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MICROBIAL LEACHING OF IRON FROM PYRITE BY MODERATE THERMOPHILE CHEMOLITHOTROPIC BACTERIA

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Abstract
The present work was aimed at studying the bioleachability of iron from pyrite by the selected moderately thermophilic strains of acidophilic chemolithotrophic and acidophilic heterotrophic bacteria. These included *Sulfobacillus thermosulfidooxidans* (chemolithotroph) and an un-identified strain of acidophilic heterotroph (code 6A1TSB) isolated from local environments. As compared to inoculated flasks, dissolution of metal (due to acid leaching) was significantly low in the un-inoculated control flasks in all the experiments in ore. A decrease in the bioleaching activity was observed at the later stages of bioleaching of metal from ore. Among the strategies adopted to enhance the metal leaching rates, a mixed consortium of the metal adapted cultures of the above-mentioned bacteria was found to exhibit the maximum metal leaching efficiency. In all the flasks where high metal leaching rates were observed, concomitantly biomass production rates were also high indicating high growth rates. It showed that the metal bioleaching capability of the bacteria was associated with their growth. Pyrite contained 42% iron.

Keywords: Acidophilic chemolithotrophic bacteria, acidophilic heterotrophic bacteria, bioleahability, *Sulfobacillus thermosulfidooxidans* (chemolithotroph).

INTRODUCTION

Due to continuing worldwide depletion of metal ore deposits and accumulation of tailing, future sustainable development requires measures to economically use the nonrenewable raw materials and to reduce the demand for primary resources. Therefore, new resources for metals must be developed with the aid of novel technologies. In addition, improvement of already existing mining techniques can result in metal recovery from sources that have not been of economical interest. Metal-winning processes based on the activity of microorganisms offer a possibility to obtain metals from mineral resources not accessible by conventional techniques. Microbes such as bacteria and fungi convert metal compounds into their water-soluble forms and are biocatalysts of this process called as Microbial Leaching or Bioleaching [Brierley 1982].

For many years, *Acidithiobacillus ferrooxidans* (formerly called *Thiobacillus ferrooxidans)* [Kelly and Wood 2000] was considered to be the most important microorganism in the bioleaching of metals from ores [Bosecker *et al*. 1997]. The ability of *Acidithiobacillus ferrooxidans, Leptospirillm ferrooxidans* and other microorganisms to solubilize metal sulfides in their habitats is successfully applied in bioleaching of metals from ores. However, the relatively slow process kinetics is the major bottleneck in the widespread use of these microorganisms at commercial level. In this regard, the use of moderately thermophilic bacteria with their ability to operate at temperatures of 45-55°C has great potential for improving the kinetics of metal extraction from sulphide minerals. *Acidithiobacillus ferrooxidans* is a mesophilic chemolithotrophic prokaryote that obtains its energy from the oxidation of ferrous iron, elemental sulfur, or partially oxidized sulfur compounds [Kelly and Harrison 1989, Hiroyoshi *et al*. 1999, Rawlings 2002]. In contrast, *S. thermosulfidooxidans* is a moderately thermophilic bacterium having a highly versatile metabolism and may grow as autotroph, heterotroph, mixotroph, or chemolithotroph [Torma 1984, Toni and Johnson 1998]. In addition, several heterotrophs can also contribute to metal solubilization by the excretion of organic acids such as citrate, gluconate, oxalate, or succinate [Brandle *et al*. 2001].
In a recent study on bioleaching of the ore/concentrate, pure adapted, un-adapted and mixed adapted strains of bacteria were employed. It was observed that moderate thermophiles displayed superior kinetics of dissolution of common metals with the other two groups of bacteria [Sand *et al*. 2003].

The main objective of the present study was to evaluate moderately thermophilic bacteria *S. thermosulfidooxidans* for bioleaching of metals from high- ore and to apply mixed microbial consortia and metal adapted cells to achieving enhanced metal bioleaching rates from the above mentioned ore.

MATERIALS AND METHODS

ANALYSIS OF ORE

Ore used in these studies was obtained from PCSIR Laboratories, Lahore. Finely powdered sample (1.0 g each) of ore was refluxed with 100 ml of aqua regia in a round bottom flask for one hour. The solution was allowed to cool at room temperature and was filtered through Whatman No.42 filter paper. Fe from ore was determined by atomic absorption spectrophotometer (Varian AA 10/20) and the percentage of metal in the sample was calculated.

PREPARATION OF ORE SAMPLE FOR BIOLEACHING STUDIES

Statistically representative sample of the respective ore was taken and larger pieces were crushed in a jaw-crusher separately. Then all pieces were ground to a relatively smaller particles using disc-grinding machine. The final grinding of the ores was carried out using ring grinder (FRITSCH Pulverisette, Germany). In order to separate the particles according to their sizes, ASTM Sieves were used. Various mesh size fractions of ore were separated and the ore particles that range from 100-270 mesh (53-150 μm) were used in the experiments.

MICROORGANISMS USED IN BIOLEACHING EXPERIMENTS

Microorganisms used in this study were local isolates from NIBGE culture collection. These included two moderately thermophilic bacterial strains, *Sulfobacillus thermosulfidooxidans* (MT-13) isolated from tailing ponds of uranium mines in D. G. Khan [Ghauri *et al*. 2003] and an unidentified acidophilic heterotroph (Code: 6A1TSB) isolated from coal heap established for biodesulfurization at Askari Cement, Nizampur, Distt, Nowshera.

GROWTH MEDIA

Following liquid media was used to grow microorganisms involved in this study:

Medium for *Sulfobacillus thermosulfidooxidans*

The liquid medium for the growth of iron-oxidizers was based upon the composition described by Postgate [1956]. It comprised of a basal salt solution containing the basal salts (amounts per liter); $MgSO_4$. $7H_2O$ 0.50 g L^{-1}; $(NH_4)_2SO_4$ 0.15 g^{-1}; KCl 0.05 g^{-1}, KH_2PO_4 0.05 g^{-1}; and Ca $(NO_3)_2$ 0.01 g^{-1}. A stock solution (10X) was prepared and stored at room temperature after adjusting its pH 3.0 with 2.0 M H_2SO_4. For further use, the aliquots of basal salt solution were diluted 10 times, the pH was adjusted to 1.8-2.0 using 2.0 M H_2SO_4 and it was supplemented with Tryptic Soya Broth (TSB) at final concentration of 0.03%. In Fe-TSB medium, ferrous sulfate solution was used as an energy source for bacteria. A 1.0 M stock solution was prepared by dissolving 278 g of $FeSO_4$. $7H_2O$ per1.0 L of distilled water. The pH was immediately adjusted to 2.0 with sulfuric acid (2M) and solution was filter sterilized by passing the solution through 0.2 μm (Millipore) nitrocellulose membrane filter using sterilized Millipore filtration assembly under vacuum. Solution was stored in a pre-sterilized bottle and kept at 4°C. To prepare growth medium, the stock ferrous sulfate solution was added to 10 times diluted (1X) basal salt solution to give final ferrous iron concentration of 50 mM.

Growth Medium for Acidophilic Heterotrophs

This medium was prepared with the above-mentioned basal salt solution with addition of 0.03% TSB or yeast extract. Glucose solution, at a concentration of 1% (w/v) was added as an energy source. Before adding to autoclaved medium, glucose medium was steam sterilized.

Growth of Bacteria and Inoculums Preparation

The stock basal salt solution was diluted 10 times and 100 mL solution was taken in Erlenmeyer flasks of 250 mL. Tryptic soya broth (TSB) at final concentration of 0.03% was added in each flask in addition to 0.4% (w/v) finely ground (<0.045mm) pyrite as energy source. The pH of the medium was adjusted to 2.0 with sulfuric acid (2.0 M). All flasks were plugged with cotton and autoclaved at 121°C and 15 psi for 15 minutes. After cooling down to ambient temperature, each flask was inoculated with 1.0 mL liquid culture of *S. thermosulfidooxidans* under sterile conditions. The inoculated flasks were incubated in a shaker (Kuhner, Switzerland) at 180 rpm and temperature was adjusted at 45°C till turbidity appeared in the flasks indicating growth. Growth was further confirmed by viewing an aliquot of the culture under phase contrast- microscopic (Zeiss Axiovert model MC80).

After obtaining rich growth, the cell mass of cultures was harvested by using centrifuge (Beckman J2-HS), with JA-14 rotor at 10,000 rpm for 20 minutes at 4°C. The cell pellet of the culture was washed twice with autoclaved distilled water having pH adjusted at 2.5 with 2.0 M sulfuric acid. Finally the pellet was suspended in sterilized distilled water and preserved at 4°C for inoculation in the further experiments.

BIOLEACHING STUDIES

Bioleaching studies on ore were carried out in three different ways:

Leaching with Un-adapted Cells of *S. Thermosulfidooxidans*

For bioleaching of pyrite, two Erlenmeyer flasks of 250 mL capacity were taken separately, one was experimental and the other acted as control. Fe-TSB medium (100 mL) was added in each flask and pH was adjusted to 2.0 with 2.0M H_2SO_4. The aerobic condition of the system was maintained by plugging the flasks with cotton plugs. All flasks were autoclaved at 121 °C and 15 psi pressure for 15 minutes. Then 1.0 g of sterilized ore was added under aseptic conditions. The pH of each flask was monitored daily and added 2.0 M H_2SO_4 in order to obtain a stable pH of 2.0 in the successive days. After adjustment of initial acid demand and pH 2.0, the experimental flask was inoculated with 1.0 mL inoculum of unadapted cells of *Sulfbacillus thermosulfidooxidans* aseptically. The control flask was not inoculated, while other conditions were kept same as that of the experimental flask. All the flasks were incubated in shaker (Kuhner, Switzerland) at 45 °C temperature and 180 rpm shaking speed.

Leaching with Adapted Cells of *S. Thermosulfidooxidans*

Repeated sub-culturing in the medium containing gradually increasing concentrations of metal ions developed metal-adapted cells. Cells were considered metal ions adapted when their growth rate was similar to that of un-adapted cells .The cell yield of the adapted cells was approximately the same as that obtained for un-adapted cells grown in the absence of metal ions. (~0.019 mg/L. cell dry mass). Metal-adapted cells were used as inoculum in this experiment. Other procedure was same as described in the preceding section.

Leaching with Mixed Culture

A mixed consortium (1:1 v/v) containing cells of *S. thermosulfidooxidans* and acidophilic heterotrophs, adapted to metal ions, was used as inoculum in these experiments, while rest of the procedure is same as described above.

SAMPLING PROCEDURE

During the course of the leaching experiments, 3.0 mL of sample was collected from the shake flask periodically and immediately frozen in order to stop the bacterial activity. The samples were thawed and filtered through Whatman No. 1 filter paper to remove ore particles and then centrifuged at 10,000 rpm for 10 minutes in order to remove bacterial cells. After suitable dilutions, the resulting clear samples were then analyzed for zinc, lead and iron, by atomic absorption spectrophotometry. The volume in the flask was maintained by adding equal volume autoclaved distill water of pH 2. This way, at least 10 samples were collected at a 48 h time interval.

A 1.5 mL aliquot of each sample was taken in eppendorf tube and centrifuged at 10,000 rpm for 10 minutes. Then pellet was removed and 1mL of supernatant was taken in falcon tubes and 8.0 mL water and 1.0 mL concentrated HCl was added to obtain total volume of 10 ml. Samples were further diluted as required and analyzed on atomic absorption spectrophotometer for metals ion concentration.

PROTEIN ESTIMATION

Growth of the microorganisms during bioleaching was monitored by determining total protein in the culture flasks periodically by using bovine serum albumin as standard. Dry biomass was calculated from the total protein by multiplying it with a factor of 0.019.

RESULTS AND DISCUSSION

CHEMICAL ANALYSIS OF ORE

Chemical analysis of the ores and electronic scrap used in these studies was carried out to determine the concentrations of various metals present in these materials. Pyrite contained 42% iron.

BIOLEACHING OF PYRITE

Experiments were conducted on bioleaching of iron from pyrite using pure un-adapted and adapted cultures of *Sulfobacillus thermosulfidooxidans* as well as its consortium with acidophilic hetertrotroph (6A1TSB). Maximum pyrite leachability was exhibited by the mixed consortium, which leached out about 47% of iron from pyrite in 120 h @ 18.30 $mgL^{-1}h^{-1}$. Whereas, pure adapted culture leached out a maximum of 37% of iron from pyrite @ 12.20 $mgL^{-1}h^{-1}$, and the un-adapted culture solubilized about 29% iron @ 9.0 $mgL^{-1}h^{-1}$ (Fig. 1). It may be mentioned that only about 13% of iron was solubilized in 120 h in the sterile control flasks.

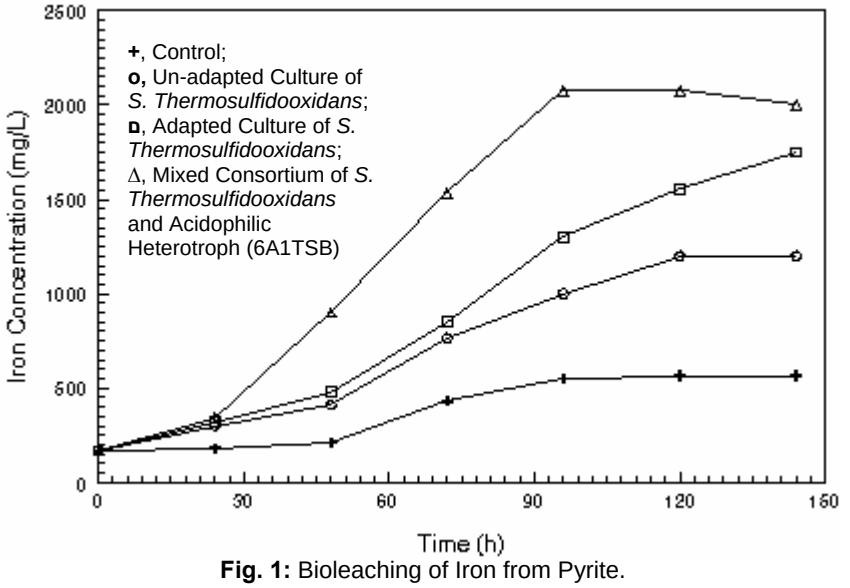

Fig. 1: Bioleaching of Iron from Pyrite.

DETERMINATION OF GROWTH

Growth of the microorganisms during bioleaching was determined by their dry biomass production rates. The biomass production followed the same pattern as shown in the bioleachability, with the mixed consortium having the highest biomass production rate of 0.021 $gL^{-1}h^{-1}$. In case of pure un-adapted and adapted cultures, biomass production rates were 0.017 $gL^{-1}h^{-1}$ and 0.019 $gL^{-1}h^{-1}$ respectively (Fig. 2).

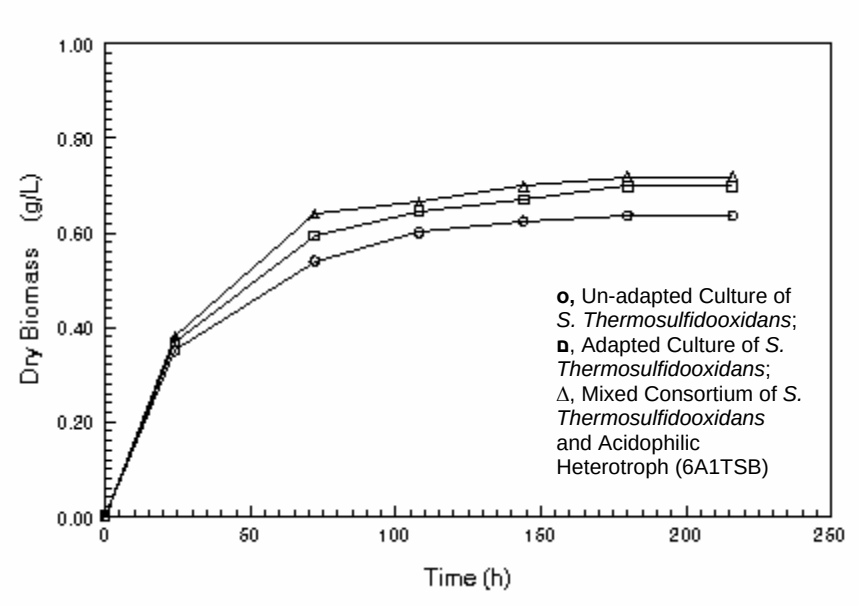

Fig. 2: Dry Biomass Production during Bioleaching of Pyrite.

CHANGES IN pH

Changes in pH were also observed and they followed the same pattern (Fig. 3).

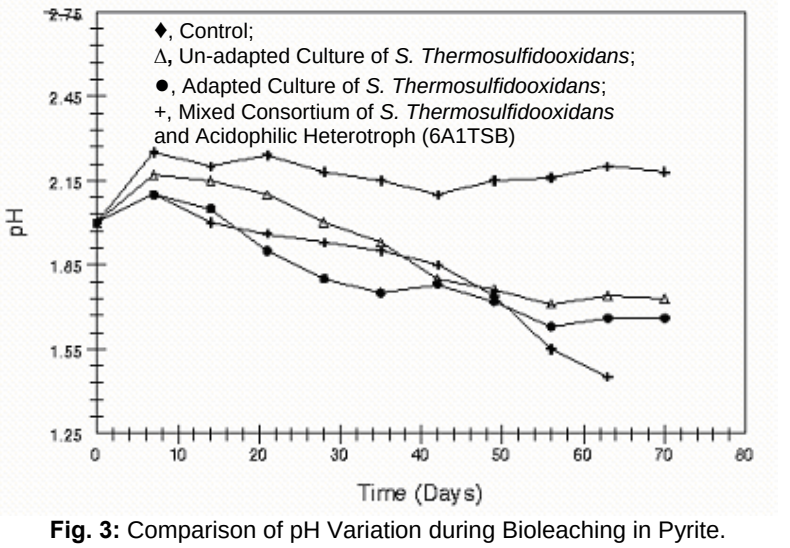

Fig. 3: Comparison of pH Variation during Bioleaching in Pyrite.

The present work was aimed at studying the bioleachability of iron metal from pyrite ore by the selected moderately thermophilic strains of acidophilic chemolithotrophic and acidophilic heterotrophic bacteria. These included *Sulfobacillus thermosulfidooxidans* (chemolithotroph) and an un-identified strain of acidophilic heterotroph (code 6A1TSB) isolated from local environments. A mixed consortium of the metal adapted cultures of the above-mentioned bacteria was found to exhibit the maximum metal leaching efficiency. It is likely that the acidophilic heterotrophs contribute to the stability of the mixed mineral-oxidizing population by consuming organic excretion products produced by the mineral oxidizers [Harrison 1984]. This group of organisms uses extra-cellular metabolites and cell lysates from autotrophs as carbon source resulting in the removal of an inhibitory excess of carbon and stimulating, therefore, growth and iron oxidation of chemolithotrophs [Butler and Kempton 1987, Acevedo *et al*. 1998, Fournier *et al.* 1998]. In addition, several heterotrophs can also contribute to metal solubilization by the excretion of organic acids such as citrate, gluconate, oxalate, or succinate. Biological oxidation of zinc sulfide and lead sulfide has been investigated separately high-grade ores or concentrate [Boon 1996, Groudev 1999, Roderguez *et al*. 2003, Sand *et al*. 2003].

In all the flasks where high metal leaching rates were observed, concomitantly biomass production rates were also high indicating high growth rates. It shows that the metal bioleaching capability of the bacteria was associated with their growth. It is due to the reason that from the oxidation of metals, chemolithotrophic bacteria obtain energy for their growth [Sand *et al*. 2003].

As compared to inoculated flasks, dissolution of metals (due to acid leaching) was significantly low in the un-inoculated control flasks in all the experiments. It showed that the bioleaching of metals is not merely an acid leaching process; rather it involves some enzymatic factors that enhance the metal leaching rates. This finding is not fully in agreement with the arguments that certain microorganisms are able to mobilize metals from solid materials (minerals, ores, wastes) by the formation of organic or inorganic acids (protons), by oxidation and reduction reactions; and by the excretion of complexing agents [Bosecker *et al*. 1997]. Some other investigators have shown that sulfuric acid is the main inorganic acid found in leaching environments [Rawlings 2002]. It is formed by sulfur-oxidizing microorganisms such as *Acidithiobacillus* species. A series of organic acids have also been reported to be produced by bacterial (as well as fungal) metabolism resulting in organic acidolysis, complex and chelate formation [Brandl *et al*. 2001].

A decrease in the bioleaching activity was observed at the late stages of bioleaching of metals from ore. It might be due to the reason that during bioleaching processes, co-precipitation of metals with mineral phases such as jarosites reduced the leaching efficiencies as reported by Hiroyoshi *et al.* [1999]. In addition, the precipitation of compounds present in the leachates on the minerals to be leached can make the solid material inaccessible for bacterial leaching.

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