Role of Acidothermophilic Autotrophs in Bioleaching of Mineral Sulphide Ores

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Living organisms synthesize a wide array of enzymes, which catalyze a myriad of reactions both inside and outside the cell. The acidothermophilic iron-oxidizing bacteria represent a group of obligately autotrophic chemolithotrophs. They include mesophilic *Thiobacillus thiooxidans*, *Leptospirillum ferrooxidans* and thermophilic *Sulfolobus* and *Acidianus* species. Several studies have shown the importance and feasibility of microbiological prospecting for sulphide ore deposits. Acidothermophilic autotrophic bacteria are now considered as an ideal source to exploit more unusual commercial applications of Geo-biotechnology, especially for metal and mining industry. The mining of copper, uranium, molybdenum, zinc, silver, gold, etc, from their sulphide ores is successfully possible with these microbes. The predominant characteristic of chemolithotrophs is their ability to survive and flourish in a completely inorganic aqueous environment with a supply of oxidizable substrate and CO$_2$.

A number of different species have now been isolated from high temperature regions and their potential for the rapid leaching of some ores. The ability of such isolates to tolerate high concentration of toxic heavy metals makes them excellent tools for accumulation and/or for biochemical transformation of metals. The outline of such applications are described in the present review.

Keywords: acidothermophiles, bioleaching, mineral sulphide ores, bio-oxidation, chemolithotrophs

Introduction

Microorganisms play a predominant role in the solubilization, transport and deposition of metals and minerals in the environment. A better understanding of these processes has allowed scientists to further characterize bacterial leaching of metals from ores and to propose innovative microbe based technologies for metal reclamation. Microbial technology presents an economic alternative for mining and waste water treatment at a time when high grade mineral resources are depleting, energy costs are increasing and adverse environmental effects are becoming more apparent as a result of few limitations of conventional technologies.

The recovery of metals from their ores has been an object of man’s activities for centuries. The role of bacteria in dissolving metallic sulphides from their ores has been known since Roman times. The practice of percolating acidified water through heaps of low grade ores to remove the metal sulphide formed by bacterial activity within the dump ore was carried out in Anglesey in the 16$^{th}$ century and in Spain in the 18$^{th}$ century. But this process, known as bacterially assisted leaching, has only been developed on a large-scale in this century principally in the USA, Chile and Romania for copper recovery. Extraction of uranium is currently being carried out using this technique in Canada. Further development of this technology may lead to the recovery of metals from underground regions inaccessible to man by conventional techniques and to the extraction of metals from the ash and slag left after the burning of coal containing significant amount of metallic ores.

The use of acidophilic, chemolithotrophic iron- and sulphur-oxidizing microbes in processes to recover metals from certain types of copper, uranium and gold-bearing minerals or mineral concentrates is now well established. During these processes insoluble metal sulphides are oxidized to soluble metal sulphates. Mineral decomposition is believed to be mostly due to chemical attack by ferric iron, with the main role of the microorganisms being to reoxidize the resultant ferrous iron back to ferric iron. Currently operating industrial biomining processes have used bacteria that grow optimally from ambient to 50°C, but thermophilic microbes have been isolated that have the potential to enable mineral biooxidation to be carried out at temperatures of 80°C or higher. The development of higher-temperature processes will extend the variety of minerals that can be commercially processed (Rawlings, 2002).
The interaction between microorganisms and minerals (or metals) is extremely varied but mainly microbiological processes related to three different types are of importance in the mineral biotechnology (Karavaiko & Groudev, 1985).

1. Oxidation of sulphide minerals, elemental sulphur, ferrous iron and some other metals in their reduced valency forms by chemolithotrophic bacteria.

2. Formation by heterotrophic microorganisms of organic (organic acids, polysaccharides, etc.) and inorganic (peroxides, etc.) compounds causing degradation of the mineral structures by solubilization of their compounds to the relevant ions or as complexes and chelates. In some cases, the degradation is connected with an enzymatic oxidation or reduction of individual chemical elements.

3. Formation by heterotrophic microorganisms and algae of large amounts of biomass or of some metabolites (mainly organic compounds but also some inorganic compounds such as hydrogen sulphides), which are capable of accumulating or precipitating metal ions from solutions.

The processes of the first type are the most specific and at the same time are largely used for the practical purposes. The need of bacterial leaching is felt in the ore beneficiation processes mainly due to the reasons such as:

1. The high-grade mineral resources are depleting continuously and new ores are not being located so frequently.
2. The conventional pyrometallurgical or other processes are becoming more and more costly.
3. The mining industries generally create a lot of pollution, which add to environmental imbalances (Brierley, 1982).

The principal benefits of bacterial leaching are low operating costs and mitigation of air pollution.

### Chemistry of the Leaching Processes

The role of *T. ferrooxidans* and thermophilic bacteria in leaching is complex and not precisely defined. Some researches supports the concept that their function may be “indirect”, whereby the microbes generate ferric iron, which oxidizes the mineral. Other investigations indicate “direct” leaching in which the microbes contact and adhere to the mineral surface, oxidizing the mineral without the use of ferric iron oxidant.

The fundamental reaction for indirect leaching is the microbial oxidation of ferrous iron (equation 1) in acidic condition for the purpose of energy generation:

\[
4\text{FeSO}_4 + \text{O}_2 + 2\text{H}_2\text{SO}_4 \rightarrow 2\text{Fe}_2(\text{SO}_4)_3 + 2\text{H}_2\text{O} \quad (1)
\]

The ferric sulphate thus generated serves to oxidize minerals such as chalcopyrite (equation 2), chalcocite (3), covellite (4), and uraninite (5):

\[
\begin{align*}
\text{CuFeS}_2 + 2\text{Fe}_2(\text{SO}_4)_3 & \rightarrow \text{CuSO}_4 + 5\text{FeSO}_4 + 2\text{S} \quad (2) \\
\text{Cu}_2\text{S} + 2\text{Fe}_2(\text{SO}_4)_3 & \rightarrow 2\text{CuSO}_4 + 4\text{FeSO}_4 + \text{S} \quad (3) \\
\text{CuS} + \text{Fe}_2(\text{SO}_4)_3 & \rightarrow \text{CuSO}_4 + 2\text{FeSO}_4 + \text{S} \quad (4) \\
\text{UO}_2 + \text{Fe}_2(\text{SO}_4)_3 & \rightarrow \text{UO}_2\text{SO}_4 + 2\text{FeSO}_4 \quad (5)
\end{align*}
\]

The resulting soluble metal sulphates are recovered by solvent extraction, ion exchange, or by other methods. The iron, now reduced to the ferrous state, is reoxidized by the microorganisms according to equation (1). The sulphur, which is often present as an end product of the metal solubilization (equations 2, 3 and 4) may also be oxidized to produce sulphuric acid.

The “direct” mechanism of metal leaching takes place without ferrous iron as an oxidant. Pyrite may be oxidized directly by the microbes as per equation (6).

\[
\text{FeS}_2 + \frac{3}{2}\text{O}_2 + \text{H}_2\text{O} \rightarrow \text{FeSO}_4 + \text{H}_2\text{SO}_4 \quad (6)
\]

This results in the solubilization of iron. The iron is subsequently oxidized according to equation 1 and the ferric iron then participates in the “indirect” leaching process. Copper-containing minerals can also be leached by the “direct” process (equations 7 and 8).

\[
\begin{align*}
2\text{CuFeS}_2 + 8\frac{1}{2}\text{O}_3 & \rightarrow 2\text{CuSO}_4 + \text{Fe}_2(\text{SO}_4)_3 + \text{H}_2\text{O} \quad (7) \\
2\text{Cu}_2\text{S} + 2\text{H}_2\text{SO}_4 + 5\text{O}_2 & \rightarrow 4\text{CuSO}_4 + 2\text{H}_2\text{O} \quad (8)
\end{align*}
\]

The “direct” leaching mechanism has not been conclusively demonstrated for iron-containing
minerals such as chalcopyrite (equation 7). Because solubilized iron facilitates the “indirect” mechanisms, even minerals without iron, such as chalcocite (equation 8) are oxidized in part by the “indirect” process. The presence of any iron as a contaminant will initiate an “indirect” leaching reaction.

Pyrrhotite ($\text{Fel}_x\text{S}$; or FeS in a simplified formula) is another common iron sulphide in sulphide mineralizations, but its chemical and microbiological oxidation is not well characterized. The oxidation of this mineral has been reported to be relatively faster than that of pyrite (Ahonen et al., 1986) and its microbiological oxidation at elevated temperatures has also been reported (Norris & Parrott, 1986), but strictly comparable experimental data relative to other sulphide minerals are not available. Pyrrhotite oxidation is an acid-demanding reaction that also produces major amounts of elemental sulphur as a by-product. Ferric iron can again act as a chemical oxidant and is regenerated via bacterial oxidation:

\[
\begin{align*}
2\text{FeS} + 4.5\text{SO}_2 + 2\text{H}^+ & \rightarrow 2\text{Fe}^{3+} + 2\text{SO}_4^{2-} + \text{H}_2\text{O} \\
2\text{FeS} + 1.5\text{O}_2 + 6\text{H}^+ & \rightarrow 2\text{Fe}^{3+} + 2\text{SO}_4^{0} + 3\text{H}_2\text{O} \\
\text{FeS} + 8\text{Fe}^{3+} + 4\text{H}_2\text{O} & \rightarrow 9\text{Fe}^{2+} + \text{SO}_4^{2-} + 8\text{H}^+
\end{align*}
\]

The microbiological leaching of chalcopyrite is a relatively slow reaction by comparison with biological leaching rates of secondary Cu-oxide and Cu-sulphide minerals. Complete oxidation of chalcopyrite can be represented by equation (7).

Iron plays a central catalytic role in the chemical and biological oxidation of mineral sulphide mine tailings and its oxidation state is the main factor that determines the prevailing redox potential. After iron gets oxidized to the ferric oxidation state, it tends to hydrolyze in solution and this reaction leads to a net increase in acid formation in the environment. The ferric hydroxide can further interact with various sulphates to form Fe(III) hydroxy-sulphate and oxyhydroxide complexes.

\[
\begin{align*}
2\text{Fe}^{3+} + 2\text{H}_2\text{O} & \rightarrow 2\text{FeOH}^{2+} + 2\text{H}^+ \\
2\text{FeOH}^{2+} + 2\text{H}_2\text{O} & \rightarrow 2\text{Fe(OH)}_2^{+} + 2\text{H}^+ \\
2\text{Fe(OH)}_2^{+} + 2\text{H}_2\text{O} & \rightarrow 2\text{Fe(OH)}_3 + 2\text{H}^+ \\
2\text{Fe}_2(\text{SO}_4)_3 + 6\text{H}_2\text{O} & \rightarrow 2\text{Fe(OH)}_3 + 3\text{H}_2\text{SO}_4
\end{align*}
\]

**Thermophiles in Leaching**

Beck (1967) observed temperature of 60 to 80°C in leach dump environments and concluded that one may have “to consider the role of microbial activity in leaching operation where these temperatures occur.” Murr and Briereley (1978) observed increase in temperature during copper leaching. In pyrite leaching, significant differences occurred with *T. ferrooxidans* and *A. brierleyi*; the thermophile extracted about 1.3 times more iron over an equivalent period of time (Ngubane & Baecker, 1988).

A wider temperature range can be very important for organisms in various ways, since it makes an organism more versatile with regard to changes in the environment (Wiegle, 1990). As thermophiles can function at elevated temperatures, the kinetics of biological and most of the chemical reactions are influenced favourably with increases in temperature. The presence and importance of thermophiles in commercial leaching operation has been described. Both these factors are important in leaching operations. Faster reaction rates reduce the time for which ores and waste must be processed to extract metal values. These thermophilic acidophiles possess the ability to bioleach over a wide range of temperatures. They are potentially well suited to industrial leaching applications where considerable temperature fluctuations limit the growth of other non-thermophilic bioleaching microorganisms. (Plumb et al., 2002).

**Sulfolobus and Related Organisms**

A microorganism that grows at high temperature and low pH was isolated first from an acid thermal region of Yellowstone National Park by Brierley in 1966 and further characterized by Brierley and Brierley in 1973. The genus *Sulfolobus* was first described by Brock et al (1972). These organisms grow in a temperature range of 50 to 80°C under acidic conditions. As these organisms oxidize sulphur as well as ferrous iron they become excellent candidates for use in microbial leaching. The isolation of *Sulfolobus* species from hot acid springs and soils around the world has been reported in the book “Thermophiles” (Brock, 1978).

Weiss (1974) speculated that survival of the organism in extreme environments may depend on the unique features of their cell envelopes. Several of the peptides of *Sulfolobus* are unique (Langworthy et al,
and only inositol-containing phospholipids are present. He proposed that thermophily is related to the long chain of isopranols, and acidophily is correlated with ether lipids. Millonig et al (1975) characterized two strains of acidiphilic thermophiles isolated by De Rosa et al (1975) from volcanic hot springs near Naples, Italy. Using the transmission electron microscope, these organisms and *Sulfolobus* (Brock et al, 1972; Brierley & Brierley, 1973) were compared and characterized. The presence of pili was noted in these organisms. Shivvers and Brock (1973) also reported on sulphur oxidation by *S. acidocaldarius*. De Rosa et al (1975) studied six microbial strains, designated MT, similar to *Sulfolobus* (Brock et al, 1972). These bacteria isolated from pools with a temperature range of 74 to 89°C and a pH of 1.4 to 2.6 were cultured in spring water amended with 0.1% yeast extract.

A number of strains of spherical bacteria, lacking cell walls and resembling *S. acidocaldarius* have been shown to be able to grow at temperatures up to about 80°C using sulphur and iron oxidation for energy. Such bacteria are able to leach recalcitrant minerals such as chalcopyrite and molybdenite at 60°C more efficiently than mesophilic bacteria (Brierley & Murr, 1973; Brierley, 1974, 1975, 1977, 1978; Brierley & Brierley, 1978). Zilling et al in 1980 established taxonomic relationship among several strains of *Sulfolobus* and proposed three species of *Sulfolobus*: *S. acidocaldarius*, *S. brierleyi* and *S. solfataricus*. The isolation of *Sulfolobus* from metal leaching environments was not reported until 1983. Marsh and Norris (1983) found these microbes in sample of a drainage channel (pH 1.5, 37°C) emanating from coal pile at the Birch Coppice Colliery, Warwickshire, UK.

The mechanisms for CO₂ assimilation by *S. brierleyi* were determined by Kandler and Stetter (1981). The autotrophic CO₂ fixation occurs via reductive carboxylic acid pathway. The Calvin-Benson cycle is apparently not present in *Sulfolobus* species. In the last decade, comparative microbial leaching studies by Marsh and Norris (1983), Kargi and Robinson (1985), Brierley and Brierley (1986), Norris and Parrot (1986) have shown that appreciably faster rates of leaching can be obtained with thermophilic *Sulfolobus* than *T. ferrooxidans* operating at 30 to 37°C.

In 1989, Gertrud et al, obtained and proposed three novel strains of spherical thermoacidophilic metal-mobilizing archaeabacteria from a Solfataric field in Italy. These new isolates grew aerobically on single sulphidic ores like pyrite, chalcopyrite and sphalerite and on combinations of them (ore mixture G6 and G1N). Growth was also obtained on the synthetic sulphides CdS, SnS, and ZnS and on S°. Arsenopyrite, bornite, cinabar, chalcocite, covellite or galena and the synthetic sulphides CuS, FeS, MoS₂ and Sb₂S₃ did not serve as substrates. During growth on S°, sulphuric acid was formed by the isolate TH-2. The presence of yeast extract (0.005%) did not change the production rate of sulphate significantly. The new isolates were able to grow on complex organic substrates such as beef extract, casamino acids, peptone, tryptone and yeast extract but growth was not obtained on sugars.

The effects of organic substances on growth and inorganic substrate oxidation by *Sulfobacillus thermosulfidooxidans* and *Asporogenes* species were studied by Vartayan et al (1990). A new genus-*Sulfothrichococcus*, thermoacidophilic archaebacteria, which oxidizing sulphur, ferrous iron and sulphide minerals have been reported by Karavaiko et al (1993). In the same year, Concetta and Teresa reported about chemolithotrophic, sulphur-oxidizing bacteria from a marine shallow hydrothermal vent of volcano (Italy). Fatty acid composition of the lipids in thermoacidophilic bacteria of the genus *Sulfobacillus* and carbon metabolism in *S. thermosulfidooxidans* strain 1269 has been reported by Zakhar蝙t et al (1994). Konishi et al (1995) studied bioleaching of pyrite by acidophilic, thermophilic, *Acidithiobacillus brierleyi*. They reported that the specific growth rate on pyrite for *A. brierleyi* was about four times more than that of the mesophilic *T. ferrooxidans*.

Another lob-shaped thermophilic isolate *Sulfolobus hakonensis* was obtained by Takayanagi et al (1996) from a geothermal area in Hakone, Japan. The isolate was found to be aerobic, facultative chemolithotrophic grew on S° and reduced sulphur compounds optimally at pH 3.0. In the same year, Williams et al reported a new strain of aerobic thermophilic bacteria from hot springs in Portugal and were identified as *Thermus oshimai*. A study on the enhanced stability of carboxypeptidase from *S. solfataricus* at high pressure was carried out by Bec et al (1996). In the same year, Wright et al did work on cloning of a potential cytochrome P450 from the *S. solfataricus*. Masullo et al also in the same year did work on purification and characterization of NADH oxidase from two species.
of *Sulfolobus*, *S. acidocaldarius* and *S. solfataricus*. A study was carried out by Ianniciello *et al* in 1996 on the expression of thermostable elongation factor I-α in *E. coli* from *S. solfataricus*. Immunochemical detection of ADP-ribosylating enzymes in this organism was also carried out (Faraone-Mennella *et al*, 1996). A study on a sec Y homologous gene in the crearchaeaean *S. acidocaldarius* was carried out by Kath and Schaefer (1996). Santin *et al* in the same year studied enzymatic synthesis of 2-β-D-galactopyranosyloxy-ethyl methacrylate (GaLEMA) by this thermophilic archaeon. Knapp *et al* (1996) did thermal unfolding of the DNA-binding proteins S97d from the same hyperthermophile.

**Rod-shaped Thermophilic Thio* bacillus *like Microbes**

The facultative, thermophilic, iron-oxidizing microbes catalyze important reactions for leaching certain metals from low-grade ores and mine wastes. Several reports have been published, which deal specifically with mineral leaching by facultative, thermophilic, iron-oxidizing microbes. Le Roux and Wakerely (1988) were the first to report leaching using facultative, thermophilic bacterium, TH-1, growing at 50°C were more effective than *T. ferrooxidans* in leaching nickel from volarite (Ni$_2$Fe$_3$S$_8$).

Observation of rod-shaped organisms, which grew at temperatures up to 55°C was made by Kaplan (1956), Schwartz and Schwartz (1965), Brierley (1966), and Schoen and Ehrlich (1968). In an ecological study of hot, acid soils, Fliermans and Brock (1972) observed the presence of *Thio* bacillus *at temperatures of 55°C*. In a study of bacterial survival at high temperatures and low pH, Weiss (1973) observed rod-shaped bacteria at concentration of 10$^7$ to 10$^8$ cells/ml in environment at pH 2 to 3 and at 75 to 80°C. Mosser *et al* (1974) noted that some hot springs in Yellowstone National Park contain thermophilic *Thio* bacilli. Bohlool (1975) observed rod-shaped bacteria in New Zealand hot springs ranging from 43 to 84°C. *Sulfolobus* and the rod-shaped bacteria coexist in some springs, but generally where both organisms are found, the rods predominate. Le Roux *et al* (1977) isolated several thermophilic, rod-shaped bacteria on ferrous iron and thiosulphate media from hot springs in South West Iceland where temperature ranged from 58 to 86°C and a pH of 4.1 to 8.9. However, the moderate, iron-oxidizing, thermophilic bacteria are recognizably larger than *T. ferrooxidans*; cell sizes range from 1.6 to 4.2 mm in length and about 1mm in diameter (Brierley, 1978). Research by Noguchi *et al* (1977) on the acidostability of *T. ferrooxidans* spheroplasts may be applicable to the stability of acidophilic thermophiles. Spheroplasts lack the peptidoglycan layer but remain acid stable.

*Thiobacillus* TH-2 isolated from a test leach facility required organic supplement for the growth. The same was found to be true for *Thiobacillus* TH-3 isolated from a copper leach dump. *T. thermosulfidooxidans* was found to be heterotrophic growing on sugars (Golovacheva & Kavavaiko, 1977). Marsh and Norris (1983) demonstrated chemolithoautotrophic growth of several strains of facultative thermophiles using ferrous iron and minerals sulphides. The data provide increasing evidence for the ability and potential for use of facultative thermophiles in mineral leaching.

Sugio *et al* (1985, 1987) carried out a systematic study of the sulphur-oxidizing systems of various *T. ferrooxidans* and *T. thiooxidans* strains, i.e. involving a large number of strains, three different substrates and three different activities. They obtained results which indicated the existence of variability among different strains not only in their ability to grow on certain substrates but also in their response to different substrate, by changing Fe$^{2+}$ and S$^0$ oxidative activities. This variability in adaptation was in agreement with the possible mechanism as described earlier by Holmes *et al* (1988). In 1996, Shooner *et al* obtained and proposed a novel species of facultatively autotrophic, moderately thermophilic bacterium, *T. thermosulfidatus* from sewage sludge samples enriched with elemental sulphur. This isolate showed production of various intermediates during growth on thiosulphate. These included tetrathionate, trithionate, and sulphate. Robertson *et al* (2002) isolated seven Fe$^{2+}$ oxidising acidophilic bacterial strains at 50°C from a pyritic coal from Collie, Australia and from a Fe$^{2+}$ oxidising fluidised bed reactor running at 60°C, which was originally inoculated with the Collie coal. The 16S rRNA gene of five of the strains was partially sequenced. The strains isolated from the reactor were closely related (99% similarity in gene sequence) to *Sulfobacillus thermosulfidooxidans* and the strains isolated directly from the coal had a 97% gene sequence similarity to *Sulfobacillus yellowstonensis*. *Thiobacillus* like acidothermophilic organisms could solubilize pyrrhotite in the presence
of 14 heavy metals at pH 2.5 and 60°C after their adaptation with all the toxic heavy metals in natural industrial soils (Umrania et al, 1998).

Factors Affecting Bioleaching

Following mineral sulphide dissociation, the chemical oxidation of the reduced valence state ionic species is greatly enhanced by the catalytic activity of the ubiquitous mixed communities of microorganisms. A major contributor to the microbial consortia is the chemosynthetic bacterium, *T. ferrooxidans*, the other sulphur- and iron oxidizing thermophilic bacteria include *Sulfolobus* species as well as Th-1, Th-2 and Th-3, *Acidianus brierley* (Brierley & Brierley, 1973, 1978; Le Roux et al, 1977). The degradation of these mineral materials is a dynamic process involving a succession of microbial populations, which develop according to the prevailing environmental conditions. They, in turn, are controlled by a complex array of physico-chemical conditions, which are generally site-specific.

Temperature Profile

There have been few attempts to measure heat profiles in mine tailings. Temperature distributions have been measured in waste rock dumps to evaluate heat source distributors as indicators of mineral sulphide oxidation sites. Harries and Ritchie (1983) showed that surface temperature changes affected temperature distributions in a waste rock dump down to a depth of approximately 6 m. Fluctuations in ambient temperature are more likely to affect airflow through waste materials thus influencing the oxygen level. The oxidation of pyrite, a common component of the minerological assemblages associated with mine tailings and waste materials, is an exothermic reaction (Harries & Ritchie, 1983). Indeed, temperature can increase up to 56 to 59°C where purely chemical reactions involving sulphides, copper bearing waste and copper ore have been studied (Murr & Berry, 1976; Murr & Brierley, 1978). Even higher temperatures in excess of 80°C have been observed in low-grade copper dumps in Bulgaria (Grudnov et al, 1978) and in the USA (Beck, 1967).

Thus, it is now known that groups of specialized thermophilic chemolithotrophic bacteria may contribute to the biological oxidation of tailings and wastes at significantly higher temperatures than those favoured by the mesophilic *Thiobacilli*.

As reported by Brierley and Le Roux (1977), ferrous iron oxidation occurred 50% at 30°C, 97% at 40°C and 94% at 50°C after five days of incubation. The apparent greater decrease of ferric iron at 50°C was attributed to its decreased solubility at the higher temperature, shorter incubation periods used at 55 and 60°C assuming that the rate of any biological oxidation would be greater.

Growth Factors

Brierley and Le Roux (1977) suggested the requirement of yeast extract by moderate thermophiles, which made it difficult to use these bacteria in metal leaching from cost point of view. But supplementing the media with partially reduced sulphur compounds (e.g. NiS, FeS, S2O3, etc.) supported the growth of microbes without addition of yeast extract. However, reduced sulphur source was not required by all moderate thermophiles. In the presence of yeast extract and Fe12+, TH-1 incorporated 1% carbon from CO2, while *T. ferrooxidans* derived 78% of its carbon from CO2, suggesting that moderate thermophiles do not fix CO2 (Brierley et al, 1978), which was proved wrong by incorporating 14CO2 and comparing CO2 fixation of TH-3 and *T. ferrooxidans*, which was almost same (Schacklett, 1983). May be, moderate thermophiles require higher concentration of CO2, as it was shown by him by increasing CO2 concentration from ~ 0.03% v/v to 10% v/v.

As mentioned earlier, thermophilic *Thiobacilli* TH-1 is an acidophilic chemolithotrophic heterotroph growing on media containing ferrous iron or pyrite when supplemented with yeast extract or glutathione (Brierley et al, 1978). Virtually, CO2 fixation is not taking place during growth on iron. The growth rate was maximum with 0.005 to 0.02% (w/v) yeast extract and it was reduced with lower or higher yeast extract concentration. Growth was completely inhibited by 0.1% (w/v) glucose in the presence of 0.02% (w/v) yeast extract. FeSO4 -grown or pyrite-grown inoculum of the thermophile were reported to grow rapidly at 50°C when transferred to elemental sulphur, copper sulphide, pyrite, chalcopyrite, or a copper concentrate when yeast extract was also provided. However, serial subculture of the organism on sulphur resulted in successively less growth with each transfer and eventual failure to grow.

A similar thermophile (Brierley & Lockwood, 1977) could grow rather slowly at pH 2.6-3.0 when medium employed with cysteine and cystine (100 mg/l) in the absence of yeast extract. For continued successful subculture in a cysteine supplemented Fe
medium, TH-3 appeared to require a further growth factor, which could be supplied by a trace amount (e.g. 0.5 mg/l) of yeast extract. At the lower yeast extract concentration only TH-3 was able to maintain the initially high rate of ferrous iron oxidation (Norris et al, 1978).

By itself, yeast extract will serve as a sole energy source for these organisms but in manometric studies, oxygen was not used by the bacteria when yeast extract was the only substrate. Most investigators have given this need for yeast extract only a superficial examination, but Shivvers and Brock (1973) reported that supplementing inorganic substrates with yeast extract has a complex effect on chemoaotrophic metabolism. They proposed that yeast extract affects both carbon assimilation and energy generation. Sulphur oxidation was greatly inhibited by yeast extract, but because of increased cellular production, the total sulphur oxidized was only reduced by approximately one third. Possibly, enzymes responsible for inorganic substrate oxidation were repressed and CO₂ assimilation was likewise suppressed.

The thermophilic Thiobacillus, isolated by Le Roux et al (1977) has been studied by Brierley and Le Roux (1977). Acidothermophilic Thiobacillus grew on ferrous iron at 30 to 50°C, but 0.02% yeast extract was required. Growth did not occur at higher temperatures. Oxygen uptake was not enhanced when the bacterium oxidized iron in the presence of yeast extract, and oxygen uptake increased with increasing iron concentration to 81 mM (4.9 g/l). The growth of acidothermophilic Thiobacillus on pyrite requires yeast extract, and growth was observed at 40 to 55°C; pyrite oxidation occurred from pH 1.1 to 2.6. Increasing pyrite concentrations enhanced oxygen uptake. The author measured growth on pyrite by pH decline, which infers oxidation of the sulphide moiety; iron dissolution was not measured, so it was not known whether the iron moiety was biogenically oxidized. When the organism was supplied with ferrous iron, pyrite, and yeast extract as substrates, ferrous iron was not entirely oxidized. Brierley and Le Roux (1977) suggested that this might be due to the microbe having a two-enzyme system; one for pyrite oxidation and the second for ferrous iron oxidation. The authors suggested that these systems function independently. Restriction profiles of chromosomal DNA were studied by Kondrat’eva et al (2002) in different Acidithiobacillus ferrooxidans strains grown on medium with Fe²⁺ and further adapted to another oxidation substrate (S⁰, FeS₂, or sulphide ore concentrates).

**pH Profile**

The oxidative dissolution of a sulphide mineral is commonly associated with an increasing acidification of the surrounding medium. This alone provides a heavy selection pressure on the development of the microbial succession since a range of pH conditions are encountered. Variations in pH also affect the development and viability of microbial populations through the availability of electron donors such as ferrous iron, the oxidation of which is sensitive to pH (Kelly & Tuovinen, 1988). The minerological composition of the tailings or waste material influences both mineral sulphide dissociation and bacterial establishment. Highly silicaceous or carbonaceous gangue associations consume acid; thereby displacing conditions beyond the pH range suitable to many leaching organisms. The chemical stability of iron and its compounds is very sensitive to conditions of pH and eH and will to a large degree determine the types of biological populations that develop. Iron oxidation is usually rapid and is sensitive to both pH and oxygen concentration, particularly at pH values above 3.5 (Ackman & Kleinmann, 1984). Thus, it could be said proved that the growth of autotrophic iron bacteria are largely dependent on the establishment of appropriate acidic environments.

**Agitation Profile**

The availability of oxygen in leach dump is undoubtedly one factor, which controls bacterial metal extraction. CO₂ solubility is low in acid solutions and therefore may be a limiting factor in growth of chemolithotrophic acidophilic bacteria (Brierley, 1978). These aerobic bacteria require adequate supply of oxygen, which can be achieved in the laboratory by aeration or shaking.

**Ore Characteristics**

The effect of particle size on leaching has been extensively studied for chalcopyrite and sphalerite. The size of the particles to be leached is critical. Torma et al (1970) found highest zinc extraction rate (17.6 mm/hr) with the finest particle size of sphalerite concentrate. If the total available surface area of the particle is increased, the rate of metal extraction is increased to a point. Pinches et al (1976) also
concluded that the most important factor affecting the extraction of copper from a concentrate was the size of mineral particles. They also discovered as did Torma (1976) that regrinding allows additional copper to be solubilized from chalcopyrite concentrate. They found that the rates increase linearly with decreasing particle size but it was less dependent when the particle size was very small. They were working with particle sizes ca. 4 to 20 μm. When the surface area was increased, either by decreasing the particle size or by increasing the solid concentration, each had similar effects on the leach rate. Therefore, surface area concentration is a real variable in leach rates. These findings correspond to the studies on sphalerite by Torma et al (1970) and on arsenopyrite concentrates by Pinches (1975). It was shown that as the extraction of metal increases exponentially, the growth of bacteria also increases exponentially. It was suggested that as the particle surface area decreases because of bacterial attachment and reaction products, leaching decreases. It was further suggested that particle-particle collision results in bacterial attrition and reduce the effective number of bacteria taking place in the reaction. These studies strongly indicate that mineral particle size and distribution influence the bacterial growth rate and hence leaching of metal sulphide mineral. Brierley (1977) reported greatest copper extraction from the smallest sieve sizes (−16+48 mesh; 1.00 mm −300 μm), of low grade porphyry ores with chalcopyrite. These results were similar to the results obtained by Ehrlich and Fox (1967) that the particles with greater surface areas leached faster. Examination of the leaching of chalcopyrite by Sulfolobus BC at increasing pulp densities was carried out by Le Roux and Wakerley (1988). They also found marked influence of pulp density on the rate of leaching. Maximum leaching rate of approximately 50 mg Cu/l/hr was attained at about 15% w/v pulp density and good bacterial growth occurred up to 25% w/v pulp density.

Considerable potentiality of Sulfolobus was also reported by Norris & Parrot (1986). A Sulfolobus strain has been shown to be very effective at solubilization of nickel from pyrrhotite and pentlandite concentrates. Soluble nickel reached about 0.7 g Ni/l with a medium initially containing 1% concentrate pulp density increased to about 10% pulp density. The Sulfolobus solubilized iron at a much greater rate than T. ferrooxidans. The effect of pulp density (w/v) on bioleaching culture capacity with respect to this copper concentrate was studied by Rubioa and García Frutos (2002). The results of the batch tests show that it is possible, operating at 10% of pulp density to attain copper extraction of 94% in 10 days and, at higher pulp densities (20%), to attain good copper extraction (80%) in only 14 days. In the same way, the culture has been amply tested with different chalcopyritic ores and copper concentrates.

**Bioleaching Activity on Natural Ores**

In bioleaching, chemical and minerological characteristics of an ore is important and hence must be established for each ore (Tuovinen, 1990). Bioleaching becomes a method of choice when CuS, FeS, PbS, ZnS, etc. occur together and are to be recovered quantitatively. Pyrite is the most abundant sulphide mineral and is associated with other sulphide minerals, such as those of copper, nickel and zinc or with uranium ores. Pyrite and arsenopyrite may also occur in the same mineralizations, as in precious-metal-containing sulphidic ore materials (Lawrence & Marchant, 1988).

The first successful leaching of molybdenum by a microorganism was accomplished using Sulfolobus (Brierley & Murr, 1973). The mineral molybdenite MoS₂ is particularly refractory to leaching. A second problem with leaching of molybdenite is toxicity of the resultant soluble molybdenum to the leaching organism. Although, mesophilic T. ferrooxidans is inhibited by 5 to 90 mg Mo/l (Tuovinen et al, 1971), Sulfolobus has a remarkable resistance to this metal. Molybdenite serves as an energy source for growth of Sulfolobus (Brierley, 1974) respiration, using sulphur as an energy source, occurred at 2000 mg Mo/l; cell growth was inhibited near a concentration of 750 mg Mo/l.

Sulfolobus was examined for possible extraction of copper from copper sulphide minerals. Preliminary studies (Brierley & Murr, 1973) indicated that the microbe could oxidize a chalcopyrite (CuFeS₂) concentrate with copper solubilization occurring at rate of 10 to 16 mg Cu/l/day over a thirty day period at 60°C. Another study of copper leaching (Wyckoff & Davidsons, 1977) suggested that microbes resembling Sulfolobus were more effective than "Sulphur bacteria" (presumably T. ferrooxidans) in leaching chalcopyrite.
In a more detailed study, *S. acidocaldarius* was used for copper leaching from porphyry copper ore with chalocopyrite as the primary mineral (Brierley, 1977). The ore sample, obtained from Duval Sierrita Corporation, AZ, possessed a mineralization of primarily chalocopyrite, with some digenite (Cu₉S₅) and covellite (CuS). The ore assayed 0.31% Cu, 0.05% Mo, 0.02% Zn, 5.7% Fe, 0.01% Pb, and 0.01% Ni. The amount of copper leached by *S. acidocaldarius* was 38% at an average rate of 21 mg/l/day; only 4% of the copper was leached from the control column at an average rate of 1.9 mg/l/day. Similar leach results obtained using a chalocopyrite ore obtained from the Pinto valley Mines, cities Service Corporation AZ by Brierley in 1980. *Sulfolobus* appears to be advantageous in extracting copper from the refractory mineral, chalcopyrite. *T. ferrooxidans* and *Sulfolobus* were compared for copper leaching from various copper containing minerals including chalcocite, covellite and chalcopyrite (Brierley et al. 1978). Marsh et al. (1983) made a comparative study of *Sulfolobus* species with regard to their ability to oxidize minerals. The rate and extent of mineral dissolution were found to be strain dependent. A study by Acevedo et al. (1983), involving the leaching of Chilean copper ores, suggested that the facultative thermophile TH-3 was the most effective microbe for solubilizing copper. Mineral concentrate dissolution at high temperatures was carried out by Norris and Parrot (1986).

Thermophilic microbial treatment of precious metal ores was carried out by Hutchins et al. (1988) and reported considerable improvement in the economics of gold recovery. Barrett et al. (1988) isolated and characterized a moderately thermophilic mixed culture of autotrophic bacteria and their application to the oxidation of refractory gold concentrates and reported their results in Perth International Gold Conference. Ngubane and Baecker (1988) also reported successful utilization of thermophilic pyrite leaching by *Sulfolobus brierleyi*. Lawrence and Marchant (1988) made the comparison of mesophilic and thermophilic oxidative systems for the treatment of refractory gold ores and concentrates. As reported by Brierley (1990) *Sulfolobus* and *Acidianus* species can be used for solubilization of copper, molybdenum and nickel as well as other metals, which occur in sulphide minerals (such as pyrite, arsenopyrite).

From an economic point of view, microbial metal extraction would become more attractive if the metal could not be easily extracted by conventional methods, or if these were too costly. Bioleaching recovery system for cobalt, nickel, molybdenum, gold, etc could be attractive because of the value of these metals. The main disadvantage of these processes is linked with their relatively slow leaching rates than conventional pyrometallurgical processes, because of the slow growth rates of microbes. It can be further optimized with faster growing and more specifically their metal solubilizing ability for maximum conversion of insoluble metal sulphides.

A simple membrane dialysis bioreactor was developed for a large-scale axenic culture of *Symbiobacterium thermophilum*, a symbiotic thermophile that requires co-cultivation with an associating thermophilic *Bacillus* strain S for normal growth (Ueda et al., 2002). A new type of microfiltration (MF) bioreactor has been developed for improving efficiency of the production of extremophilic enzymes (Schiraldi et al., 2001). In spite of the difficulties in cultivating hyperthermophiles, they achieved, in 300 hrs of fermentation, more than 38 g/l dry weight of *Sulfolobus solfataricus* using a MF technique, and demonstrated that the activity of alcohol dehydrogenase (ADH), as the reporter enzyme, was not affected by cell density. However hyperthermophile cultivation is difficult to scale up because of evaporation and the very low growth rate. Thus, to achieve high productivity they cultivated, in the MF bioreactor, recombinant mesophilic hosts engineered for the production of two thermophilic enzymes, namely, trehalosylsucrose-forming enzyme (*SsTDFE*) and trehalose-forming enzyme (*SsTFE*) from *Sulfolobus solfataricus*. An electric water heater has been modified by Worthington et al. (2003) for the large-scale cultivation of aerobic acidophilic hyperthermophiles to enable recovery of secreted proteins. Critical changes include thermostat replacement, redesign of the temperature control circuit, and removal of the cathodic anticorrosion system. These alterations provided accurate temperature and pH control. The bioreactor was used to cultivate selected strains of the archaeon *Sulfolobus solfataricus* and other species within this genus.

The published literature is also available on a few bacterial strains resembling *T. ferrooxidans* and possessing thermophilic, acidophilic or/and iron-oxidizing characteristics. They include *Sulfolobus* BC, *Sulfolobus solfataricus*, *S. acidocaldarius*, *S. hakonensis*, *Acidianus brierleyi*, TH-1, TH-2, TH-3.
**Metal Tolerance**

Metal tolerance among microorganisms is well known with several areas now receiving intensive attention at the molecular and genetical level. Various microbial mechanisms are implicated for survival in the presence of potentially toxic concentrations of metal species. A given organism often relies directly and/or indirectly on several survival strategies. Many of such organic and inorganic metal species can be accumulated by microbial cells as a result of physicochemical mechanisms and transport systems of varying specificity, independent of, or directly and indirectly dependent on metabolism. The pervasive nature of metals in the environment has resulted in the widespread appearance of metal resistance in microorganisms (Duncan et al, 1994).

Microbial metal resistance is heterogeneous in both their genetic and biochemical bases and may be chromosomally-, plasmid- or transposon encoded with one or more genes being involved (Duncan et al, 1994). At the biochemical level microorganisms demonstrate a diversity in the type of resistance mechanisms they have evolved. This includes six different fundamental types. These different mechanisms may occur singly or in various combinations to produce resistance. The five mechanisms generally proposed are illustrated as follows:

1. **Exclusion of the metal by a permeability barrier**
2. **Exclusion by active export of metal from the cell**
3. **Intracellular physical sequestration of the metal by binding proteins to prevent it from damaging metal sensitive cell materials**
4. **Extracellular sequestration**
5. **Extracellular detoxification where the metal is chemically modified to render it less active.**

In addition to the five general mechanisms the specific reduction in metal sensitivity of cellular targets for metal damage provides a sixth mechanism of resistance. The specific maintenance of a metal sensitive cell component may be achieved in the following four ways:

1. **By mutation altering the component to decrease its sensitivity, without unduly affecting its normal role**
2. **By increasing the amount of the affected cell component, if inactivation is not total**
3. **By repair of the component, in general only feasible for DNA**
4. **By bypassing it, either through utilizing a plasmid-encoded metal resistant form of the component to bypass the metal sensitive chromosomal component, analogous to the common mechanism of trimethoprim resistance (Amyes & Gemell, 1992), or through increasing activity in an alternative (shunt) pathway that is relatively metal-resistant.**

Metal toxicity could reduce industrial application of bioleaching processes (Norris & Kelly, 1978). A notable tolerance to heavy metals is found in common leaching bacterium, *T. ferrooxidans* (Ingledew, 1986; Boscecker, 1987) and this property is clearly exhibited during iron oxidation (Agate, 1982).
Bioleaching process is affected by the toxicity of metal like Ag, Co, Cu, Hg, Ni and Zn (Garcia & Da Silva, 1991). Metals like mercury and silver are very toxic to the bacteria even at low concentration (Mahapatra & Mishra, 1984; Garcia & Da Silva, 1991). According to Kazutumi et al (1975) silver ion had the most harmful effect on growth and the iron oxidizing activity of T. ferrooxidans even at $10^{-3}$ M concentration.

Silver is frequently associated with base metal sulphide such as Cu, Pb and Zn (Gupta & Ehrlich, 1989). Available literature reports inhibition of organism at Ag concentration as low as 0.005 mM (Norris & Kelly, 1978). Roy et al (1981) have also reported 0.1 ppm Ag concentration inhibitory for the growth of T. ferrooxidans culture. Tuovinen (1990) observed a lag phase longer than 3 weeks for the four strains of T. ferrooxidans tested by them.

T. ferrooxidans can tolerate Aluminium, 0.37 M; Zinc, 0.15 M; Cobalt, 0.17 M; Manganese, 0.18 M; Copper, 0.16 M; Chromium, 0.10 M; Uranium, 0.001 M; Mercury, 0.05 M; Silver $10^{-9}$ $M$-$10^{-3}$ M; and Molybdenum, 0.03 M; while oxides of Selenium, Tellurium and Arsenic are inhibitory (Brierley, 1978).

On one hand, a very few reports are available on the metal tolerance by moderate thermophilic chemolithotrophic bacteria and on the other hand, the importance of thermophiles in metal recovery processes at mine sites are getting more applicability. The heavy metal sensitivity restricts the application of thermoacidophiles in metal extraction processes. The ability of acidothermophilic bacteria to oxidize inorganic substrate makes them potential microbes for use in the leaching of metallic sulphides and reduce organic load.

The leaching of molybdenite (MoS$_2$) by Sulfolobus was first reported in 1973 by Brierley and Murr and further described thoroughly by Brierley in 1974. The leaching of molybdenite by the chemoautotrophic thiobacilli is limited because of their inability to tolerate high concentration of soluble molybdenum. But metal tolerant Sulfolobus can be developed which can tolerate 2000 ppm (=21 mM) hexavalent molybdenum, thus toxicity of Mo is not a problem with this Sulfolobus. A molybdenite concentrate is leached at a maximum rate of 6.6 mg Mo solubilized/l/day; this rate was maximized by supplementing the medium with 0.02% yeast extract and 1% iron (II) sulphate. The tolerance to high concentration of soluble molybdenum is unique to this organism. Several copper sulphide ores and concentrates were leached in stationary batch reactors using Sulfolobus. The first evaluation of the potential of A. brierleyi for bioleaching of metals indicated that this microbe could grow on the common copper sulphide minerals chalcocite and chalcopyrite. (Brierley & Brierley, 1978).

Further studies considered the use of extreme thermophiles for copper leaching. Sulfolobus BC developed tolerance for copper from a limit of about 3 g Cu/l to about 27 g Cu/l during progressive acclimatization (Le Roux & Wakerley, 1988). Comparison of Sulfolobus BC with T. ferrooxidans for copper leaching ability from the chalcopyrite concentrate demonstrated the thermophile to be much more effective. Copper leaching by the Sulfolobus occurred at an overall rate of about 11.5 mg Cu/l/hr with 83% copper extraction at the end of the test. The T. ferrooxidans leached the copper at a rate of 2.5 mg Cu/l/hr for 19% copper extraction (Le Roux & Wakerley, 1988).

Sulfolobus are not only utilized for molybdenite, chalcopyrite and chalcocite but also for precious heavy metal recovery. These thermophiles viz. Acidianus, Sulfolobus and thermoacidophilic eubacteria have been evaluated for microbial pretreatment of the ore to degrade the encapsulating sulphide matrix. This encapsulated sulphide matrix will facilitate contact between the cyanide and the precious metal (Hutchins et al, 1988; Barrett et al, 1988).

The influence of metallic ions on the activity of Sulfolobus BC has been examined (Mier et al, 1996). The maximum tolerance to increasing quantities of metal in cultures grown on copper concentrate was determined after a period of progressive adaptation. In case of silver, mercury, ruthenium and molybdenum, only adapted organisms led to an increase in the oxidation rate of ferrous iron and sulphur.

Thus, the use of thermophilic bacteria for microbial pretreatment of precious metal ores can offer economic advantages over common bioleaching processes in terms of both increased reaction rates and a lower requirement for cooling. Additional work is required to evaluate the relative merits of the different groups of leaching bacteria as applied to precious metal ores.

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