*ECB6: Proceedings of the 6th European Congress on Biotechnology*
edited by L. Alberghina, L. Frontali, P. Sensi

# *Thiobacillus ferrooxidans*, a versatile mineworker

P. Bos, R. Meulenberg, J.T. Pronk, W. Hazeu and J.G. Kuenen

Department of Microbiology and Enzymology, Kluyver Laboratory for Biotechnology, Faculty of Chemical Engineering and Material Sciences, Delft University of Technology, Julianalaan 67, 2628 BC Delft The Netherlands

## 1. INTRODUCTION

Microbial metal leaching is based on the ability of a special group of acidophilic bacteria to oxidize sulphidic minerals. They convert these extremely insoluble compounds into water-soluble products. Their activity results in metal mobilization and sulphuric acid production. Reaction equation (1) shows the oxidation of the most common sulphidic mineral: pyrite.

$$4FeS_2 + 15O_2 + 2H_2O \dashrightarrow 2Fe_2(SO_4)_3 + 2H_2SO_4 \qquad (1)$$

The natural habitats of these bacteria are sites where metal sulphide-containing deposits and pyrite-containing coal are exposed to molecular oxygen and humidity, and where, due to spontaneous oxidation of the sulphides the environmental pH has dropped to values below 4. The microflora includes obligately or facultatively chemolithotrophic bacteria which gain energy from the oxidation of ferrous iron and/or reduced sulphur compounds. The first species isolated from such areas were *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* [1]. Originally they were considered as the causative agents of acid mine drainage, a major environmental problem in mining areas. The microflora also contains a satelite population of acidophilic heterotrophs. These bacteria contribute significantly to the stability of the metal mobilizing microflora because they consume organic compounds which might inhibit the obligate autotrophs [2].

Since ancient times the activities of acidophilic metal- mobilizing bacteria have been exploited, unknowingly. For example the Romans recovered copper from the Rio Tinto (Spain). This river, fed by runoff water from exposed copper and iron containing deposits, is characterized by its low pH and comparatively high concentrations of copper and iron. Indeed, the name of the river refers to the typical discolouration of the water caused by ferric-iron containing precipitates (jarosites). Microbial metal leaching has also been used for copper and uranium recovery from low grade ores [3].

Since the discovery in the 1940's that bacterial activity is reponsible for microbial leaching processes, applied research has been focussed on the control of mine drainage water and improvement of microbial metal leaching processes. As this technology deals with low-value bulk materials, process installations should be as simple and as cheap as possible. Copper and uranium production are mainly achieved by leaching dumps and heaps.

There is a growing interest in the use of agitated tank reactors for leaching purposes. The environmental conditions in heaps and dumps are far from optimal, especially with respect to the required oxygen supply. Nowadays, tank leaching has become economically feasible for gold recovery processes from (arseno)pyrite concentrates which contain minute amounts of this precious metal [4]. Tank leaching had also been suggested in the microbial desulphurization of coal [5].

## 2. ACIDOPHILIC METAL MOBILIZING BACTERIA

Since the isolation of the obligate autotrophs *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* awareness has grown that these organisms are representatives of an extensive group of acidophilic metal-mobilizing bacteria. Table 1 [adapted from 6] shows some species which are believed to play a role in leaching. It includes obligate and facultative autotrophs

Table 1
Some acidophilic bacteria involved in bacterial leaching

| | energy source | | | | C-source | |
|---|---|---|---|---|---|---|
| | Fe | S-comp[1] | MeS[2] | org[3] | $CO_2$ | org |
| mesophiles: | | | | | | |
| *T. ferrooxidans* | + | + | + | +[4] | + | - |
| *T. thiooxidans* | - | + | -/+ | - | + | - |
| *T. acidophilus* | - | + | - | + | + | + |
| *T. cuprinus* | - | + | + | + | + | + |
| *T. prosperus* | ± | + | + | - | + | - |
| *Leptospirillum ferrooxidans* | + | - | + | - | + | - |
| *Acidiphylium cryptum* | - | - | - | + | - | + |
| moderate thermophiles: | | | | | | |
| *Sulfobacillus thermosulfidooxidans* | + | + | + | + | + | + |
| strain BC 13 | - | + | -/+ | + | + | + |
| strain TH1/BC1 | + | + | + | + | + | + |
| strain ALV | + | + | + | + | + | + |
| strain LM2 | + | + | - | + | + | + |
| strain TH3 | + | + | + | + | + | + |
| extreme thermophiles: | | | | | | |
| *Sulfolobus* BC | + | + | + | + | + | + |
| *Sulfolobus* B6-2 | - | + | ? | + | + | + |

[1]: elemental sulphur and/or tetrathionate; [2]: pyrite and/or chalcopyrite; [3]: organic C-compounds including yeast extract; [4]: formate (see Caption 4); +: growth, -: no growth, ±: weak growth, -/+: only growth in mixed cultures.

and a representative of the satelite population. As well as mesophiles (optimum temperature between 20 and 35 °C), there are also moderate thermophiles (optimum temperature between 40 and 55 °C) and even extreme thermophiles (optimum temperature above 60 °C).

The phylogenetic relationships among acidophilic thiobacilli are now becoming clearer, thanks to RNA analysis [7]. It appears that the group is very diverse, comprising eubacteria and archaebacteria. This is probably the result of convergent evolution in bacteria able to use reduced sulphur compounds, giving rise to wide variation in the metabolism of sulphur compounds among the bacteria listed.

In the literature on acidophilic thiobacilli incorrectly described strains can often be found. Designations such as "strain ALV" and "TH3" are frequent, especially in the range of facultative autotrophs. In most cases, the main problem in characterizing isolates is the limited number of substrates that can be used in assimilation tests, and the fastidious character of the organisms in traditional taxonomical procedures. Minute concentrations, especially of organic acids, often inhibit activity because of the large difference in pH outside (e.g. 2) and inside (e.g. 6.5) the cell. This sensitivity also explains problems experienced in isolating and purifying strains using traditional techniques with agar and other solidifying agents, as these contain low-molecular weight organic impurities.

The list of metal-mobilizing bacteria is still increasing. Among the new acidophilic thiobacilli that have recently been isolated and described are *Thiobacillus prosperus* [8] and *Thiobacillus cuprinus* [9]. *Thiobacillus prosperus* is halotolerant, contrasting with *Thiobacillus ferrooxidans* which is inhibited by low concentrations of chloride ions. *Thiobacillus cuprinus* has a relatively high pH optimum, and appears to favour chalcopyrite ($CuFeS_2$) over pyrite ($FeS_2$). More new species can be expected to be isolated, especially as improved isolation techniques are developed.

In Delft, a simple method which avoids the problems of solidifying agents has been developed [10]. This technique employs hydrophobic polycarbonate filters floating on a well-defined liquid mineral medium. Using this technique, the isolation of fastidious organisms that fail to grow on solidified media appears possible. By using floating filters, the dominant organism from a coal leaching operation run at elevated temperatures has been isolated [11]. The rod-shaped, moderately thermophilic organism had a temperature optimum of 45 °C and a pH optimum of 1.5. It appeared to be a facultative autotroph, able to grow heterotrophically on yeast extract.

There is a great deal of literature suggesting that at mesophilic temperatures *Thiobacillus ferrooxidans* is the most important species. However, recent reports indicate that the role of this well-known organism has been overestimated. In coal leaching studies, Muyzer et al. [12] observed that *Thiobacillus ferrooxidans* could not be detected in the natural enrichment culture used in their studies by using immuno-fluorescence techniques. Moreover, if *Thiobacillus ferrooxidans* was added to the non-sterilized coal slurry, the cell number didn't increase significantly during leaching experiments. There was only an increase in *Thiobacillus ferrooxidans* cell numbers if it was inoculated into a sterile coal slurry. The leaching results were comparable with those obtained with the wild community. *Thiobacillus ferrooxidans* is probably unable to compete effectively with the wild community. *Thiobacillus ferrooxidans* is relatively simple to isolate, compared with most acidophilic bacteria. It is favoured by the high ferrous iron concentration in the 9K medium [13]. Other acidophiles involved in metal-mobilization do not as well at high iron concentrations. However, it should be noted that this level of ferrous iron has no relevance to a normal metal leaching operation. Sand et al. [14] reported also in their leaching experiments with a low grade ore containing pyrite, chalcopyrite, galenite, and spheralite that *Thiobacillus ferrooxidans* was not the dominating organism at higher temperatures. They suggested that *Leptospirillum ferrooxidans* might be more important.

## 3. FOCAL POINTS OF SCIENTIFIC INTEREST IN ACIDOPHILIC THIOBACILLI

As *Thiobacillus ferrooxidans* is generally considered to be the most important acidophilic metal mobilizing bacterium, most studies on the physiology, enzymology, and genetics of acidophiles have concentrated on this organism. Reviewing the older literature reveals that fundamental research has mainly been focussed on the iron-oxidizing system.

The interest in ferrous iron oxidation is based on the long-standing assumption that the attack on sulphidic minerals is mainly effected by an indirect oxidation mechanism. The basic idea underlying this mechanism is that ferric iron, produced by biological activity, serves as the chemical oxidizer of the metal sulphides. Several authors considered the biological ferrous iron oxidation as the rate-limiting step in microbial leaching. In their view, a better understanding of biological ferrous-iron oxidation could open the way to improving leaching rates. Details about the complex ferrous iron oxidizing system can be found in the review of Ehrlich et al. [15]. However, it should be stressed that the rate-limiting step in mineral sulphide oxidation has not yet been identified. Holmes [16] suggested that various other factors might be rate limiting, including biological sulphur oxidation or the capacity to oxidize non-ferrous metals. A reduced sensitivity towards organic compounds could improve the effectiveness of the organism. The same might hold for better attachment properties, tolerance to surfactants and chloride ions, resistance to bacteriophage infections and, last but not least, increased metal tolerance.

It has long been realized that the use of *Thiobacillus ferrooxidans* in leaching operations is in most cases, limited by its metal tolerance. *Thiobacillus ferrooxidans* and related organisms can withstand concentrations of heavy metals that are two to three orders of magnitude higher than neutrophilic bacteria (for copper, cobalt, manganese, nickel, and zinc several grams per liter [17]. Not all heavy metals can be tolerated at these high concentrations. Molybdenum and silver are inhibitory in concentrations in the order of magnitude of milligrams per liter. A further improvement of the metal tolerance would expand the range of applications for these organisms to ores with high metal concentrations, and would allow the production of leachates with higher, and thus economically more interesting, metal concentrations.

The genes encoding for metal tolerance are located on plasmids. Genetic studies on *Thiobacillus ferrooxidans* have therefore been started with the plasmids encoding for metal tolerance. Genetic engineering of an acidophile such as *Thiobacillus ferrooxidans* appears to be difficult. Although, it is possible to transfer genetic information into neutrophilic organisms such as *Escherichia coli*, efforts to return the genetic information to *Thiobacillus ferrooxidans* appeared, until recently, to be impossible. However, Kusano et al. [18] recently demonstrated electrotransformation of a plasmid containing a mercury resistance gene into a strain of *Thiobacillus ferrooxidans*. The transformation efficiency was low, and the mercury tolerance was only increased slightly. Nevertheless, in the near future a breakthrough in the area of genetic engineering of *Thiobacillus ferrooxidans* can be expected.

## 4. *THIOBACILLUS FERROOXIDANS*: A VERSATILE ORGANISM

As early as 1980 Arkesteyn [19] stressed that the sulphur oxidizing capacity of *Thiobacillus ferrooxidans* was involved in the oxidation of pyrite. These findings have been supported by the results of Hazeu et al. [20] which showed a higher yield per mole electron on reduced sulphur compounds and on pyrite than on ferrous iron. If only an indirect oxidation mechanism was important, approximately equal cell yields per mole electron from ferrous iron and pyrite should be obtained. It is likely that electrons from reduced sulphur compounds enter the respiratory chain at an energetically more favourable point than those from ferrous iron. With respect to the practical application of acidophilic metal-mobilizing bacteria, it therefore makes sense to investigate the sulphur-oxidizing system of *Thiobacillus ferrooxidans*. Comparatively little has been done in this area, but a start has been made at Delft University of Technology . The major problem in studying the enzymes of *Thiobacillus ferrooxidans* is the low cell yield on inorganic substrates. Normally, continuous culture cultivation results in biomass concentrations of less than 100 mg dry weight per litre. It was therefore decided some years ago to use another acidophilic *Thiobacillus* which can grow mixotrophically on a mixture of organic compounds and reduced sulphur compounds: *Thiobacillus acidophilus*. By using continuous culture techniques production of enough biomass for the enzyme studies was possible. Although this organism is believed to be unimportant in leaching, its sulphur-oxidizing system appeared to be quite similar to that of *Thiobacillus ferrooxidans*. The research has resulted in a tentative scheme for the pathways of reduced sulphur compound oxidation by *Thiobacillus acidophilus* (Figure 1) [21]. Some

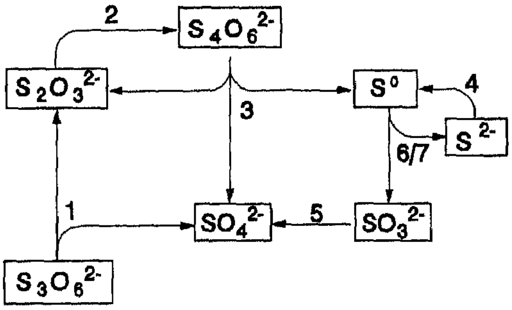

Figure 1: Hypothetic scheme for sulfur-compound oxidation in only 6 steps in acidophilic thiobacilli, assuming the presence of a sulfur oxygenase reductase. 1: trithionate hydrolase, 2: thiosulfate dehydrogenase, 3: tetrathionate hydrolase, 4: not yet characterized, 5: sulfite oxidizing enzyme, 6/7: sulfur oxygenase reductase.

enzyme steps have been studied in detail. Trithionate hydrolase and thiosulphate oxidoreductase have been purified and characterized. These two enzymes have pH optima at values around 3, suggesting a location outside the plasma-membrane, and probably in the periplasmic space. One of the enzymes, trithionate hydrolase appears to be exceptionally temperature tolerant. Activity loss was negligible if the preparation was kept for 4 hours at 70 °C.

The sulphide oxidizing system, which is more relevant to the oxidation of mineral sulphides, must now be studied and enzymological studies should be extended to *Thiobacillus ferrooxidans*. In the meanwhile, it has been found that this organism can gain energy from the oxidation of formate. It was even proved possible to grow *Thiobacillus ferrooxidans* in a chemostat on formate, resulting in high cell density cultures [22]. The formate only serves as the energy source and the biomass is built up from carbon dioxide fixation. Biomass concentrations of about 700 mg/l could be obtained in continuous cultures. Cells maintained their iron- and sulphide oxidizing capacity (see Table 2). Apart from its use in enzymological studies, this finding might also have a practical application as it will allow the bulk inoculation of tank leaching operations. This might be of special interest in those cases where special strains of *Thiobacillus ferrooxidans* are used, such as in the selective removal of pyrite and arsenopyrite from gold-bearing ores. Leaching rates in such processes often suffer from biomass limitation in the reactors.

Table 2
Oxidation of various substrates by formate- and ferrous iron-grown *Thiobacillus ferrooxidans*.

| substrate | conc. (mM) | oxygen uptake rate (nmol/min.mg) | |
|---|---|---|---|
| | | Fe-grown cells | formate-grown cells |
| formate | 0.1 | 55 | 53 |
| $FeSO_4$ | 4.5 | 630 | 750 |
| $Na_2S$ | 0.1 | 46 | 67 |

Cell suspensions from formate-limited chemostat cultures were used directly after sampling. Cells from ferrous iron-grown batch cultures were harvested by filtration, washed twice, and resuspended in mineral medium (pH 1.8). Oxygen uptake rates were measured with a Clark-type electrode at 30 °C.

From the foregoing it is clear that *Thiobacillus ferrooxidans* cannot correctly be regarded as an obligately chemolithoautotrophic organism. Recently it has also been demonstrated that *Thiobacillus ferrooxidans* is able to use molecular hydrogen as its energy source [23]. In the traditional description, *Thiobacillus ferrooxidans* is also described as an obligately aerobic organism. However, *Thiobacillus ferrooxidans* can oxidize elemental sulphur by using ferric iron as the terminal electron acceptor [24]. Recently, it has been shown that this reaction will provide useful energy. Under anaerobic conditions, the active transport of radio-labelled amino acids has been observed in the presence of ferric iron and elemental sulphur or formate [25]. Even anaerobic growth is possible on elemental sulphur [26] in the presence of ferric iron. That *Thiobacillus ferrooxidans* can also thrive under anaerobic conditions has important consequences for the role of this organism in the natural environment. One of the basic ideas to prevent acid mine drainage has always been to stop the activity of the organisms by excluding oxygen. The facultatively anaerobic nature of *Thiobacillus ferrooxidans* (and other acidophilic thiobacilli?) might explain why this has often not been successful.

## 5. CONCLUDING REMARKS

Considering *Thiobacillus ferrooxidans* as the best studied representative of acidophilic metal-mobilizing bacteria, one might speculate that other acidophiles might also be more versatile. Investigations on the physiology, enzymology and genetics of these bacteria might result in an understanding of the mechanisms of sulphidic mineral attack. These studies could result in new concepts applicable in biohydrometallurgy which in their turn could improve the economics of microbial metal leaching of high grade ores which are often hampered by the slow kinetics.

## ACKNOWLEDGEMENTS

For critical reading and correction of the English text, dr. Lesley Robertson is gratefully acknowledged.

## REFERENCES

1. Colmer, A.R. and M.E. Hinkle, Science 106 (1947) 253.
2. Harrison, A.P., Annu. Rev. Microbiol. 38 (1984) 265.
3. Ehrlich, H. and Brierley, C.L. Microbial mineral recovery, McGraw-Hill Publ. Cie, New York, 1990.
4. Lawrence, R,W., pp. 127-148, in: Ehrlich, H. and Brierley, C.L. Microbial mineral recovery, McGraw-Hill Publ. Cie, New York, 1990.
5. Bos, P., Boogerd, F.C. and Kuenen, J.G., pp. 375-403, in: Mitchell, R. (ed.), Environmental microbiology, Wiley-Liss, New York, 1992.
6. Norris, P.R., pp. 3-27, in: Ehrlich, H. and Brierley, C.L. Microbial mineral recovery, McGraw-Hill Publ. Cie, New York, 1990.
7. Robertson, L.A. and Kuenen J.G., pp. 385-413, in: Balows, A., Trüper, H.G., Dworkin, M., Harder, W. and Schleifer, K.-H. (eds.), The prokaryotes, 2nd edition, Springer Verlag, New York, 1992.
8. Huber, H. and Stetter, K.O., Arch. Microbiol. 151 (1989) 479.
9. Huber, H. and Stetter, K.O., Appl. Environm. Microbiol. 56 (1990) 315.
10. Bruyn, J.C. de, Boogerd, F.C., Bos, P. and Kuenen, J.G., Appl. Environm. Microbiol. 56 (1990) 2891.
11. Boogerd, F.C., Alphen, M.M.Q. van, Anrooij, W.J. van, Bruyn, J.C. de, Bos, P. and Kuenen, J.G., pp. 735-751, in: Salley, J., McGready, R.G.L. and Wichlacz P.L. (eds.), Proc. Intern. Symp. Biohydrometallurgy, Canmet, Ottawa (Ont), 1989.
12. Muyzer, G., Bruyn, A.C. de, Schmedding, D.J.M., Bos, P., Westbroek, P. and Kuenen, J.G., Appl. Environ. Microbiol. 53 (1987) 660.

13. Silverman, M.P. and Lundgren, D.G., J. Bacteriol. 77 (1959) 642.
14. Sand, W., Rohde, K., Sobotke, B. and Zenneck, C., Appl. Environm. Microbiol. 58 (1992) 82.
15. Ehrlich, H.L., Ingledew, W.J. and Salerno, J.C., pp. 147-170, in: Shively, J.M., and Barton, L.L., Variations in autotrophic life, Academic Press, London, 1991.
16. Holmes, D.S., pp. 29-54, in: Ehrlich, H. and Brierley, C.L. Microbial mineral recovery, McGraw-Hill Publ. Cie, New York, 1990.
17. Tuovinen, O.H., pp. 55-77, in: Ehrlich, H. and Brierley, C.L. Microbial mineral recovery, McGraw-Hill Publ. Cie, New York, 1990.
18. Kusano, T., Sugawara, K., Inoue, C., Takeshima, T., Numata, M., and Shiratori, T., J. Bacteriol. 174 (1992) 6617
19. Arkesteyn, G.J.M.W., Thesis Wageningen, 1980.
20 Hazeu, W., Schmedding, D.J., Goddijn, O., Bos, P. and Kuenen, J.G. pp. 497-499, in: Neijssel, O.M., Meer, R.R. van der, and Luyben, K.Ch.A.M., (eds.), Proc. ECB IV, Vol. 3 pp., 497-499, Elsevier, Amsterdam, 1987.
21. Kuenen J.G., Pronk, J.T., Hazeu, W., Meulenberg, R. and Bos, P., 1993. Proc.Intern. Symp. Biohydrometallurgy, Jackson Hole, 1993 (in press).
22. Pronk, J.T., Meijer, W.M., Hazeu, W., van Dijken, J.P., Bos, P. and Kuenen, J.G., Appl. Environm. Microbiol. 57 (1991) 2057.
23. Drobner, E., Huber E., and Stetter, K.O. Appl. Environm. Microbiol. 56 (1990) 2922.
24. Brock, T.D. and Gustafson, J., Appl. Environ. Microbiol. 32 (1976) 567.
25. J.T. Pronk, J.T., Liem, K., Bos, P. and Kuenen, J.G., Appl. Environm. Microbiol. 57 (1991) 2063.
26. Pronk, J.T., de Bruyn, J.C., Bos, P. and J.G. Kuenen, Appl. Environm. Microbiol. 58 (1992) 2227.