Isolation and Testing of Sulphate Reducing Bacteria and Its iron Bioleaching Efficiency on Pyrite

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ABSTRACT: *Mud sedimentsample was collected from the pond, Mathur, Trichy. Winogradskycolumnwas prepared and the black soil from the bottom layer of the column was collected. Bacterial colonies were isolated through pour plate technique followed by Serial dilution by enrichment medium. The black coloured colonies were identified as sulphate reducing bacteria and found to be Gram negative large rod shape bacterium. The growth onchemotroph (9k medium), heterotroph (glucose and yeast extract), mixotroph (yeast extract) was studied. Luxuriant growth was found at chemotroph and heterotroph, and less significant on Mixotroph. Atomic adsorption of ironleaching from pyriteby isolated sulphate reducing bacteria was found maximum of 120 mg/ Lon Chemolithotrophic medium.*

Key Words: *Sulphate reducing bacteria, bioleaching, pyrite, Heterotroph*

INTRODUCTION

Bioleaching is a simple and effective technology for metal extraction from low-grade ores and mineral concentrates. Metal recovery from sulfide minerals is based on the activity of chemolithotrophic bacteria, mainly *Thiobacillusferrooxidans* and *T. thiooxidans*, which convert insoluble metal sulphides into soluble metal sulfates. Non-sulfide ores and minerals can be treated by heterotrophic bacteria and by fungi(Akcil, 2007). In these cases metal extraction is due to the production of organic acids and chelating and complexing compounds excreted into the environment. At present bioleaching is used essentially for the recovery of copper, uranium and gold, and the main techniques employed are heap, dump and in situ leaching. direct bioleaching minerals which are susceptible to oxidation undergoes direct enzymatic attack by the microorganisms. In direct method of bioleaching of minerals bacteria produce strong oxidizing agent which reacts with metals and extract them from the ores(Kelly, 2000).Biomining is a combination of chemical and biological reactions. During metal sulphide bioleaching, metal sulphides are oxidized to metal ions and sulphate by aerobic, acidophilic Fe iron and /or sulphur compound oxidizing bacteria or Achaea (Schippers,2007). The process is more environmentally friendly than traditional extraction methods. For the company this can translate into profit, since the necessary limiting of sulfur di oxide emission during smelting is expensive. Less landscape damage occurs, since the bacteria involved grow naturally, and the mine and surrounding area can be left relatively untouched. As the bacteria breed in the conditions of the mine, they are easily cultivated and recycled.

Bioleaching is a biological process where metals and microorganisms have been interaction. At this processes microorganisms prove recovery of metals by dissolving insoluble forms of them. However this biological solubilisation is often used at recovering metals from ore mining, wastes have also considerable economic importance at metal gaining for their cheap raw material.(Omer simsek, 2007) At the recent years, it has been reported that important amount of aluminum, zinc, nickel and copper can be extracted from different kind of municipal and industrial wastes.

Bioleaching has gained increased interest as an alternative for processing zinc sulfide ores without the generation of SO2. The bioleaching of ores with mesophile microorganisms at 1% pulp density under batch experiments were carried out at 34° C and 200 rpm by many researchers. The effects of pH, concentration of Fe (II), as well as the presence of Fe (III) in the zinc extraction were assessed. Fast zinc dissolution can be achieved working with *Acidithiobacillus sp*. The best pH for bioleaching is in the 1.75–2.00 range and the presence of Fe (III) has a strong influence in zinc extraction, increasing the rate of dissolution and does not adversely affect the growth of the *Acidithiobacillu*s population(Daman, 2002).

The type strain of *Desulfovibrioprofundus* was isolated from 500 m depth in sediments of the Japan Sea. Other piezophilic isolates closely related to *D. profundus* were cultivated from 222 m deep sediments of the Cascadia margin of the Pacific Ocean. However, cultivation-based studies on the marine deep biosphere are

still limited to a few sampling sites representing pinpricks in the ocean floor. So far, isolates from the marine subsurface were obtained from sediment samples retrieved from Mediterranean sediments (Süss et al., 2004) and from various sites in the Pacific Ocean (Batzke et al., 2007).Microorganisms are preferred because of theircheap cost. When the SO42-in the solution is removed bythe microorganisms, sulfate reducing bacteria (SRB)firstly deoxidize SO42-into sulfide including H2S, S2-andHS-under anaerobic condition; after which the sulfidemay then be oxidized into elemental sulphur (S0) bysulphide oxidation bacteria (SOB) under aerobic andspecific condition (Jiang et al., 2009).In order to advance the ability to remove sulfate in theintrinsic treatment system, investigators have isolatedand selected many efficient pure SRB to treat sulphate (Kjeldsen et al., 2009). Until now, therehave been some reports about the removal of sulfate intannery wastewater by SRB (Boshoff et al., 2004) and aCompetition between SRB and methanogens withanaerobic treatment for tannery wastewater (Tadesse et al., 2003). However, there has not been any report about removing sulfate in tannery wastewater with isolated predominant SRB strains. Recently, several heterotrophic bacteria and methanogenicArchaea were isolated from up to 106 mbsf deep sediments off Shimokita Peninsula, Japan using a continuous-flow bioreactor (Imachi et al., 2011).

MATERIALS AND METHODS

Sample collection

The mud sample was collected from the pond located near mathur, Tiruchirappali, Tamilnadu India. The collected mud sample was used to set upWinogradsky column.

Winogradsky column packing

To pack the column (40 x 5 cm) is to prepare the mud or soil samples as slurry having the consistency of heavy cream. The slurry can be enriched before packing the column. Calcium sulphate (1-2 % w/w) and calcium carbonate (1-2 % w/w) are added as sources of sulphur and CO2, respectively. Finely shredded paper (a piece of newsprint about 100 cm2) provides a long term carbon source and provides CO_2 and other organic substrates such as organic acids as it is mineralized (Tamer et al, 1989).After one month, sample was retrieved from the winogradsky column.

Isolation of sulphate reducing bacteria

Thiobacillussp, were isolated from the winogradsky column and the black bottom layer after 25 days incubation. Samples taken from Winogradsky column and serially diluted up to 10^7. The soil sample was diluted and it was plated by pour plate technique. 200µl of diluted soil sample was inoculated on solidified Lyngby medium (peptone 20, Yeast extract 3, Beef extract 3,Sodium thiosulfate 0.3, Sodium chloride 5, Ferric citrate 0.3 and 15 g/L) and incubated at 37°C for 24 hours. After incubation the plates were observed for black colonies. The black colonies indicate sulphate reducing bacterial colonies.

Biochemical characterization of SRB: Isolated bacterial strain was subjected to IMViC, Catalase and Oxidase and the results were recorded.

Cell morphology by Gram staining:

Thin smear were made in a clean slide and heat fixed before stain process. Staining was done by flooding the smears with crystal violet solution for 1 minute and the smear was washed in a gentle and direct stream of tap water and the slide was flooded with iodine mordant for 1 minute. After washing with distilled water, the smear is decolorized using 95% ethanol until no more colour flows from the smear, then it is rinsed in tap water and counterstain safranin was added for 30 seconds. It was again rinsed in tap water and air dried and then it was observed microscopically under oil-immersion objective.

Growth on chmotrophic, hetrotrophic, and mixotrophic:

9k medium was prepared, yeast extract(0.1%) and glucose (1%) were added to the medium and it is kept as heterotroph. Yeast extract alone is added to the medium and act as mixotrophic. 9k medium alone is kept as chemotroph. The grown *Thiobacillus sp*., from the plate was taken and subcultured in the prepared heterotroph, chemotroph and mixotroph broth. It was incubated at 37°C for 24 hrs. After incubation, the absorbance was measured at 600 nm for every one hour upto 14 hours.

Iron leaching

A "pyrite leaching" simulation experiment was conducted in 500 ml conical flasks containing Modified Lyngby medium as described above. The medium used during these experiments was the same as used for submerged *Thiobacillus sp.* pyrite concentrate (500g) was kept in each conical flask and mixed with 50 ml of SRB culure. The flasks and their contents were incubated at 37ºC for 24 hours. Leaching of iron was determined by atomic absorption. Medium without culture kept as control.

RESULT AND DISCUSSION

At the end of 20thday incubation black color band was observed on winogradsky column. Bands were darkened at the end of 30th day on the column. The bacterial cell present in the black precipitate soil sample was isolated and the colony forming unit was 38 X10^{7}on Lyngby medium gave black coloured colonies (plate 1a). when microscopically observed were found to be rod shaped and Gram negative rod shaped cells. Bacterial colonies of *Thiobacillusferrooxidans*appeared on ferrous sulfate agar after incubation at 35ºC for 48h were observed to be smooth, circular, low convex and greater opacity of their size. The diameter of the colonies were found to be approximately (1-2) mm. further enrichment of isolates on sulphate reducing medium. During the development of colony, there was a change in colour of colony with black dots and found to be Gram negative rod (Plate 1b). Based On biochemical characters (table1) the isolate was identified as *Thiobacillus* sp. Isolate showed Indole, methyl red, Vogesproskuer,catalae, oxidase.

Table 1: Biochemical characters of isolated SRB

S.No.	TEST	RESULT
1.	Indole	Positive
2.	Methyl red	Positive
3.	Vogesproskauer	Positive
4.	Citrate	Positive
5.	Catalase	Positive
6.	Oxidase	Negative

The diffusion of H_2S from the sediment into the water column enables anaerobic photosynthetic bacteria to grow. They are seen usually as two narrow, brightly coloured bands immediately above the sediment - a zone of green sulphur bacteria then a zone of purple sulphur bacteria (Brock et al., 1994). The sulphur-reducing bacteria such as Desulfovibrio can utilise these fermentation products by anaerobic respiration, using either sulphate or other partly oxidised forms of sulphur (e.g. Thiosulphate) as the terminal electron acceptor, generating large amounts of H_2S by this process The H_2S will react with any iron in the sediment, producing black ferrous sulphide. This is why the bottom of column is frequently black (Pansona et al. 2002). However, some of the H_2S diffuses upwards into the water column, where it is utilised by other microorganisms. The purple sulphur bacteria typically have large cells and they deposit sulphur granules inside the cells. The green sulphur bacteria have smaller cells and typically deposit sulphur externally. The sulphur (or sulphate formed from it) produced by the photosynthetic bacteria returns to the sediment where it can be recycled by *Desulfovibrio*spand chemolitotrophic*Thiobacillus sp*. part of the sulphur cycle in natural waters.

Figure 1shows Bacterial growth pattern underchemolithotrophic, mixotrophic and heterotrophic condition (Plate 2). Under chemolithotrophic ($Na_2S_2O_3$& Yeast Extract) cultural condition, lag phase of *Thiobacillusferrooxidans* continued up to 2hr of incubation. The log phase started thereafter till the 8 hr of incubation after which stationary phase started. As revealed from the experimental data, initially pH of the culture medium before the bacterial growth was maintained at 5.0. However, at the end of the growth at 30 h of incubation, pH value showed a decline trend and it dropped down to 2.5. Sulfur was the only energy source in the culture and the initial pH of the culture was 3.5 to 4. Only SRB could survive in these conditions. The bacteria used sulfur to yield H_2SO_4, indicating the biochemical characteristics of *A. thiooxidans.*Such variation in pH can indicate the activity of *Thiobacillussp*was reported in many studies (Zhang et al., 2009).

Similarly growth on mixotrophic (Glucose & $Na_2S_2O_3$) growth condition, the lag phase started at 6h up to 7 h and was followed by log phase up to 14 h of incubation. The stationary phase was then initiated followed by change in pH value from 4.0 to 5.1 at 30 h of incubation. However, the heterotrophic (Glucose & Yeast Extract) growth condition showed that the lag phase of bacterium continued up to 2 h of incubation followed by log phase up to 22hr. The pH due to bacterial growth in heterotrophic culture medium increased from 4.0 to 6.17 at 48 hr of incubation.

Bioleaching of pyrite:

T. ferrooxidans caused a decrease in pH because the Fe^{3+} produced from Fe^{2+} by *T. ferrooxidans* could hydrolyze and yield black precipitation on (plate 2) heterotrophic and chemolithotrophic broth but failed in mixotrophic .Decrease in pH was recorded because it utilised sulfur to produce H_2SO_4. In contrast, Zhang et al., (2009)reported that *Thiobacillus*sp oxidizes iron well in mixotrophic. Isolated strains of heterotrophic iron-oxidizing acidophilic bacteria were examined to determine their abilities to promote oxidative dissolution of pyrite (FeS_2) when they were grown in pure cultures of sulfur-oxidizing *Thiobacillus* sp. Oxidation of pyrite when it was grown in pyrite-basal salts medium was less siginificant. However, when pyrite-containing cultures were supplemented with 0.02% (wt/vol) yeast extract, oxidation of pyrite was enhanced. Addition of yeast promoted rates of mineral dissolution similar to the rates observed with the iron-oxidizing autotroph *Thiobacillusferrooxidans*.

pyrite oxidation by pure cultures of heterotrophic iron-oxidizing was potent by the end of the 25 day leaching period. The total amount of pyrite oxidized by cultures of strain 120 mg/L followed by 12, 32 mg/L respectively on 7 and 15 day incubation(Figure 2). We also found that the final recorded pH in cultures reduced from 3 to 1.50 at the end of incubation period. In contrast to heterotrophic, chemolithotrophic bioleaching with strain *T. ferrooxidans* was maximum of 08, 12 and 60 mg/L at the end of 25th day incubation. The bioleaching of Fe at mixotrophic medium was 06, 09 and 12 mg/L .*T. acidophilus* also accelerated pyrite oxidation, the effect was relatively marginal in inorganic pyrite medium. *T. acidophilus* differs from *T. thiooxidans* in that it is mixotrophic and readily changes from using inorganic carbon sources to using organic carbon sources and leaching is less stable due to less lag phase and long log phase(Mason and Kelly, 1998)

Conclusion: the present study concludes that isolated *T.ferroxidans*is a potent bioleaching biological agent and capable to leach iron.

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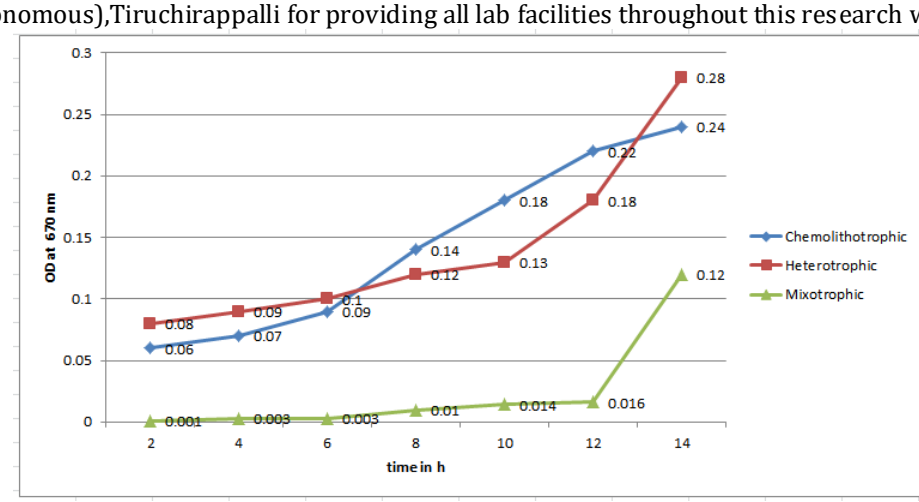


Figure 1: Bacterial growth pattern on different media

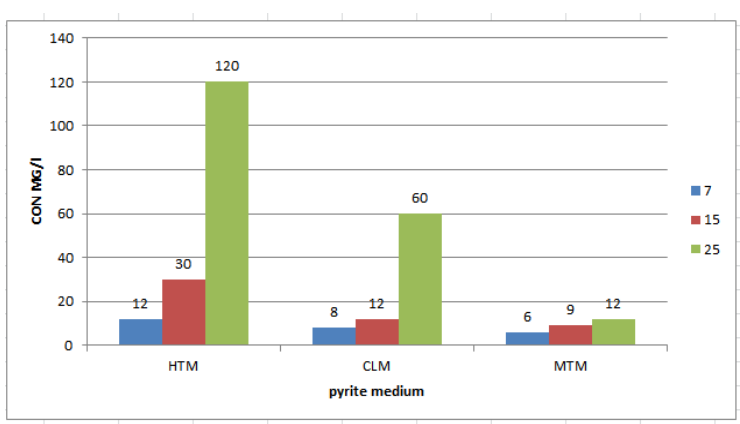
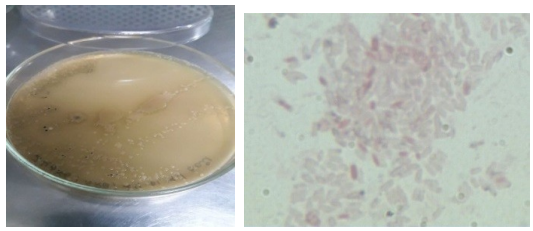


Figure 2. Iron bioleaching estimation by Atomic absorption

Plate: 1 Isolation of SR bacterial colonies



a) Isolated SRB b) Gram negative rod

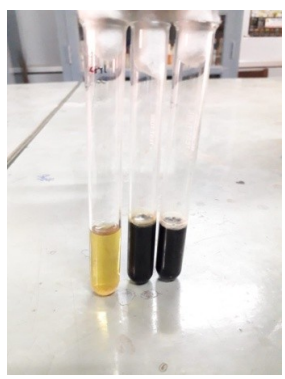


Plate 2: Oxidation of Ferrous Iron (mixotrophic, heterotrophic, chemolithotrophic,)

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