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Ferrous sulphate oxidation using *Thiobacillus ferrooxidans* cells immobilised on sand for the purpose of treating acid mine-drainage

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Abstract *Thiobacillus ferrooxidans* was immobilised on sand (size 0.85 mm to 1.18 mm) for use in a repeated batch and continuously operated packed-bed bioreactor which has not been previously reported in the literature. Repeated batch operation resulted in the complete oxidation of ferrous to ferric iron. The bacteria were active immediately after 3–4 weeks in a non-aqueous medium; i.e. the sand was allowed to dry out, demonstrating the stability of the system. A lag phase of 28 days was recorded when the sand was stored dried in a sealed container for 16 weeks compared with a lag phase of 13 days for a sample frozen for 18 weeks. After a period of 10 days, continuous operation of the reactor at a dilution rate of 0.64 h^{-1} resulted in 95–99% oxidation of ferrous iron or $0.31\text{--}0.33\text{ kg m}^{-3}\text{ h}^{-1}$. With the use of a scanning electron microscope, images were recorded of *Thiobacillus ferrooxidans* on sand.

Introduction

Thiobacillus ferrooxidans is a chemolithotrophic bacterium found naturally in acid mine-drainage water from abandoned coal mines (Jensen and Webb 1995). The impacts of untreated mine drainage on aquatic communities can be severe; ochreous precipitates smother stream beds, suffocating benthic species, plants and fish eggs, and the increase in acidity asphyxiates fresh-water fish such as trout and salmon (Pentreath 1994). *Thiobacillus ferrooxidans* catalyses the oxidation of ferrous (Fe^{2+}) ions to ferric (Fe^{3+}) ions, a reaction that facilitates the formation of ochreous precipitates present in polluted mine drainage (Eq. 1). Nonetheless, research has illustrat-

ed the potential of these bacteria and the bio-oxidation of iron in removing iron precipitates from polluted water (Nemati et al. 1998):



The conventional treatment of mine water involves the addition of lime to neutralise the acid, ferric sulphates precipitate simultaneously with the change in pH, a more acceptable neutralising agent would be calcium carbonate (Imaizumi 1986). Unfortunately, calcium carbonate cannot be used because it produces carbonic acid during neutralisation, which limits the maximum pH to about 6.5 (Murayama et al. 1987). This pH prevents the soluble ferrous ions from oxidising to ferric ions and hence the precipitation of iron from mine drainage. However, *Thiobacillus ferrooxidans* can oxidise ferrous ions to ferric ions between pH 1.0 and 4.5 (Jensen and Webb 1995), permitting the use of calcium carbonate as the neutralising agent.

The many potential applications of ferrous bio-oxidation, e.g. removal of H_2S from sour gases (Imaizumi 1986; Satoh et al. 1988; Halfmeier et al. 1993a, b; Pagella et al. 1996), biohydrometallurgy (Brierley and Brierley 1997), bioleaching (Kai et al. 2000), and treatment of acid mine-drainage (Murayama et al. 1987), and *Thiobacillus ferrooxidans*' natural tendency to adhere to surfaces (MacDonald and Clark 1970) have led to numerous immobilisation studies (Murayama et al. 1987, diatomaceous earth; Grishin and Tuovinen 1988; glass beads, ion-exchange resin and activated carbon; Armentia and Webb 1992, polyurethane foam; Halfmeier et al. 1993b, ceramic supports, activated carbon, porous glass rings and quartz sand; Wakao et al. 1994, calcium alginate, κ -carrageenan and gelrite).

A cheap, readily available support matrix such as sand would be ideal in a treatment system for acid mine-drainage, where pollution from abandoned mines is a problem for water authorities. Halfmeier et al. (1993b) used quartz sand (0.2–0.3 mm) in a three-phase fluidised bed, air-lift reactor in an unsuccessful attempt to immobilise *Thiobacillus ferrooxidans*. It is likely that the high

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shear forces within the reactor inhibited bacterial growth and not the sand itself, although the authors also reported no growth in controlled experiments with static cultures. In the following work, *Thiobacillus ferrooxidans* cells have been successfully immobilised on sand (size 0.85 mm to 1.18 mm) using shake flasks before being transferred to a glass column that operated as a packed-bed bioreactor. The immobilisation of *Thiobacillus ferrooxidans* on sand and their use in a packed bed for the oxidation of ferrous sulphate have not been previously reported in the literature.

Materials and methods

Strain and growth conditions

The strain of *Thiobacillus ferrooxidans* used in the study was a freeze-dried culture, NCIMB 9490 (originally isolated from Britannia Mine water, British Columbia), obtained from The National Collections of Industrial and Marine Bacteria, Aberdeen. For the cultivation of *Thiobacillus ferrooxidans*, the 9K media developed by Silverman and Lundgren (1959) with a reduced iron content (4 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}/\text{l}$) was utilised; the pH was adjusted to 2 with concentrated H_2SO_4 . *Thiobacillus ferrooxidans* cultures were shaken at 150 rpm on an orbital shaker at room temperature (19–22 °C). Subcultures were carried out every 4–5 days to keep the bacteria in the logarithmic phase of growth. The presence of *Thiobacillus ferrooxidans* was considered positive when the media changed in colour from pale green to orange/red due to the oxidation of the green ferrous ions to the yellow/orange ferric ions.

Immobilisation procedure

The following procedure was adapted from the immobilisation procedure used by Nemati and Webb (1996) to immobilise *Thiobacillus ferrooxidans* in polyurethane-foam biomass support particles. Twenty-five grams of quartz sand was washed with 0.1 M H_2SO_4 and added to a conical flask containing 100 ml media and 1 ml of *Thiobacillus ferrooxidans* culture in the logarithmic phase of growth. Control flasks contained 25 g quartz sand washed with 0.1 M H_2SO_4 and 100 ml uninoculated media. The flasks were placed on an orbital shaker at 100 rpm for 4–5 days at room temperature (19–22 °C) to allow almost complete oxidation of ferrous iron. The sand was rinsed several times with distilled water before being transferred to a clean flask with 100 ml of fresh media. No further inoculation with *Thiobacillus ferrooxidans* was required after the initial set-up, as growth was confirmed by a colour change within the media and by microscopic examination. This procedure was repeated a further four times, in accordance with the method by Nemati and Webb (1996), to ensure adequate build-up of a *Thiobacillus ferrooxidans* biofilm.

Evaluating the number of *Thiobacillus ferrooxidans* cells adsorbed on 1 g 0.85 mm–1.18 mm quartz sand

A culture in the stationary phase of growth was used for this experiment. One g of dry quartz sand washed with 0.1 M H_2SO_4 and rinsed with distilled water was added to a 50-ml Pyrex beaker containing 10 ml of *Thiobacillus ferrooxidans* culture with a known concentration of cells. A second 50-ml Pyrex beaker was set up containing 10 ml of the same culture and no sand. Each beaker was set up in triplicate. The number of *Thiobacillus ferrooxidans* cells in the liquid medium was counted daily using a Neubauer Improved Cell Counter of 0.1 mm depth and 1/400 mm² area; the cells were counted until the log of the number of cells varied less

than 5%. As the culture was in the stationary phase of growth, the number of cells immobilised in the Pyrex beaker and sand could be evaluated.

Storage of immobilised *Thiobacillus ferrooxidans* cells

Two methods of storing *Thiobacillus ferrooxidans* cells immobilised on quartz sand were tested: (1) 25 g of 0.85–1.18-mm sand with *Thiobacillus* on its surface and 25 g of control sand were washed with 0.1 M H_2SO_4 and rinsed with distilled water. The samples were dried in the open air and stored in a sealed plastic container for 16 weeks. (2) Twenty-five g of 135–180- μm sand with *Thiobacillus* on its surface and 25 g control sand were washed with 0.1 M H_2SO_4 and rinsed with distilled water. The samples were removed from culture flasks containing 9K medium and drained. The wet sand was then frozen for 18 weeks. At the end of the storage period, the sand samples were placed in 100 ml 9K medium and shaken on an orbital shaker at 100 rpm at room temperature (19–22 °C) for 28 days.

Iron analysis

Ferrous iron and total iron were measured using a Hach DR/2000 direct reading spectrophotometer. Ferrous iron powder-pillows containing 1,10 phenanthroline and FerroZine reagent solution pillows were used to treat the samples using the method described by Hach.

Mini-reactor experiment

Inoculated sand and acid-washed sand were packed into two separate glass columns, 15 cm in length and 1.8 cm wide, to evaluate whether media flowing through the packed bed would result in the oxidation of ferrous iron. Three hundred ml 9K media containing 1 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}/\text{l}$ was set to flow through the columns at a rate of 0.54 l h⁻¹. Iron concentrations in each batch were recorded on a daily basis (after volume correction with 0.1 M H_2SO_4 to compensate for evaporation) and continued until 0 mg ferrous iron/l was recorded.

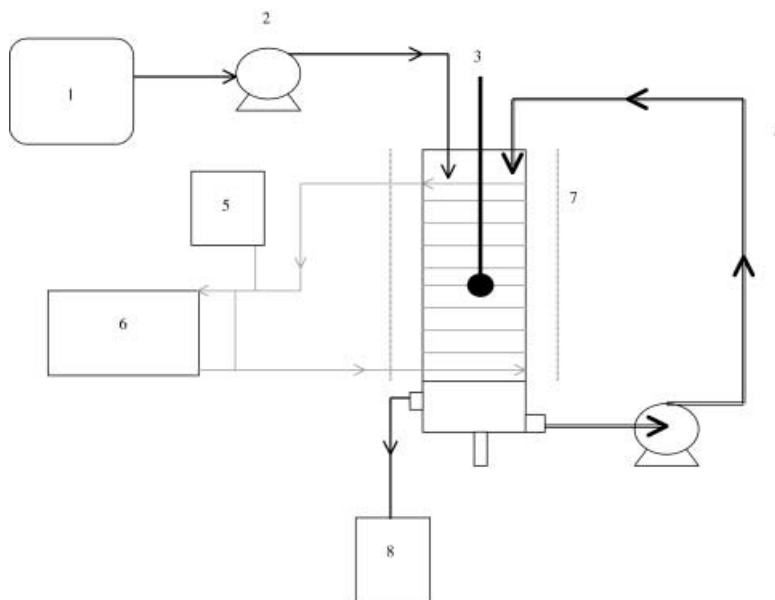
Batch and continuous experiments

Inoculated sand (900.7 g) was packed into a glass column 45 cm in length with a diameter of 5.5 cm. Initially, 1 l of 9K medium containing 3 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}/\text{l}$ was recycled continuously through the column to stimulate bacterial growth. The column was ran in batch and continuous mode (Fig. 1) with 9K medium containing 3 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}/\text{l}$ at a flow rate of 0.12 l h⁻¹ at 20 °C. A working volume of 188 ml was maintained throughout all batch and continuous experiments. Samples were taken from the inlet and effluent pipes and analysed for ferrous and total iron.

Preparation of samples for scanning electron microscopy

Sand samples were removed from the top of the column a few centimetres below the inlet flow. Control sand (sand not exposed to *Thiobacillus ferrooxidans*) was removed from a packed column that had been exposed to 9K medium. The 135–180- μm control and inoculated sand samples were removed from flasks containing 9K medium after 2 weeks of being exposed to the medium. In accordance with the method used by Grishin and Tuovinen (1989), the samples were gently rinsed three times with 0.11 N sulphuric acid and fixed for 2.5 h with 2.5% v/v glutaraldehyde dissolved in 0.11 N sulphuric acid. After fixation, the samples were gently rinsed three times with 0.1 M sulphuric acid, dehydrated and air-dried. The samples were then fixed on specimen stubs, gold-coated and examined by a SEM at 20 kV.

Fig. 1 Packed-bed reactor containing sand inoculated with *Thiobacillus ferrooxidans*: 1 Fresh medium, 2 feed pump, 3 thermometer, 4 recycle, 5 reservoir, 6 flow heater, 7 insulation jacket, 8 effluent vessel



Results

Immobilisation

Preliminary shake-flask experiments comparing the oxidation of Fe^{2+} by a liquid culture and an immobilised culture revealed little difference (Fig. 2). Using linear regression on the first three points (over 48 h), the immobilised culture oxidised Fe^{2+} at a rate of $0.183 \text{ mmol l}^{-1} \text{ h}^{-1}$ whereas the liquid culture oxidised Fe^{2+} at a rate of $0.157 \text{ mmol l}^{-1} \text{ h}^{-1}$.

Evaluation of the number of *Thiobacillus* cells adsorbed per gram of sand

After a period of 38 and 1/4 h, from an original culture containing 2.75×10^7 *Thiobacillus* cells/ml, an average of 1×10^7 cells had adsorbed on 1 g quartz sand and 1.8×10^6 cells to the Pyrex beaker. Twenty-five grams of sand can potentially adsorb 2.5×10^8 cells. A typical culture containing 200 mg Fe^{2+} maintained at room temperature may only have 2.75×10^7 cells/100 ml after the inoculant is added.

Storage of immobilised *Thiobacillus ferrooxidans*

Both control sand samples showed no signs of iron oxidation within the media. After a period of 13 days the bacteria on the previously frozen 135–180- μm sand were active and the ferrous ions within the media were oxidised, indicating growth of *Thiobacillus ferrooxidans* (Fig. 6a, b). Cells immobilised on 0.85–1.18-mm sand had a lag phase of 28 days before iron oxidation became prevalent in the media.

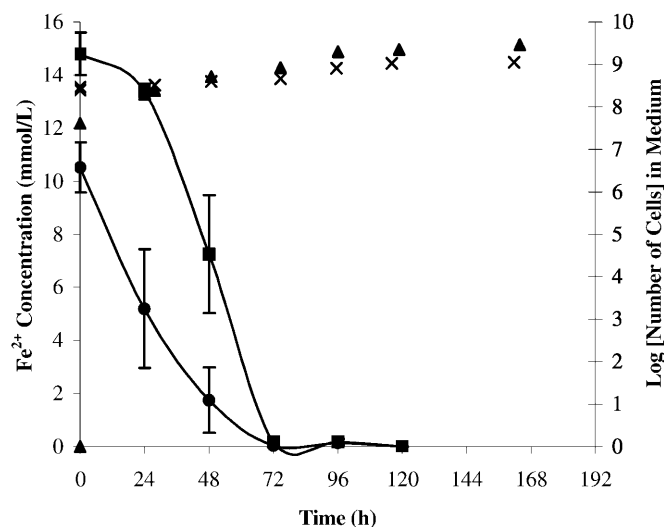


Fig. 2 Liquid culture oxidation compared with immobilised cell oxidation. Error bars show the standard deviation over three experiments. ■ $[\text{Fe}^{2+}]$ in liquid culture, ● $[\text{Fe}^{2+}]$ in immobilised culture, ▲ Cells in liquid culture, × cells in immobilised culture

Mini-reactor

Batch operation of the mini-reactor containing *Thiobacillus ferrooxidans* resulted in the complete oxidation of ferrous iron after six batches, whereas the control column (Fig. 3) showed no signs of oxidation and by the end of the fifth batch no ferrous ions had been oxidised.

Batch and continuous operation of rig

Due to the favourable results from batch operation (Fig. 4), the rig was switched to continuous operation.

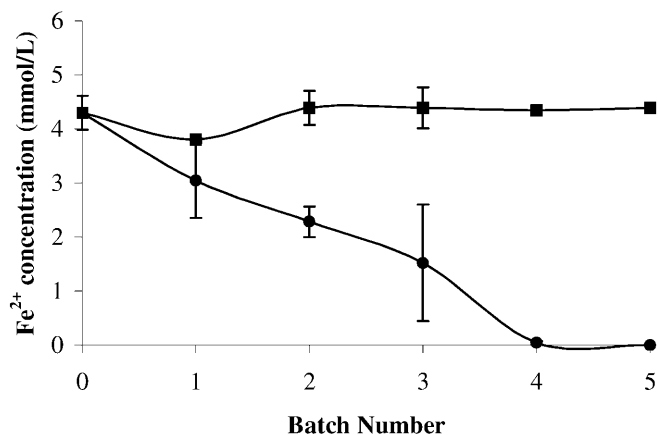


Fig. 3 Batch results from mini reactor experiment. Error bars show the standard deviation over two experiments. ■ Control sand, ● immobilised culture

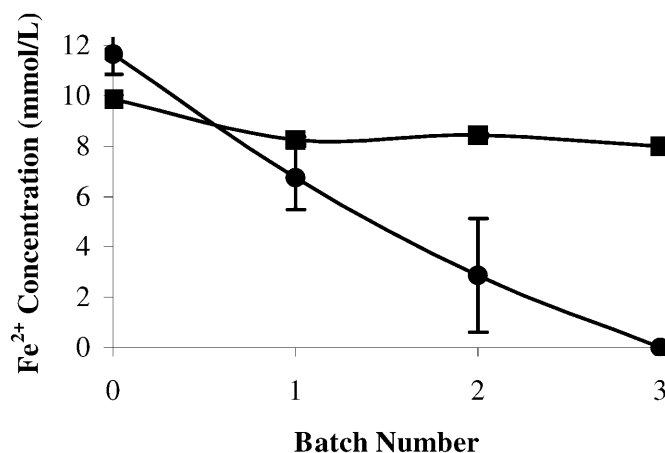


Fig. 4 Batch results from packed bed reactor. Error bars show the standard deviation over three experiments. ■ Control sand, ● immobilised culture

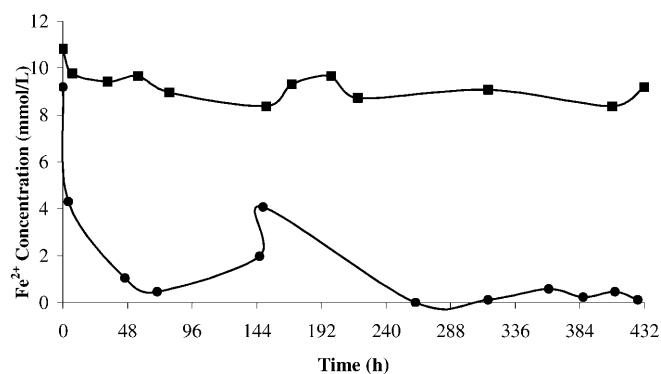


Fig. 5 Continuous results from packed bed reactor. ■ Control sand, ● immobilised culture

In continuous experiments (Fig. 5) the reactor was operated at a dilution rate of 0.64 h^{-1} and an average inlet value of $0.33 \text{ kg Fe}^{2+} \text{ m}^{-3} \text{ h}^{-1}$, resulting in the oxidation of $0.31\text{--}0.33 \text{ kg Fe}^{2+} \text{ m}^{-3} \text{ h}^{-1}$. Oxidation rates were calculated using the retained volume, 0.188 l . Sand from

the column was examined using a scanning electron microscope and showed a build-up of iron precipitates and *Thiobacillus ferrooxidans* on the sand surface. Yeast-like cells were present on some sand particles (Fig. 6a, b), but as these were not present on control sand it is likely that they are secondary to *Thiobacillus ferrooxidans*. Control sand not exposed to *Thiobacillus ferrooxidans* exhibited no signs of bacteria on its surface.

The reactor took 10 days to stabilise, i.e. when the ferrous-ion effluent concentration varied by less than 5% during a period of more than two to three retention times starting from the period when the column was filled with the treated sand. As the sand was dry before the filter was switched on, the bacteria would need to re-adapt to their environment – hence the length of time before stabilisation of the reactor transpired.

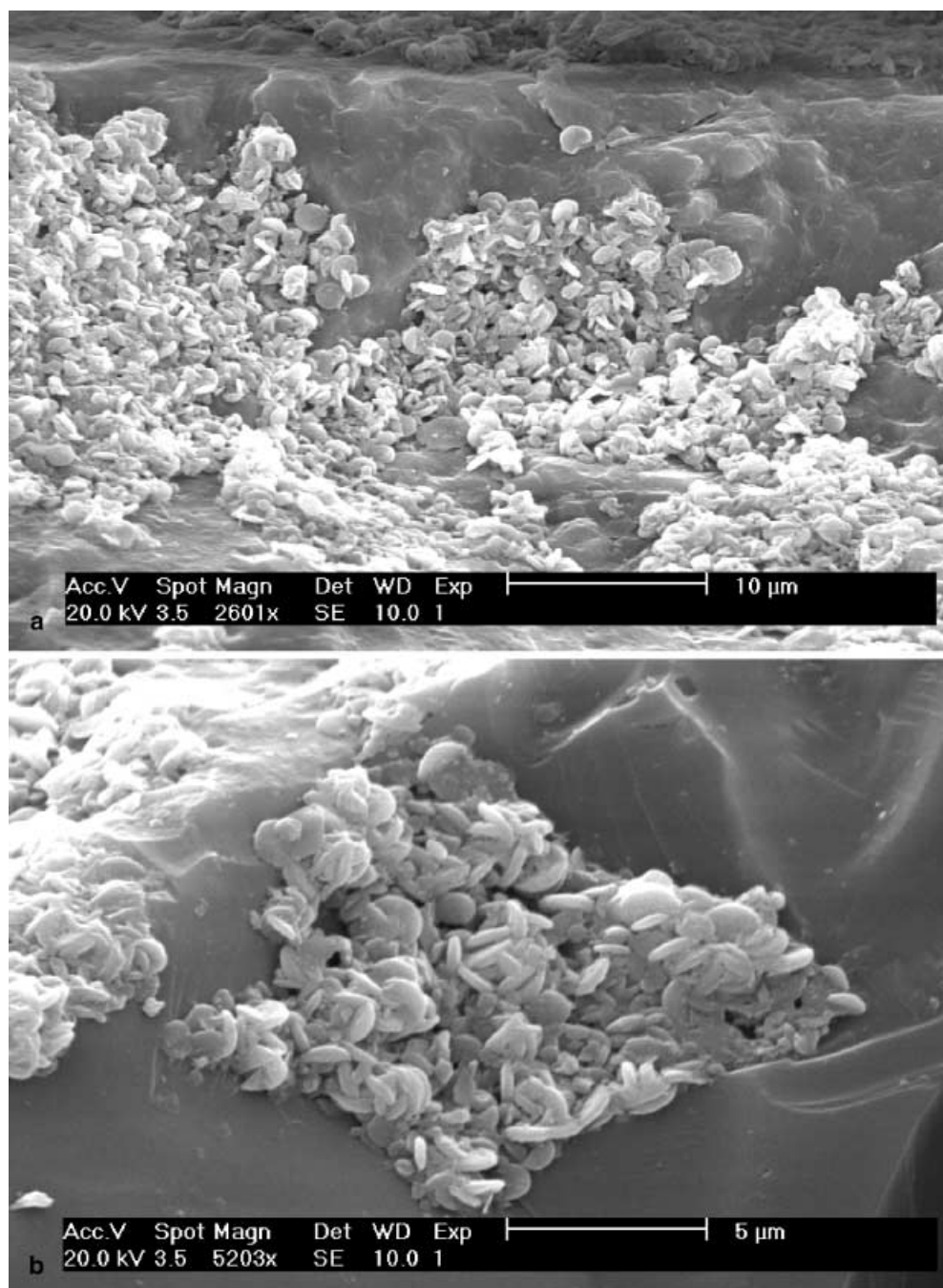
Discussion

Contrary to the results published by Halfmeier et al. (1993b), *Thiobacillus ferrooxidans* has been immobilised on quartz sand particles. The method simply requires introducing the sand to a culture and the cells soon adhere to the particles shortly afterwards. The immobilised culture oxidises iron (Fig. 2) at a slightly faster rate than the liquid culture, but the variation is probably due to a difference in population rather than an effect of immobilisation. The number of cells adsorbed per gram of sand is lower than on other carrier matrices previously investigated: $250\text{--}500\text{-}\mu\text{m}$ activated carbon, 6.8×10^9 cells/g (Kai et al. 1990); $<63\text{-}\mu\text{m}$ coal, 4.09×10^{10} cells/g; $120\text{--}180\text{-}\mu\text{m}$ pore glass, 2.18×10^{10} cells/g (Myerson and Kline 1983); calcium alginate gel, 2×10^{10} cells/ml gel, κ -carrageenan gel, 3.3×10^9 cells/ml gel; gelrite gel, 6.1×10^9 cells/ml gel (Wakao et al. 1994). However, the cell concentration could be increased by using smaller particles of sand.

It is not possible to accurately compare the packed sand column with other systems due to the low Fe^{2+} concentrations used to test the column, the low flow rate and lack of aeration. However, comparing the oxidation rate with other studies, i.e. chemostat free cells at 28°C , $0.42 \text{ kg m}^{-3} \text{ h}^{-1}$ oxidised, inlet loading $0.49 \text{ kg m}^{-3} \text{ h}^{-1}$ (MacDonald and Clark 1970); an aerated packed bed with sintered glass at 30°C , $3.6 \text{ kg m}^{-3} \text{ h}^{-1}$ oxidised, inlet loading $4 \text{ kg m}^{-3} \text{ h}^{-1}$ (Halfmeier et al. 1993b); packed bed with calcium alginate at 25°C , $0.53 \text{ kg m}^{-3} \text{ h}^{-1}$ oxidised, inlet loading $0.56 \text{ kg m}^{-3} \text{ h}^{-1}$ (Lancey and Tuovinen 1984), shows that sand is not inhibitory to the oxidation of Fe^{2+} by *Thiobacillus ferrooxidans*. Using an aerated column at higher temperatures may produce results comparable with other systems.

The packed column used in this study was stable for up to 1 month when the sand was allowed to completely dry out and could be reactivated immediately simply by the introduction of 9K medium. Longer periods of shut-down would require a greater length of time to allow the

Fig. 6 Scanning electron microscopy of 135–180- μm sand particles with *Thiobacillus ferrooxidans* on the surface at (a) 2600 $\times$ and (b) 5200 $\times$ magnification



bacteria to adapt to the changed environment. For long-term storage, the inoculated sand particles could be frozen and introduced to 9K medium when required and recovered within 2 weeks. Both methods, drying and freezing of *Thiobacillus* cells, would be advantageous to industry where it may be necessary to transport inoculated sand to various sites.

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References

- Armentia H, Webb C (1992) Ferrous sulphate oxidation using *Thiobacillus ferrooxidans* cells immobilized in polyurethane foam support particles. *Appl Microbiol Biotechnol* 36:697–700
- Brierley CL, Brierley JA (1997) Microbiology for the metal mining industry. In: Hurst CJ, Knudsen GR, McInerney MJ, Stetzenbach LD, Walter MV (eds) *Manual of environmental microbiology*, ASM, Washington DC, pp 830–841
- Grishin SI, Tuovinen OH (1988) Fast kinetics of Fe^{2+} oxidation in packed-bed reactors. *Appl Environ Microbiol* 54:3092–3100
- Halfmeier H, Schäfer-Treffendorf W, Reuss M (1993a) Potential of *Thiobacillus ferrooxidans* for waste gas purification. Part 1. Kinetics of continuous ferrous iron oxidation. *Appl Microbiol Biotechnol* 40:416–420

- Halfmeier H, Schäfer-Treffenfeldt W, Reuss M (1993b) Potential of *Thiobacillus ferrooxidans* for waste gas purification. Part 2. Increase in continuous ferrous iron oxidation kinetics using immobilized cells. *Appl Microbiol Biotechnol* 40:582–587
- Imaizumi T (1986) Some industrial applications of inorganic microbial oxidation in Japan. *Biotechnol Bioeng Symp* 16:363–371
- Jensen AB, Webb C (1995) Ferrous sulphate oxidation using *Thiobacillus ferrooxidans*: a review. *Process Biochem* 30:225–236
- Kai T, Takahashi T, Shirakawa Y, Kawabata Y (1990) Decrease in iron oxidizing activity of *Thiobacillus ferrooxidans* adsorbed on activated carbon. *Biotechnol Bioeng* 36:1105–1109
- Kai T, Suenaga Y, Migita A, Takahashi T (2000) Kinetic model for simultaneous leaching of zinc sulfide and manganese dioxide in the presence of iron-oxidizing bacteria. *Chem Eng Sci* 55:3429–3436
- Lancey E D, Tuovinen O (1984) Ferrous ion oxidation by *Thiobacillus ferrooxidans* immobilised in calcium alginate. *Appl Microbiol Biotechnol* 20:94–99
- MacDonald DG, Clark RH (1970) The oxidation of aqueous ferrous sulphate by *Thiobacillus ferrooxidans*. *Can J Chem Eng* 48:669–676
- Murayama T, Konno Y, Sakata T, Imaizumi T (1987) Application of immobilized *Thiobacillus ferrooxidans* for large-scale treatment of acid mine drainage. In: Mosbach K (ed) *Methods in enzymology*, vol 136. Academic, New York, pp 530–540
- Myerson AS, Kline P (1983) The adsorption of *Thiobacillus ferrooxidans* on solid particles. *Biotechnol Bioeng* 25:1669–1676
- Nemati M, Webb C (1996) Effect of ferrous iron concentration on the catalytic activity of immobilised cells of *Thiobacillus ferrooxidans*. *Appl Microbiol Biotechnol* 46:250–255
- Nemati M, Harrison STL, Hansford GS, Webb C (1998) Biological oxidation of ferrous sulphate by *Thiobacillus ferrooxidans*: a review on the kinetic aspects. *Biochem Eng J* 1:171–190
- Pagella C, Perego P, Zilli M (1996) Biotechnological H₂S gas treatment with *Thiobacillus ferrooxidans*. *Chem Eng Technol* 19:79–88
- Pentreath RJ (1994) The discharge of waters from active and abandoned mines. In: Hestor RE, Harrison RM (eds) *Issues in environmental science and technology. Part 1. Mining and its environmental impact*. Royal Society of Chemistry, Cambridge, pp 121–131
- Silverman MP, Lundgren DG (1959) Studies on the chemoautotrophic iron bacterium *Ferrobacillus ferrooxidans*. *J Bacteriol* 77:642–647
- Satoh H, Yoshizawa J, Kametani S (1988) Bacteria help desulfurize gas. *Hydrocarb Process* 67: 76D–76F
- Wakao N, Endo K, Mino K, Sakurai Y, Shiota H (1994) Immobilization of *Thiobacillus ferrooxidans* using various polymers as matrix. *J Gen Appl Microbiol* 40:349–358

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