp. and the sulfide oxidizing aerobic bacteria *Beggiatoa alba* and *Thiothrix sp.* were reported in ancient gold mine. Natarajan et al. (1997) inferred from the presence of the members of the bacterial genera *Thiobacillus*, *Bacillus*, and *Pseudomonas* in the

Jamnagar bauxite mines in Gujarat, India, that these microorganisms are involved in bauxite formation (Ehrlich 2009a).

Acidophiles are distinguished on the basis of their nutritional characteristics (energy and carbon sources). Four groups may be recognized:

(i) Chemolithotrophic acidophiles–autotrophic prokaryotes that use an inorganic energy source;
(ii) phototrophic acidophiles (micro-algae such as Euglena mutabilis) that use solar energy;
(iii) heterotrophic acidophiles that use organic carbon as both carbon and energy source;
(iv) mixotrophic acidophiles that use inorganic electron donors and assimilate organic carbon (Johnson 2009, Temple et al 1951).

'Heterotrophically inclined' microbes are believed to assist the growth of *A. ferrooxidans*. This is thought to be due to their ability to provide essential nutrients or to remove toxic organic compounds or other inhibitory substances. How much this ability contributes to the overall mineral biooxidation efficiency of a microbial consortium in practice is still unclear (Willscher et al 2003).

Heterotrophic bacteria do not have as high a demand for NAD(P)H as their carbon source is more reduced than CO_2 and hydrogen atoms removed from their source of nutrition may be used to satisfy their lower NAD(P)H requirement. Chemolithotrophic autotrophs require a large transmembrane proton gradient to generate the required proton motive force to energize the synthesis of NAD(P)H. This process is known as reverse electron transport or the 'uphill' electron transfer pathway (Rawlings 2005).

Table 1 List of some aerobic sulphur-oxidizing bacteria (Ehrlich 2009b)

Autotrophic	Mixotrophic	Heterotrophic
Acidithiobacillus albertensis[c]	*Pseudomonas* spp.	*Beggiatoa* spp.
Acidithiobacillus caldus[c]	*Thiobacillus intermedius*	*Thiobacillus perometabolis*
Acidithiobacillus ferrooxidans[c]	*Thiobacillus organoparus*	
Acidithiobacillus thiooxidans[c]	*Thiobacillus versutus*[d]	
Acidianus brierleyi[e]		
Alicyclobacillus disulfidooxidans[f,g]		
Alicyclobacillus tolerans[f,g]		
Beggiatoa alba MS-81-6		
Sulfolobus acidocaldarius[e]		
Thermothrix thiopara[f]		
Thiobacillus denitrificans[h]		
Thiobacillus neapolitanus		
Thiobacillus novellus[g]		
Thiobacillus tepidarius		
Thiobacillus thioparus		

[a] A more complete survey of aerobic sulfur-oxidizing bacteria can be found in Balows et al. (1992) and Dworkin (2001).

[b] All members of the domain Bacteria in this table are gram-negative except for *Alicyclobacillus disulfidooxidans* and *A. tolerans*.

[c] Formerly assigned to the genus *Thiobacillus* (see Kelly and Wood, 2000).

[d] Can also grow autotrophically and heterotrophically.

[e] Archeon.

[f] *Alicyclobacillus disulfidooxidans* formerly known as *Sulfobacillus disulfidooxidans* and *Alicyclobacillus tolerans* formerly known as *Sulfobacillus thermosulfidooxidans* subsp. *thermotolerans* (see Karavaiko et al., 2005).

[g] Facultative autotroph.

[h] Facultative anaerobe.

Oxidation of Sulphur compounds is not restricted to the true Sulphur bacteria; this process also occurs in heterotrophic bacteria. Heterotrophic bacteria able to oxidize thiosulphate to tetrathionate are widely distributed in different environmental samples. Such bacteria dominate among Sulphur-oxidizing communities in stratified bodies of water where there are low sulfide concentrations in the interface layer, such as the Black Sea. Large numbers of tetrathionate-forming bacteria have been found in freshwater lakes and specialized environments such as soda lakes and sulfide-oxidizing bioreactors (Dimitry et al. 1999).

Most of the heterotrophic bacteria demonstrating Sulphur oxidation activities belong to the genera Pseudomonas, Mycobacterium, *Arthrobacter, Flavobacterium*, and *Xanthobacter* strains. However, few of these heterotrophs have the ability to generate biologically useful energy from the oxidation of reduced Sulphur compounds. An exception is *Paracoccus denitrificans*, which also grows autotrophically in the presence of thiosulphate. Only a few heterotrophic bacteria, such as *Thiobacillus sp.* strain Q and *Catenococcus thiocyclus,* have been shown to obtain energy from the oxidation of Sulphur compounds. This type of nutrition has been designated chemolithoheterotrophy. (Das et al. 1996)

To study the potential importance of chemolithoheterotrophs in leaching, attempts were made to isolate chemolithoheterotrophic bacteria from mine waters by enrichment and isolation culture techniques. The aim was to identify possible habitat related thiosulphate utilizing heterotrophic dominant bacteria and determine differences in physiology and phylogenetic relationship of these bacteria.

Heterotrophic bacteria were isolated from various water samples collected from Ambamata multi metal mine site on the basis of their potential to utilize thiosulphate under aerobic conditions. Eleven strains were isolated from Ambamata mine water samples, and this strains were designated TH1 to TH11. Strains oxidizes reduced inorganic Sulphur compound (thiosulphate) both in the presence and absence of organic substrate. All the isolates were analyzed for their colony morphology diversity, biochemical characteristics, phylogenetic affiliation based on the results of a 16s ribosomal DNA sequence analysis, thiosulphate oxidation and pH shifting patterns.

2. Materials and Methods

2.1Sampling and sample characterization

Five water samples were collected from Ambamata multimetal mine, Ambaji, Gujarat F. Physico-chemical characteristics of collected samples were evaluated in terms of pH, redox potential (RP), total dissolved solids (TDS) and conductivity. The pH and mV were measured using μ-pH meter (Systronics, India; model 361). Total dissolved solids (TDS) were measured by portable TDS meter (Eutech, Singapore, model TDSTestr 11^+). Also, samples were analyzed for presence of soluble metals like Cu, Zn and total iron using atomic absorption spectrophotometer (Elico Ltd, model BL-194, India).

2.2 Isolation and characterization of heterotrophic Sulphur utilizers

2.2.1 Microbiological media for heterotrophic Sulphur utilizers

Thiosulphate medium for Sulphur-utilizing bacteria contained (per liter): 3 g $(NH_4)_2SO_4$, 0.1g KCl, 0.5g K_2HPO_4, 0.5g $MgSO_4 \times 7H_2O$, 0.15g tryptone soya broth (TSB). Two milliliters of 0.5% phenol red solution was added per liter of medium as pH indicator. The pH was adjusted to 7.0 and medium was sterilized at 121^0C at 15 lbs pressure for 20 minutes. Sodium thiosulphate solution was filter sterilized with 0.22 µm nylon membrane filter (Pall Life Sciences, India) and added to mineral medium to get final concentration of 10 mM thiosulphate. The broth medium was used for quantification of sulphate produced by oxidation of thiosulphate. Negative control did not contain thiosulphate, but otherwise contained same salts, TSB and pH indicator to check any pH shift without utilization of thiosulphate. All the reagents and chemicals used in the study were of analytical grade.

2.2.2 Isolation of heterotrophic Sulphur utilizers

Heterotrophic thiosulphate utilizers were first enriched in liquid 9K medium with 1% sodium thiosulphate and 0.15 g/L TSB. For the enrichment of thiosulphate oxidizing organisms 10 mL sample was inoculated in 250 mL Erlenmeyer flask containing 90 mL 9K medium as mentioned above. All the flasks were incubated for 30 d on environmental orbital shaker (Newtronics, India) rotating at 150 rpm at 32 ± 2 °C. The samples were withdrawn at regular intervals of time for the analysis of remaining thiosulphate. Further they were isolated and purified on agar medium of the same composition (thiosulphate agar plates) and identified by 16S rRNA gene sequence analysis.

2.2.3 Thiosulphate utilization study of heterotrophic Sulphur utilizers

Unless otherwise mentioned, for thiosulphate biooxidation studies, all experiments were carried out in triplicate in 250 mL Erlenmeyer flasks with 90 mL working volume of basal 9K medium. The flasks were supplemented with 1%(w/v) filter sterilised thiosulphate salt and inoculated with each actively growing isolate (TH1 to TH11) to have a final cell density of 1.8×10^5 cells/mL. All the systems were incubated on an environmental orbital shaker at 32 ± 2°C with agitation at 150 rpm. At regular time interval, samples were withdrawn and analyzed for the remaining thiosulphate. The remaining thiosulphate was estimated by standard iodimetric method using starch as indicator. (Thiosulphate estimation (Vogel, 1962).

2.2.4 Sulphate estimation, pH measurement and cell count

Sulphate concentration in the medium was estimated with standard spectrophotometric analysis. pH of the medium was analyzed using Systronic 361 µ pH meter. Cell count was done directly using Nuber's chamber.

2.2.5 Genetic relationship of strains

Representative strains were identified by 16S rRNA sequencing. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary analyses were conducted in MEGA5.

2.2.6 Physiological characteristics of the isolates

Isolated organisms were analyzed for diversity in colony morphology under 40X microscope magnification. Also the isolates were analyzed for their "C" substrate pattern using BioLOG analysis systems. Heterotrophic growth on different carbon sources was studied on BioLOG GN2 and GP2 plates (BioLOG Hayward, CA, USA) as per described standard procedures for gram negative and gram positive strains respectively. Plates were incubated and monitored for 5 days at 32 ^{0}C.

2.2.7 Phylogenetic analysis

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length 0.44 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. The analysis involved 6 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 396 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Felsentein 1985, Kimura 1980, Saitou et al 1987, Stephenson 2003, Zeng et al 2008).

2.3 Isolation and characterization of autotrophic iron and Sulphur oxidizers

2.3.1 Microbiological media for isolation of autotrophic Sulphur and Iron Oxidizers

Growth medium for isolation of bacteria contained 9K medium (($(NH_4)_2SO_4$-3g/L, K_2HPO_4-0.5g/L, $MgSO_4.7H_2O$-0.5g/L, pH-1.9±0.2). The medium was sterilized for 20 minutes at 121 ^{0}C at 15 lbs pressure. Ferrous sulphate and sodium thiosulphate solutions were filter sterilized with 0.22 µm nylon membrane filter (Pall Life Sciences, India) and added to mineral medium to get final concentration of 10.0 g/L as energy source. Sulphur powder was U.V. sterilized and used in isolation. All the reagents and chemicals used in the study were of analytical grade. The medium pH was kept 4.5, 3.5, and 1.9 in case of thiosulphate, Sulphur and iron oxidizers respectively.

2.3.2 Isolation of autotrophic Iron and Sulphur oxidizers

Iron and Sulphur oxidizers were isolated from water samples by serial dilution tube technique and selective solid medium method. In serial dilution tube technique, tube inoculated with the highest dilution and showing positive growth on 14th d of incubation at 32 ± 2 °C was plated on selective solid medium to get well isolated single colony (Dave, 1980).

2.3.3 Iron and Sulphur biooxidation study by isolated autotrophs

Unless otherwise mentioned, for biooxidation studies, all experiments were carried out in triplicate in 250 mL Erlenmeyer flasks with 90 mL working volume of basal m9k medium. The flasks were supplemented with filter sterilised ferrous sulphate to get different required concentrations ranging from 50-600mM and inoculated with each actively growing isolate (DA, DB, DC) to have a final cell density of 1.2×108 cells/mL. Thisulphate oxidation study was carried with final 1%(w/v) sodium thiosulphate concentration. Sulphur oxidation study was carried out using 1% (w/v) Sulphur powder. All the systems were incubated on an environmental orbital shaker at 32 ± 2 °C with agitation at 150 rpm. At regular time interval, samples were withdrawn for the analysis of remaining ferrous iron or thiosulphate or pH measurements respectively.

2.3.4 Analysis

At regular interval of time, samples were withdrawn for remaining ferrous (to determine the iron oxidation rate (IOR)) or thiosulphate estimation or pH measurement as the case may be. Ferrous biooxidation was determined by measuring unoxidized ferrous using standard diphenylamine titrimetric method. (Ferrous estimation (Vogel, 1962)) Thiosulphate oxidation was determined by estimating the left out thiosulphate using standard iodimetric method. (Thiosulphate estimation (Vogel, 1962)) Sulphur oxidation was measured in terms of change in pH using Systronic 361 µ pH meter.

3. Results and discussion

3.1 Physico-chemical Characterization of mine water samples

All the water samples collected from the mine site showed pH values in range of 3.53 to 4.10, indicating presence of Sulphur and iron oxidizers, whereas presence of solubilized metals indicated activity of leaching microbes (Table 2).

Table 2 Physico-chemical Characterization of mine water samples

Sample No.	pH	Conductivity (mS)	TDS (ppt)	Redox potential (mV)	Cu (ppm)	Zn (ppm)	Total Fe (ppm)
1	3.77	2.1	1.59	360	29.4	35	0.25
2	3.78	0.37	0.26	307	26.3	19	0.00
3	3.53	2.38	1.69	341	61.0	57	3.13
4	4.1	2.14	1.52	332	50.2	56	0.00
5	3.8	2.4	1.70	352	19.8	45	0.00

3.2 Isolation and characterization of heterotrophic Sulphur utilizers

3.2.1 Isolation of heterotrophic Sulphur utilizers

Changes of pH (colour changes of phenol red indicator) were used as a preliminary indicator of thiosulphate-oxidizing activity. Bacterial growth never reached the level of visible turbidity; the indicators of microbial activity were pH changes so, microscopically visible cells were obtained by centrifuging 100 to 500-ml portions of the medium whenever needed. Over the course of 2 months, different bacterial populations developed in the liquid samples that increased or lowered the pH. An interpretation of all pH changes as positive would have been simplistic and of no value to account for the obviously diverse bacterial populations in these samples. Therefore, pure cultures were isolated individually which showed distinct pH changes. This approach allowed the identification and cultivation of numerically dominant, but slow-growing or fastidious, bacteria. Strains obtained in this way were marked as TH1 to TH11. Individual colonies of all strains were obtained by restreaking on agar plates and continued incubation. The plates were checked for pH changes using phenol red as indicators. Among eleven strains (TH1 to TH11) of heterotrophic thiosulphate utilizers, five were identified as *Pseudomonas stutzeri* and one as *Bacillus boroniphilus*.

A: Acid production B: Base production C: Control

Fig 1 Acid production by thiosulphate utilizers in presence of phenol red as indicator during enrichment

3.2.2 Characterization of heterotrophic Sulphur utilizers

A) Acid producing thiosulphate oxidizing bacteria

As previously described, the cultivation survey for heterotrophic thiosulphate utilizers yielded, in general, pH increasing and pH decreasing thiosulphate utilizing bacterial isolates (Teske et al, 2000). They have been most consistently found in 10^3 and 10^4 dilutions of collected samples Isolates increased the medium moderately by 0.082 to 1.2 pH units (Table 3). The isolate TH5 constitutes an exception. It was obtained from 103 dilution of the sample and was the only isolated gram positive bacteria in the study. This acid producing thiosulphate utilizer showed phylogenetic and physiological similarities to *Bacillus boroniphilus* Over 30 days of aerobic incubation at 32^0C isolates produced 0.09 to 2.0mM (Fig 2 to Fig 5) sulphate corresponding to oxidation of approximately 32.2 % to 68.8 % of the original 10mM thiosulphate in the medium to sulphate (Table 3).

Parallel incubation in unsupplemented and 0.15 g/L TSB supplemented medium show different thiosulphate oxidation patterns for the isolated bacteria. In most cases, TSB inhibits thiosulphate oxidation (Table 3) and sulphate production (Fig 2 to 5), indicating that these strains prefer organic substrates over inorganic electron donors such as thiosulphate.

Fig 2 Sulphate production in medium with TSB by isolatesTH1, TH2, TH3, TH4, TH5 and TH6

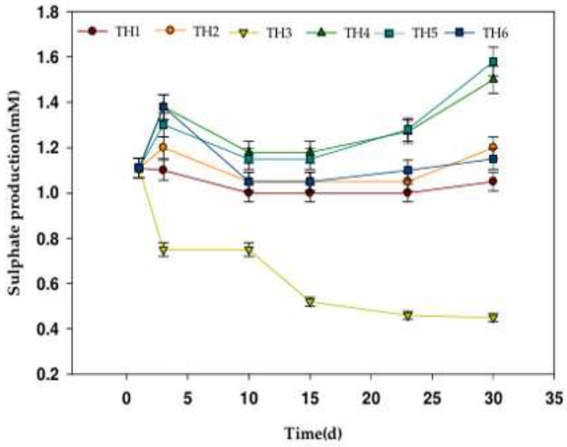


Fig 3 Sulphate production in medium with TSB by isolatesTH7, TH8, TH9, TH10, and TH11

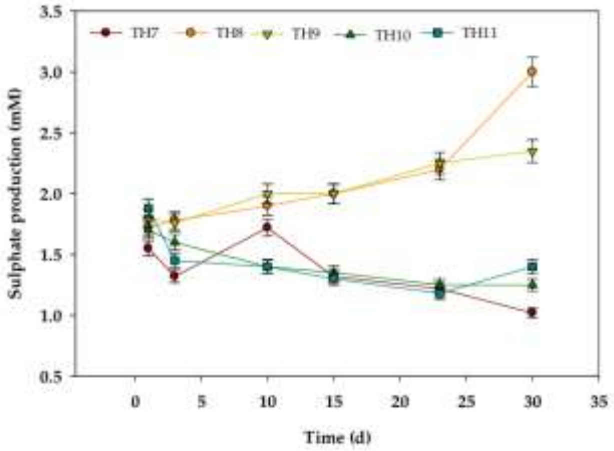


Table 3 Growth, pH shifting and thiosulphate utilization by heterotrophic thiosulphate utilizers

Isolate	With TSB					Without TSB				
	Cell count		pH		Thiosufate oxidation (%)	Cell count		pH		Thiosufate oxidation (%)
	Initial	Final	Initial	Final		Initial	Final	Initial	Final	
TH1	1.8×10^5	12×10^7	7	7.12	23.30	1.8×10^5	2.0×10^7	7	6.52	36.6
TH2	1.4×10^5	8×10^7	7	6.98	26.60	1.4×10^5	1.2×10^7	7	6.92	32.2
TH3	1.8×10^5	8×10^7	7	8.10	10.00	1.8×10^5	0.8×10^7	7	7.83	13.3
TH4	1.8×10^5	6×10^7	7	6.61	33.30	1.8×10^5	0.8×10^7	7	6.50	37.7
TH5	1.8×10^5	4×10^7	7	6.43	35.10	1.8×10^5	0.4×10^7	7	6.81	31.1
TH6	1.8×10^5	2×10^7	7	6.98	25.50	1.8×10^5	0.4×10^7	7	6.79	31.1
TH7	1.8×10^5	5.2×10^7	7	7.30	23.30	1.8×10^5	4.2×10^7	7	5.84	66.6
TH8	1.8×10^5	8×10^7	7	5.82	66.60	1.8×10^5	4.4×10^7	7	5.80	68.8
TH9	1.8×10^5	4×10^7	7	6.20	52.20	1.8×10^5	4.0×10^7	7	5.80	68.8
TH10	1.8×10^5	3×10^7	7	8.08	27.70	1.8×10^5	1.6×10^7	7	6.33	44.4
TH11	1.8×10^5	4.8×10^7	7	8.05	31.10	1.8×10^5	2.0×10^7	7	6.25	50.0

Fig 4 Sulphate production in medium without TSB by isolates TH1, TH2, TH3, TH4, TH5 and TH6

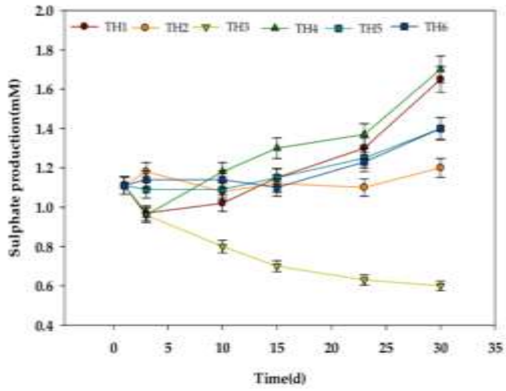


Fig.5 Sulphate production in medium without TSB by isolates TH7 to TH11

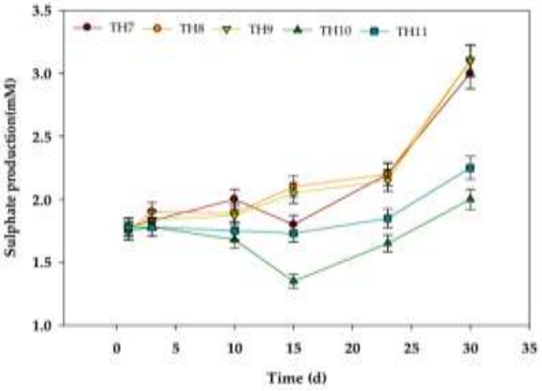
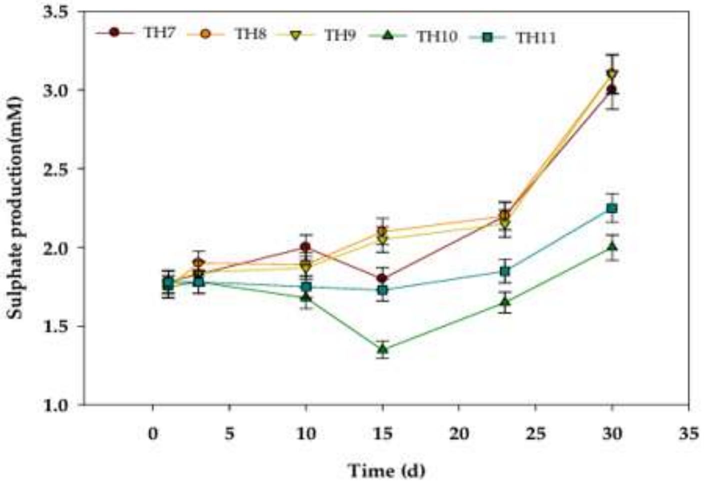


Table 4 Substrates utilized as carbon sources by the thiosulphate utilizing isolates as determined by Biolog® microplate assays

Substrate	Isolate TH										
	1	2	3	4	5	6	7	8	9	10	11
2,3-Butanediol	H	-	-	-	-	-	H	H	H	-	-
2-Aminoethanol	H	-	-	-	-	-	-	H	H	-	-
α cyclodextrin	-	-	-	-	-	-	-	-	-	-	-
α -D-Glucose	H	-	-	-	+	-	H	H	H	-	-
α -D-Lactose	H	-	-	-	-	-	H	-	-	-	-
α Hydroxybutyric acid	H	-	H	-	-	-	H	H	H	-	-
α Ketobutyric acid	H	-	-	-	-	-	H	H	H	-	-
α Ketoglutaric acid	H	-	H	-	+	-	H	H	H	-	-
α Ketovaleric acid	H	-	H	-	-	-	H	H	H	-	-
Acetic acid	H	-	-	-	+	-	-	H	H	-	-
α-D-Glucose-1-Phosphate	H	-	-	-	-	-	-	H		-	-
Adonitol	H	-	H	-	-	-	H	H	H	-	-
β Hydroxybutyric acid	H	-	-	-	-	-	H	H	H	-	-
β Methyl D Glucoside	H	-	-	-	-	-	-	-	-	-	-
Bromosuccinic acid	H	-	-	-	-	-	-	H	H	-	-
Cis- Aconitic acid	H	-	-	-	-	-	H	H	H	-	-
Citric acid	H	-	H	-	+	-	H	H	H	-	-
D,L,a-Glycerol Phosphate	H	-	-	-	-	-	H	H	H	-	-
D,L-carnitine	H	-	H	-	-	-	-	H	H	-	-
D,L-Lactic acid	H	-	H	-	-	-	H	H	H	-	-
D-Alanine	H	-	H	-	-	-	H	H	H	-	-
D-Arabitol	H	-	-	-	-	-	-	H	H	-	-
D-Cellobiose	-	-	-	-	-	-	-	H	-	-	-
Dextrin	H	H	H	H	+	H	-	H	H	H	H
D-Fructose	H	-	-	-	+	-	H	H	H	-	-
D-Galactonic acid lactone	H	-	-	-	+	-	H	H	H	-	-
D-Galactose	H	-	-	-	+	-	H	H	H	-	-
D-Galacturonic acid	H	-	-	-	+	-	-	H	H	-	-
D-Gluconic acid	H	-	H	-	+	-	H	H	H	-	-
D-Glucosaminic acid	H	-	H	-	-	-	H	H	H	-	-
D-Glucose-6-Phosphate	H	-	-	-	-	-	H	H	H	-	-
D-Glucuronic acid	H	-	H	-	-	-	H	H	H	-	-
D-Mannitol	H	-	H	-	+	-	H	H	H	-	-
D-Mannose	H	-	-	-	-	-	H	H	-	-	-
D-Melibiose	H	-	-	-	+	-	-	-	-	-	-
D-Psicose	H	-	-	-	-	-	H	H	H	-	-
D-Raffinose	H	-	-	-	+	-	H	-	-	-	-
D-Saccharic acid	-	-	H	-	-	-	-	H	H	-	-
D-Serine	-	-	-	-	+	-	-	H	H	-	-
D-Sorbitol	H	-	-	-	-	-	H	H	-	-	-
D-Trehalose	H	-	-	-	+	-	H	H	H	-	-
Formic Acid	H	-	H	-	-	-	H	H	H	-	-
g Hydroxybutyric Acid	H	-	-	-	-	-	H	H	H	-	-
g-Aminobutyric Acid	H	-	H	-	-	-	-	H	H	-	-
Gentiobiose	H	-	-	-	+	-	H	-	-	-	-
Glucuronamide	H	-	H	-	-	-	H	H	H	-	-
Glycerol	H	-	H	-	-	-	H	H	H	-	-
Glycogen	H	H	H	H	+	H	H	H	H	H	H
Glycyl-L-Aspartic acid	H	-	-	-	-	-	H	H	H	-	-
Glycyl-L-Glutamic acid	-	-	H	-	-	-	-	H	H	-	-
Hydroxy- L-Proline	H	-	-	-	-	-	-	H	H	-	-
I-Erythritol	H	-	H	-	-	-	H	-	-	-	-
Inosine	H	-	-	-	-	-	H	-	-	-	-
Itaconic acid	H	H	H	H	-	H	H	H	H	H	H
L- Alanine	-	-	H	-	-	-	H	H	H	-	-
Lactulose	H	-	-	-	-	-	H	H	-	-	-
L-Alaninamide	-	-	-	-	-	-	H	H	H	-	-
L-Alanyl-Glycine	H	-	H	-	-	-	H	H	H	-	-
L-Arabinose	H	-	-	-	+	-	-	H	-	-	-
L-Asparagine	H	-	H	-	+	-	-	H	H	-	-
L-Aspartic acid	H	-	H	-	-	-	H	H	H	-	-
L-Fucose	H	-	-	-	-	-	H	H	H	-	-
L-Glutamic acid	H	-	-	-	-	-	H	H	H	-	-
L-Histidine	-	-	-	-	-	-	-	H	H	-	-
L-Leucine	H	H	H	H	-	H	H	H	H	H	H
L-Ornithine	H	-	-	-	-	-	-	H	H	-	-
L-Phenylalanine	H	-	-	-	-	-	-	H	H	-	-
L-Proline	H	-	H	-	-	-	-	H	H	-	-
L-Pyroglutamic acid	H	-	-	-	-	-	-	H	H	-	-
L-Rhamnose	H	-	-	-	-	-	H	-	-	-	-
L-Serine	H	-	-	-	-	-	-	H	H	-	-
L-Threonine	-	-	-	-	-	-	-	H	H	-	-
Malonic acid	H	-	H	-	-	-	-	H	H	-	-
Maltose	H	-	H	H	+	H	H	-	H	H	H
m-Inositol	H	-	-	-	-	-	-	H	H	-	-
N-acetyl D-Galactosamine	H	-	-	-	-	-	-	-	-	-	-
N-acetyl D-Glucosamine	H	-	H	-	+	-	H	H	H	-	-
Phenylethylamine	H	-	-	-	-	-	H	H	H	-	-
p-Hydroxyphenylacetic acid	H	-	H	-	-	-	H	H	H	-	-
Propionic Acid	-	-	H	-	-	-	-	H	H	-	-
Putrescine	H	-	-	-	-	-	-	-	-	-	-
Pyruvic acid methyl ester	H	-	-	-	-	-	H	H	H	-	-
Quinic acid	-	-	H	-	+	-	H	H	H	-	-
Sebacic acid	H	-	H	-	-	-	H	H	H	-	-
Succinamic acid	H	-	H	-	-	-	H	H	H	-	-
Succinic acid	H	-	H	-	-	-	H	H	H	-	-
Succinic acid mono-methyl ester	H	-	-	-	-	-	-	H	H	-	-
Sucrose	H	-	-	-	+	-	H	H	H	-	-
Thymidine	H	-	-	-	-	-	H	H	H	-	-
Turanose	H	-	-	-	+	-	H	H	H	-	-
Uridine	H	-	-	-	-	-	-	-	-	-	-
Urocanic acid	H	-	-	-	-	-	-	H	H	-	-
Water	-	-	-	-	-	-	-	-	-	-	-
Xylitol	H	-	-	-	-	-	H	H	H	-	-
Tween 40	H	-	-	-	-	-	H	H	H	-	-
Tween 80	H	-	-	-	-	-	-	H	-	-	-

Cell count was higher when the isolates were grown in medium with TSB than the medium without TSB (Fig 6). This results also supported the obtained lesser thiosulphate oxidation in presence of TSB. On the other hand, isolate TH5 shows increased thiosulphate oxidation after TSB supplementation, indicating that this organism use thiosulphate and organic substrate in parallel. Isolates TH1, TH3 and TH9 grew as aerobes but could also reduce nitrate to nitrite. Organic acids and amino acids such as acetate, propionate, butyrate, glutamate, pro line and citric acid cycle intermediates can serve as sole carbon sources but sugars generally cannot. (Table 4).

Fig 6 Cell count comparison in medium with and without TSB during thiosulphate oxidation by isolates TH1 to TH11



These acid producing thiosulphate utilizers showed phylogenetic and physiological similarities to the previously isolated hydrothermal vent thiosulphate utilizers. Sulphate production from thiosulphate was consistent feature of these isolates although the extent of sulfate production varied considerably from strain to strain. *Pseudomonas stutzeri* isolated from mine site, is of high genetic diversity (Fig 8) (Lalucut et al 2006). They showed strain and colony morphology diversity suggesting strong influence of mutability (Fig 7) (Galleguillos et al 2009).

Fig.7 Colony diversity of heterotrophic thiosulphate utilizers isolated and identified

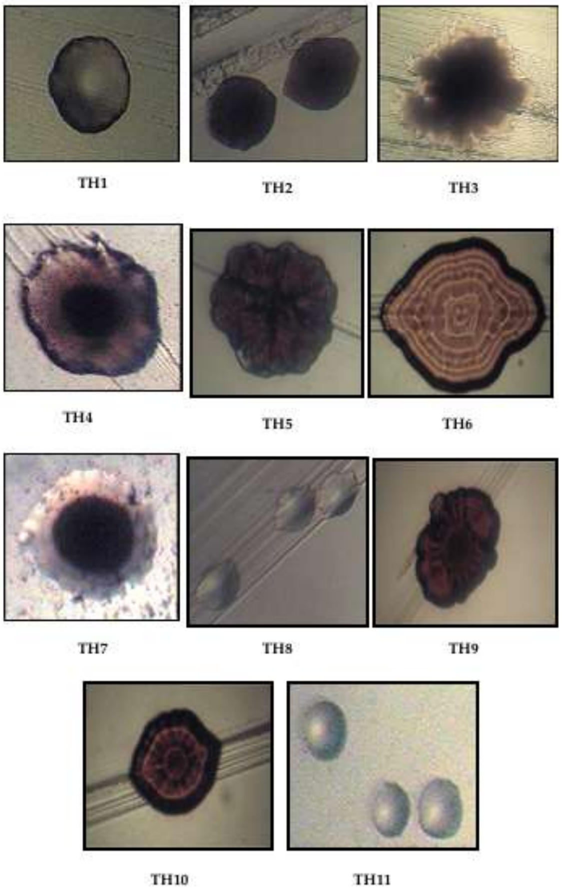
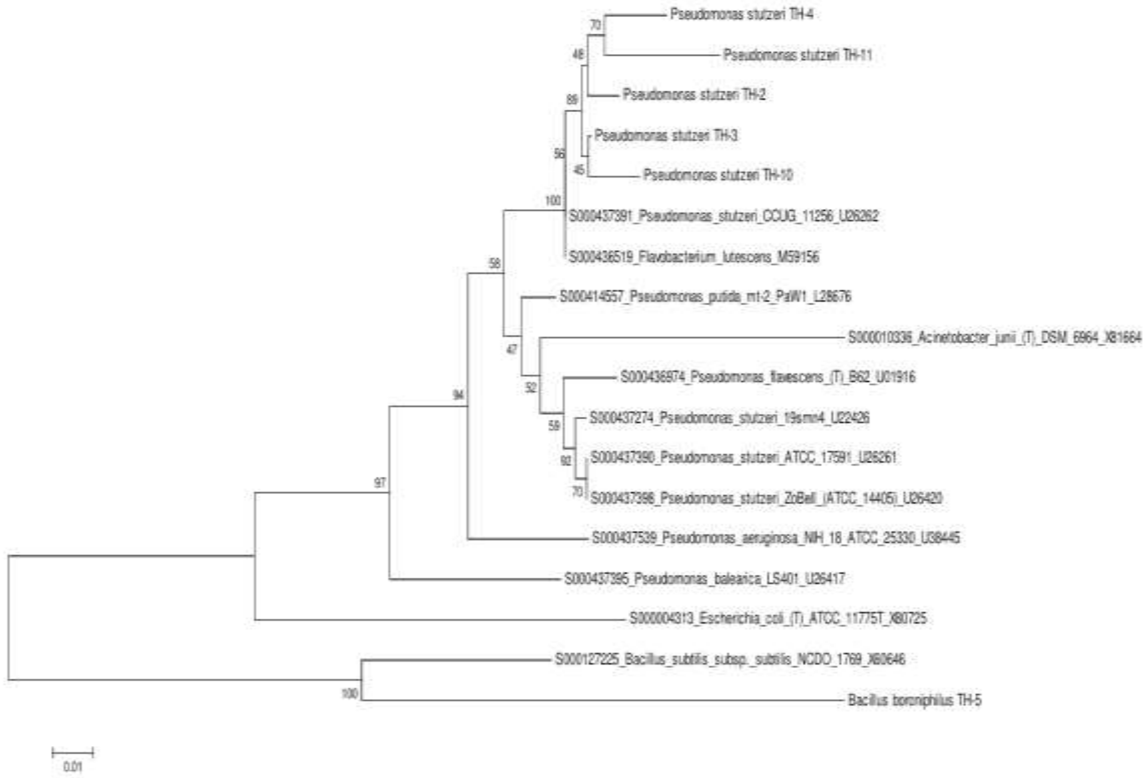


Fig 8 Phylogenetic analysis of the heterotrophic thiosulphate utilizing isolates



B) Base producing thiosulphate utilizer

Base producing isolate TH3 were isolated from 103 dilutions of samples. After growth in the same thiosulphate medium for 30 days under the same conditions as the acid producing thiosulphate utilizers, they increased the medium pH up to 1.1 in TSB supplemented medium, while up to 0.83 in unsupplemented medium.

The acid producing alpha proteobacterial thiosulphate utilizers from mine sites are phylogenetically related and physiologically similar; their differences appear to be in degree rather than in principle and show their importance in the mine environment.

Heterotrophic thiosulphate utilizers can make versatile and quick use of the Sulphur and carbon sources in the mine environment. Thiosulphate is supplied by chemical oxidation of metal sulfides. Visible acidification by thiosulphate oxidation as well as heterotrophic growth and colony formation could be seen after 10-12 days of incubation.

Dissolved organic carbon and particulate organic carbon levels are several hundred times less in waters of mine environment. This supports the preferable use of thiosulphate in absence of supplemented TSB by the isolates.

Sequence analysis the 16S rRNA gene of the selected isolates showed their affiliation to specific genomovars of. Cladera et al in 2004. He had also reported great strain diversity among *Pseudomonas stutzeri*. High strain and colony morphology diversity suggests strong influence of mutability due to extreme environment of the mine. The data suggest that thiosulphate utilizing bacteria, in particular those belonging to *Pseudomonas stutzeri* can play a significant role in bioleaching. Bilog analysis showed varied physiological characters of the isolated thiosulphate utilizers. This supports the reported physiological diversity among *Pseudomonas stutzeri* strains (Lycely 2001).

3.2.2 Isolation and characterization of autotrophic iron and Sulphur oxidizers

Three iron oxidizers were isolated from the collected water samples and named DA, DB and DC. One thiosulphate oxidizer named TH and one Sulphur oxidizer named DS were also isolated The data shown in Fig 9 to 11 describe the iron biooxidation activity of isolates DA, DB and DC under different concentration of Fe^{2+} iron.

Fig.9 Iron biooxidation study of autotrophic isolate DA

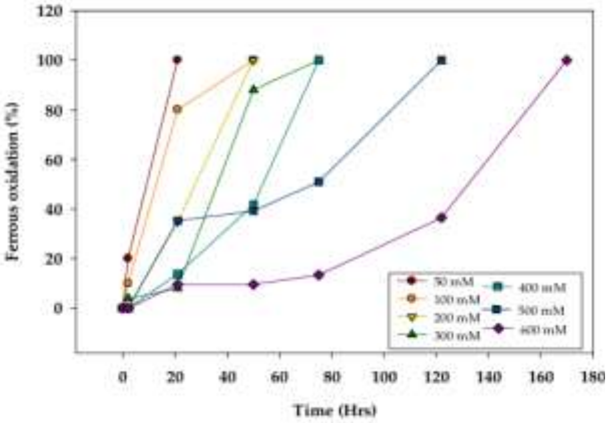
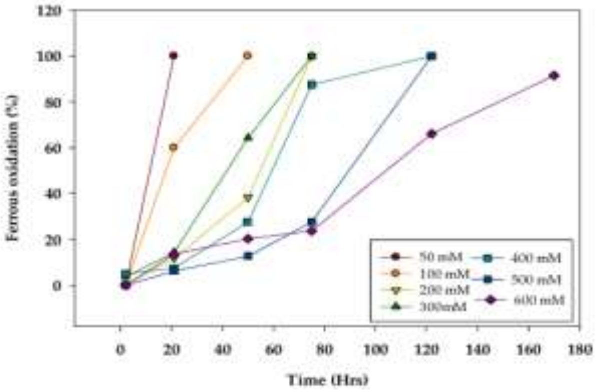


Fig.10 Iron biooxidation study of autotrophic isolate DB



Ferrous oxidation at ferrous concentration 500mM and above affect oxidation in case of all the three isolates DA, DB and DC. From the data shown in Fig 2.12, isolate DC is fastest growing among the three. Iron oxidizers DA, DB and DC showed Vmax mg/L/h of 0.287, 0.262and 0.303as well as Km mM of 70, 60 and 100 (Fig 13 to 15), respectively, confirming DC as the fastest grower, so was selected as inoculums for further flask and heap study.

Fig .11 Iron biooxidation study of autotrophic isolate DC

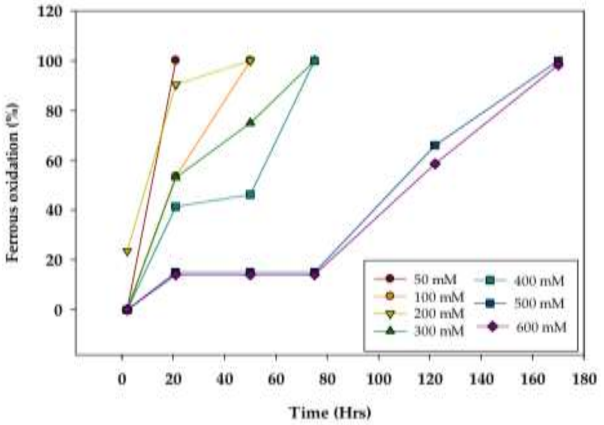


Fig.12 Comparative ferrous biooxidation study of autotrophic isolates DA, DB and DC

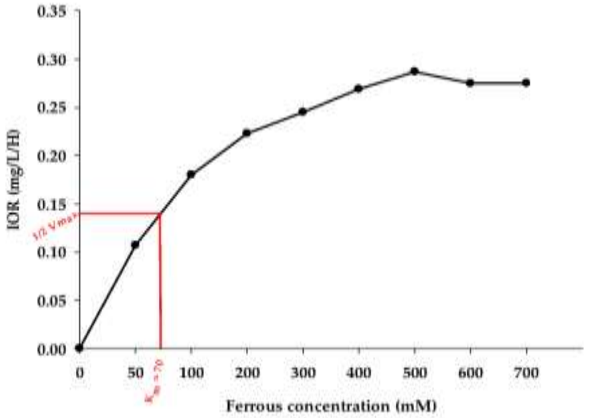


Fig.13 Average IOR and Km values of iron oxidizing autotrophic isolate DA

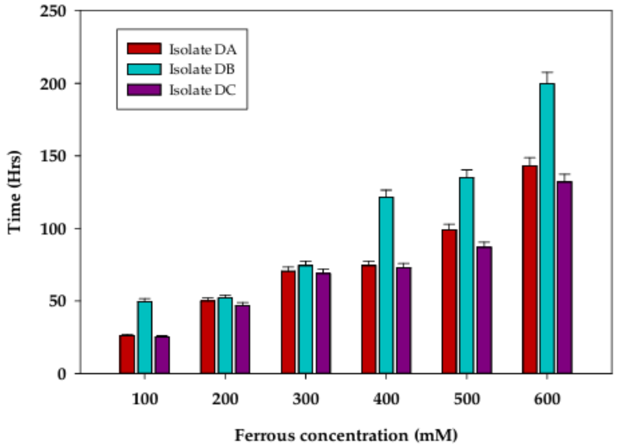


Fig.14 Average IOR and Km values of iron oxidizing autotrophic isolate DB

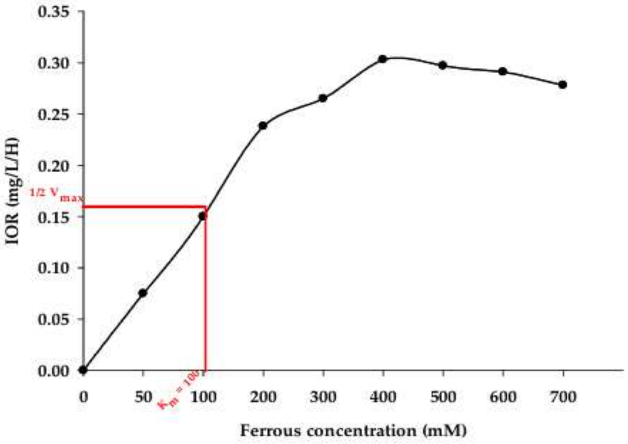


Fig.15 Average IOR and Km values of iron oxidizing autotrophic isolate DC

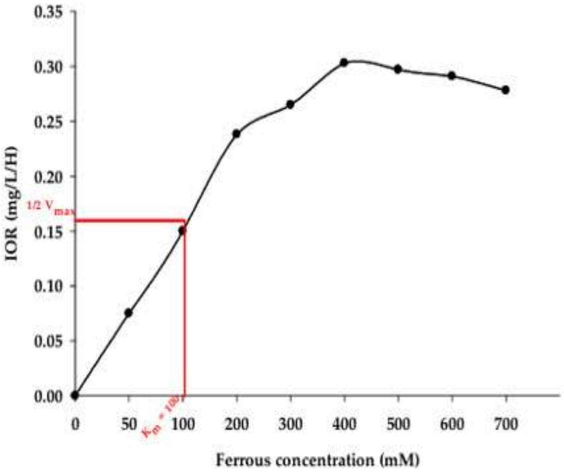
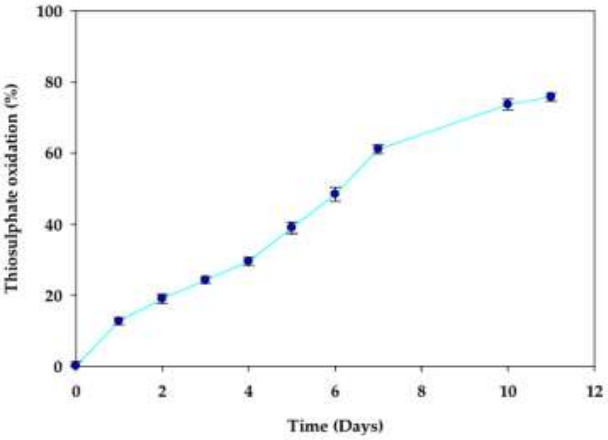
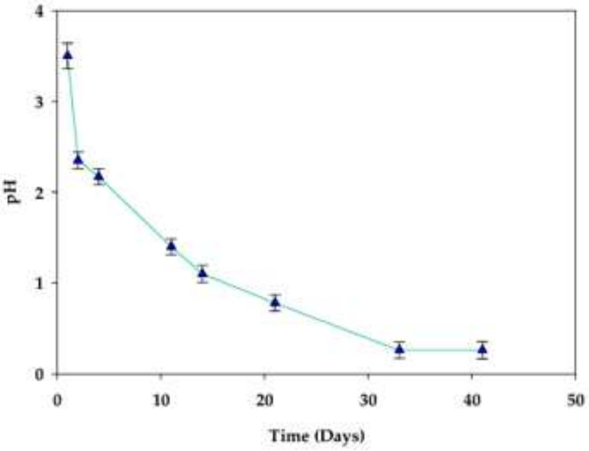


Fig.16 Thiosulphate biooxidation by autotrophic isolate TH



Thiosulphate oxidizing isolate TH1 showed 78% thiosulphate oxidation within 11 days and decreased the medium pH from 4.5 to less than 3.0, indicatin it the potential acid generator (Fig.16). Sulphur oxidizing isolate DS decreased the medium pH from 3.5 to 0.4, indicating it as potent acid generator (Fig. 17).

Fig.17 Sulphur biooxidation by autotrophic isolate DS



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