Isolation and identification of moderately Thermophilic Iron-Oxidizing & Sulphur-Oxidizing Bacteria at Indian Sub-Continent

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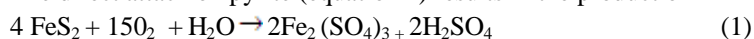(*Corresponding Author Yuvneet Rai*)

Abstract: *The aim of this study was to investigate the iron- and sulphur-oxidizing activities of thermophilic bacteria with reference to the possible use of such bacteria in the extraction of metals from mineral sulphides. The initial characterization of a range of isolates was based on growth studies with iron and sulphur substrates and on the comparison of whole cell. Three groups of bacteria were isolated and studied moderately thermophilic iron- and mineral sulphide-oxidizing bacteria, moderately thermophilic sulphur oxidizers and extremely thermophilic Sulfolobus-like organisms. Both moderately and extremely thermophilic acidophiles were isolated from hot spring and coal pile samples. The moderately thermophilic iron-oxidizing bacteria and the extreme thermophiles which were examined were sub-divided into three and four sub-groups respectively, moderate thermophiles did not produce higher rates of ferric iron production than the mesophile T. ferrooxidans but iron oxidation was less sensitive to inhibition by chloride in a vessel containing a thermophile than in a vessel operating with the mesophile. Iron oxidation during autotrophic growth of moderately thermophilic acidophiles and the rapid dissolution of mineral sulphides during the autotrophic growth of both the moderate and the extreme thermophiles were demonstated, thus considerably increasing the potential industrial significance of these bacteria. The yield of soluble copper from a chalcopyrite concentrate was shown to increase with temperature from relatively low yields with the mesophile T. ferrooxidans, through moderate yields with the moderately thermophilic bacteria to almost complete mineral solubilization with the newly isolated Sulfolobus strains.*

Keywords- *thermophilic, heterotrophic, oxidizing, isolates, autotrophically, mixotrophic, chalcopyrite*
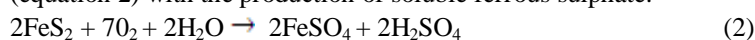
I.   Introduction

In contemporary era, the research and development of bioleaching have been always focused on achieving effective recovery of valuable metals by improving the efficiency of bioleaching microorganisms (Brierley, 2008), which is related to sulfur oxidation activities of sulfur-oxidizing microbes and the speciation of intermediate compounds formed during bioleaching processes (Akcil et al., 2007; Jordan et al., 2006; Vilcáez et al., 2008; Xia et al., 2010a).The initial characterization of isolates was based on growth studies with iron and sulphur substrates and on the comparison of whole cell. Three groups of bacteria were isolated and studied: moderately thermophilic iron-mineral sulphide-oxidizing (Juwarkar et al., 2004; Machulla et al., 2005; Gogoi et al., 2007; Daniels et al., 2009) bacteria, moderately thermophilic sulphur oxidizers and extremely thermophilic .Sulfolobus-like organisms. Both moderately and extremely thermophilic acidophiles were isolated from hot spring and coal pile samples. There are four basic systems of leaching. Dump leaching involves the use of low grade ore containing a variety of ore fragment sizes. The leach solution is sprinkled on and allowed to percolate through the dump and recovered in basins. The target metal is then removed and the solution recycled back to the dump. Leaching on this scale usually occurs over a period of years which is in contrast to the second method, heap leaching, which has a leach cycle measured in months. Khalil, 2002; Mendo et al., 2004; Walczak and Donderski, 2004; Khalil et al., 2006; Bushra et al., 2007; Awais et al., 2008; Muhammad et al., 2009) Heap leaching occurs with crushed or uncrushed ore but of a higher grade than in dump leaching. The bacterial leaching of metal sulphides can proceed via a direct attack on the metal sulphide or an indirect attack as a result of the bacterial production of ferric iron during oxidation. Both the direct and indirect mechanisms can operate simultaneously in mineral dissolution.
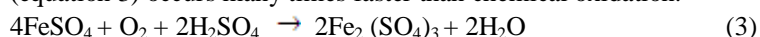
The direct attack on pyrite (equation 1) results in the production
$$4\,FeS_2 + 15O_2 + H_2O \rightarrow 2Fe_2(SO_4)_{3} + 2H_2SO_4 \qquad (1)$$
of soluble ferric sulphate and sulphuric acid. Pyrite chemically degrades slowly in the presence of oxygen (equation 2) with the production of soluble ferrous sulphate.
$$2FeS_2 + 7O_2 + 2H_2O \rightarrow 2FeSO_4 + 2H_2SO_4 \qquad (2)$$
The ferrous sulphate can then be oxidized by bacteria to produce ferric sulphate (equation 3) which will participate in the further chemical oxidation of pyrite (equation 4). The oxidation of ferrous sulphate by bacteria (equation 3) occurs many times faster than chemical oxidation.
$$4FeSO_4 + O_2 + 2H_2SO_4 \rightarrow 2Fe_2(SO_4)_3 + 2H_2O \qquad (3)$$

$$FeS_2 + Fe_2(SO_4)_3 \rightarrow 3FeSO_4 + 2S \qquad\qquad (4)$$

The ferrous iron produced (equation 4) can again be reoxidized by bacteria resulting in a ferric iron leaching cycle which is driven by the bacterial oxidation of ferrous iron. The sulphur produced as a result of the chemical oxidation of pyrite by ferric sulphate can itself be oxidized by bacteria to produce sulphuric acid (equation 5).

$$2S + 3O + 2H_2O \rightarrow 2H_2SO_4 \qquad\qquad (5)$$

A theoretical overall equation for the direct bacterial attack on a divalent metal sulphide is

$$MS + 2O_2 \rightarrow MSO_4 \qquad\qquad (6)$$

although it has been suggested (Torma et al., 1974; Tomizuka & Yagisawa, 1978) that an initial acid solubilization of metal sulphides could be important in the absence of iron with the bacteria ensuring continuous acid production (equation 7).

$$2MS + 2H_2SO_4 + O2 \rightarrow 2MSO_4 + 2S + 2H_2O \qquad\qquad (7)$$
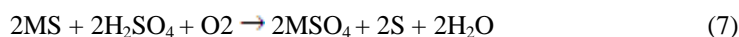
The solubilization of chalcopyrite to copper sulphate illustrates this general equation (equation 8)

$$4CuFeS_2 + 17O2 + 2H2SO_4 \rightarrow 4CuSO_4 + 2Fe_2(SO_4)_3 + 2H_2O \qquad (8)$$

the solubilization of chalcopyrite can also occur as a result of chemical leaching by ferric sulphate (equation 9), so a combination of direct' and 'indirect' bacterial attack on such sulphides is likely.

$$CuFeS_2 + 2Fe_2(SO_4)_3 \rightarrow CuSO_4 + 5FeSO4 + 2S \qquad\qquad (9)$$
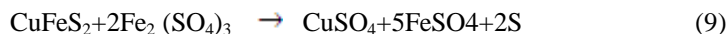
II.   Materials and Methods

Sources of Samples for Organism Isolation

Coal sediment and water samples were collected from coal spoil heaps at Sambalpur (sample pH 3.0) and at Bilaspur (pH3.0) in Orissa, INDIA. & at Shahdol (sample pH 2.8) Kymore. Drainage water from a 2 year old, washed-coal pile was collected from the Gondegaon, Maharashtra coal washeries (sample pH 1.5, water temperature 370C). All these samples were transferred to culture media on the day of their collection .A hot spring water and sediment sample (pH 1.8) was obtained from Lake Manikaran, H.P, INDIA and a nickel/copper mine water and sediment sample (pH 3.0) was obtained from CETP, Industrial Area, Ludhiana, Punjab, INDIA. The mine water sample was transferred to culture medium within a month of collection and the hot spring sample was used to establish cultures two weeks after its collection.

Growth Medium for Thermophilic Bacteria

Sulfolobus acidocaldarius isolated at optimum growth conditions of approximately 80°C and pH 3 the medium was adjusted to pH 3 with 10 M H_2SO_4 and filter sterilized with 0.2-μm-pore-size bottle-top filters. Cultures were grown to the mid-exponential growth phase (4×10^9 cells/ml) and stored as frozen glycerol stocks at −70°C. For tracking experiments, a frozen aliquot of 150 μl was thawed and delivered to a 250-ml shake flask containing 60 ml of medium. The inoculated medium was grown at 72°C in a model 655G Iso-temperature oven (Fisher Scientific) while being shaken on a model G_2 Gyrotory shaker set at 100 rpm ($0.48 \times$ g). Cells were grown to an approximate cell density of 10^7 cells/ml. To test the effect of pH on motility, cells were filtered from the pH 3 growth medium with a 0.45-μm-pore-size filter (Millipore Corporation, Bedford, Mass) on the basis of motility, S. acidocaldarius was grown to 10^4 cells/ml, filtered with a 0.45-μm-pore-size filter, and rinsed three times with motility buffer. Cells were then resuspended in pH 3 motility buffer, incubated at 72°C, and qualitatively observed with the tracking microscope.

Growth on Ferrous Iron

Chemolithoheterotrophic growth of thermophile TH1 measured as the rate of ferrous iron oxidation in OD (Figure-1) followed the sigmoidal curve characteristic of bacterial growth. This was accompanied by a rise in pH from 1.7 to 2.5 which caused some of the ferric iron produced to precipitate. Measurement of the growth rate on ferrous iron by determinations of iron oxidation, protein, cellular carbon and optical density showed that all methods were reliable indicators of growth during the early stages of a growth curve. The aim of this work with moderately thermophilic iron oxidizing bacteria was to isolate organisms from a range of sites, thermophilic, iron-oxidizing organisms and then to compare the organisms with a view to a preliminary classification of the 'group' and an assessment of their potential use in applied biohydrometallurgy (Johnson, 2003; Ren et al., 2010) TH1, expressed as a percentage of dry weight, was composed of 42.42% carbon, 6.25%

hydrogen and 9.3% nitrogen. With the same preparation and analysis procedures, T. ferrooxidans contained 42.15% carbon, 6.49% hydrogen and 9.42% nitrogen.
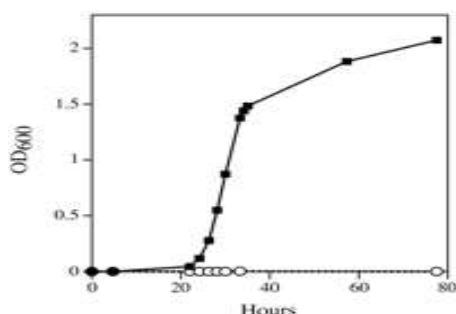

Figure.1 OD of thermophilic iron oxidizing bacteria

Heterotrophic Growth on Yeast Extract

TH1 grew heterotrophically in a yeast extract medium at 50°C with concentrations of 0.1 to 0.8g yeast extract per litre. Maximum optical density occurred with 0.8g yeast extract 1-1 although there was a longer lag phase than that observed at lower concentrations. A level of 1.0g yeast extract per litre was inhibitory to growth. A decrease in optical density occurred once the maximum had been reached indicating that the cells had metabolized the available yeast extract and had begun to lyse. The pH remained at 1.8 during the growth on yeast extract. Under the light microscope, differences were observed in the morphology of the isolates which were dependent on their growth substrate and type of growth. When grown on ferrous iron in the presence of yeast extract, all of the isolates appeared as long, fat, rod-shaped bacteria. TH1 grown on iron in the presence of yeast extract was 1.5-4.5 μm by 1.0 μm but when grown autotrophically its size was reduced to 1.3-2.5 pm by 0.8 pm. When TH1 was grown on yeast extract alone it appeared as short chains of almost spherical organisms surrounded by an optically refractile coat. The cells measured 0.8-1.6 μm by 0.8-1.0 pm. The outer layer, which was clearly visible under the light microscope was not easily detected under the electron microscope due, presumably to the dry nature of the preparation. The obligately heterotrophic bacterium isolated at 50°C

III. Results and Discussion

Isolation of Moderately Thermophilic Iron-Oxidizing Bacteria

Organisms from ferrous iron enrichment cultures, grown in the absence of organic nutrients, and were purified on ferrous iron plates at 50°C by single colony isolation. Organisms from ferrous iron enrichment cultures grown in the presence of yeast extract from the above sites were purified at 50°C on yeast extract supplemented ferrous iron plates and yeast extract plates by single colony isolation. An organism from an autotrophic iron enrichment culture at 37°C was isolated and purified by P. Norris. Enrichment cultures growing on ferrous iron and on pyrite at 50°C in the presence of yeast extract from CETP, Industrial Area, Ludhiana, Punjab nickel mine were not purified and are referred to as enrichment cultures of the sample sites growing on ferrous iron at 50°C in the presence of yeast extract.
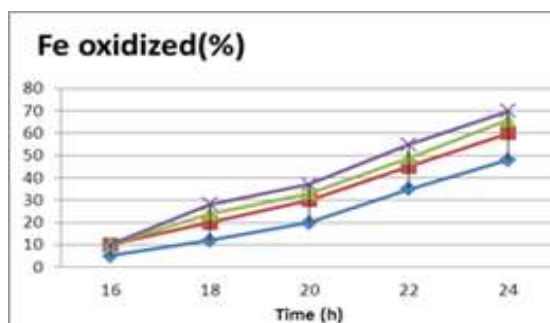

Figure.2 Ferrous iron oxidation during growth of thermophile TH1 at 50°C in the presence of yeast extract (0.2 gl-l) in different salts medias

Isolation of Sulphur-Oxidizing Bacteria at 37°C & 50°C

Organisms were isolated from the Sambalpur and Lake Manikaran, CO_2-enriched sulphur enrichment cultures at 50°C.(Image1,2) The isolates were purified via thiosulphate agar plates but the Lake Manikaran isolate growing at 50°C was only able to be grown on tetrathionate agar plates. Further work was carried out on isolates designated BC2, BC4 and LM2 as these appeared morphologically different under the microscope.

Organisms from CO_2 enriched sulphur enrichment cultures at 37°C were isolated and purified on thiosulphate agar plates. Two isolates, designated as BC13 and LM7, have been used for comparative purposes in this study. Enrichment cultures on sulphur at 50°C only be maintained in the presence of yeast extract and were not purified or studied further. Growth of sulphur-grown TH1 cultures could not be established on thiosulphate or tetrathionate agar plates. Cultures of TH1 were serially sub-cultured three times on sulphur before being used in experimental work.

Image.1

Image.2

Moderately Thermophilic Sulphur-Oxidizing Bacteria

Rod-shaped bacteria growing auto trophically on elemental sulphur up to a temperature of 55°C have been isolated from hot springs and soils around the world and described as high temperature strains of Thiobacillus thiooxidans. They have frequently shown poor growth or failed to grow without a carbon dioxide enriched atmosphere. Such organisms are thought to contribute to the production of sulphuric acid in acidic, thermal habitats and occupy a temperature niche between that of the lower temperature T. thiooxidans strains and the extremely thermophilic Sulfolobus organisms. However, beyond the initial isolations of such organisms little work has been reported on any further characterization. The iron-oxidizing moderate thermophile TH1 has been growing poorly on elemental sulphur in the presence of yeast extract with less growth being observed at each serial sub-culture. Oxygen uptake could not be detected with elemental sulphur in an oxygen electrode by a cell suspension of TH1. The growth on sulphur of the moderately thermophilic, iron-oxidizing (Figure.3).TH group of organisms and reports the isolation of new sulphur-oxidizing strains.
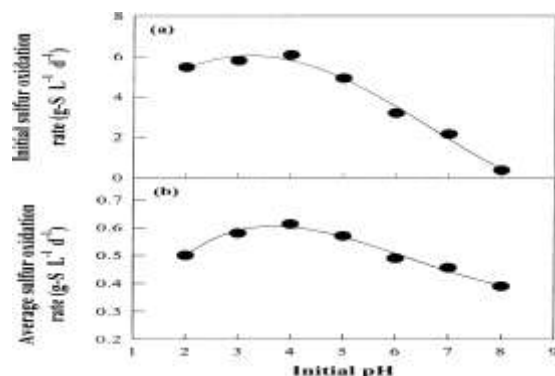

Figure.3 Curves shows growth rate of Initial and average sulfur oxidation

Selection Medium for The Isolation of Organisms and The Growth Studies

The growth of thermophile TH1 on sulphur at 50°C in the presence of yeast extract (180mg 1-1) in five different salts media was compared with the growth of T. ferrooxidans at 30°C in the same media. T. ferrooxidans was chosen in preference to T. thiooxidans so that organisms which had the capacity to oxidize both iron and sulphur were involved in the comparison. Growth by TH1 on sulphur was often difficult to establish over several sub-cultures. Successive growth did the greatest fall in culture pH was observed using the low salt concentration medium of sulphur salts in which the pH fell from 3.1 to 1.5. Growth in the higher salt concentration media of 9K salts produced a smaller drop in culture pH from about 3.2 to 2.7. During growth of T. ferrooxidans the pH fell from about 3.0 to 1.2 in all the salts media. The lag phase before growth commenced was longer than TH1 and in all media.
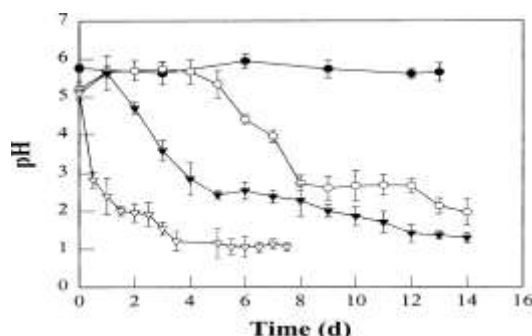
Figure.4 Growth of thermophile TH1 on sulphur at different pH scales.

Growth on Sulphur

Autotrophic growth on sulphur by LM7 showed an optimum temperature for growth at 55°C with a doubling time of about 10 hours as estimated by sulphate production. At 50°C there was a rapid decline in growth rate. The influence of temperature on growth followed a similar pattern with BC2, BC4 and BC13, all of which had an optimum temperature for growth of 45°C and maximum doubling times, when measured by sulphate production, from 8.3 to 10.5 hours In contrast, LM2 showed an optimum temperature of 50°C for growth on sulphur of and a much slower doubling time of 25 hours. At 37°C there was a long lag phase before the faster rate of growth was obtained and at 55°C growth was inhibited. All results were obtained from shake flask and slightly faster rates have been observed when these organisms were grown in 250 ml stirred culture vessels. Isolates BC2, BC4, BC13 and LM7 were unable to oxidize ferrous iron at any temperature but LM2 readily showed the capacity to oxidize iron. The newly-isolated, moderately thermophilic iron-oxidizing bacteria did not oxidize elemental sulphur on transfer to the sulphur salts medium from iron-oxidizing cultures.

Comparison Of Substrate Utilization By Sulfolobus Strains

	Substrates			
Organisms	**Yeast Extract**	**Fe2⁺**	**S**	**GROUP**
S. acidocaldarius 98-3	+	-	-	
S. solfataricus	+	-	-	**1**
Sulfolobus (Lake Manikaran) (grown on yeast extract)	+	-	-	
Sulfolobus B6-2	N. D.	-	+	**2**
Sulfolobus (Lake Manikaran)	N. D.	+	+	**3**
Sulfolobus (Sambalpur)	N. D.	+	+	
S. brierleyi	N. D.	+	+	
S. acidocaldarius	N. D.	+	+	**4**

IV. Conclusion

The increase in culture pH which occurred when *S. acidocaldarius* 98-3 and *S. solfataricus* were grown in the presence of elemental sulphur and yeast extract indicates that they were not growing on the sulphur but on the yeast extract alone. The inability of these strains to oxidize either sulphur or ferrous iron indicates that they are obligate heterotrophs a mixed population of autotrophs and heterotrophs giving the appearance of a mixotrophic culture. The doubling time for growth on yeast extract by S. acidocaldarius 98-3 was slightly higher than that previously reported but rates have been shown to be strain dependent. When the enrichment culture of Sulfolobus was grown on-yeast extract over several sub-cultures a 'heterotrophic-type' of organism, similar to S. acidocaldarius was selected. This would account for its subsqunt inability to revert back to oxidizing ferrous iron. Even though the two different mixed populations of Sulfolobus were morphologically indistinguishable, to S. acidocaldarius 98-3 and S. solfataricus. It is possible that S. acidocaldarius 98-3, while being kept in the presence of yeast extract, has undergone the same selection procedure as occurred with Sulfolobus in the short term, and that the organisms responsible for sulphur oxidation in this particular culture have been lost from the population. The observation that yeast extract is required by 'S. brierleyi' for sustained growth on ferrous iron confirms previous reports that yeast extract enhanced growth. However, even in the presence of yeast extract the

doubling times for growth on ferrous iron were slow when compared with the mesophile T. ferrooxidans and the moderately thermophilic iron-oxidizing bacteria. The rate of iron oxidation by Sulfolobus was similar to that observed using Sulfolobus strain 36-1.The growth rate of Sulfolobus on sulphur was similar to that observed by the moderately thermophilic thiobacillus-type bacterium LM7 isolated from the same site but faster than that observed by the moderately thermophilic iron-oxidizer LM2. The rate of sulphur oxidation was more rapid than previously reported for some Sulfolobus strains. Sulfolobus B6-2 was the only strain capable of growing on sulphur but not on ferrous iron. Although detailed physiological characteristics were not determined, comparison confirms that it is probably a different species of Sulfolobus to either of the other sulphur-oxidizers, Sulfolobus and S. brierleyi. S. brierleyi appeared during all observations to be identical to S. acidocaldarius. Some differences might be expected in view of the different characters of the isolation sites.

All observations suggest that at present, the physiological characteristics that there are 4 'groups' of Sulfolobus like organisms, Group1, characterised by *S. acidocaldarius 98-3* are obligate heterotrophs. Group 2, characterised by Sulfolobus B6-2, can maintain growth on sulphur but not on ferrous iron. Group 3, characterised by *Sulfolobus*, can maintain autotrophic growth on iron and sulphur although whether such organisms will also grow on organic substrates has not been proven. Group 4, characterised by *S. brierleyi*, will grow on sulphur and ferrous iron but autotrophic growth on iron cannot be sustained even in the presence of a reduced sulphur source. This study has outlined some of the problems when comparing the present Sulfolobus strains and indicated that S. acidocaldarius 98-3 in one collection might not be identical to that in another collection. An effective method of obtaining pure cultures must be found before further comparative studies can be undertaken.

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