The Effect of Temperature on the Attachment of *Metallosphaera hakonensis* to a Copper Sulphide Concentrate with Application to Heap Bioleaching

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Abstract

Temperatures in excess of 60°C are required for efficient bioleaching of chalcopyrite. Within heaps, colonisation of the mineral with thermophilic archae is important in reaching and maintaining high temperatures. The effect of temperature and culture history on the attachment of *Metallosphaera hakonensis,* an extreme thermophile, to sulphide concentrates and low grade ore was investigated in shake flask and column experiments. Attachment studies were conducted at 25°C, 45°C and 65°C. The results show a clear relationship between increasing temperature and attachment efficiency. Attachment at 25°C was low. Increasing the temperature to 45°C improved attachment efficiency by between 50% and 100% while a further increase to 65°C improved attachment by an additional 20% to 50%. Cells maintained on elemental sulphur showed a 1.3 times greater affinity for the mineral concentrate. In contrast to previous studies using mesophilic organisms the selective attachment of *Metallosphaera* to sulphide minerals, relative to gangue, was less pronounced. Cell surface properties (surface charge and hydrophobicity) and metabolic activity were investigated to provide insight into the observed phenomena. The data suggest that retention of thermophiles within the heap could be enhanced by a secondary inoculation following elevation of the temperature above 40°C by the mesophilic pioneer species.

1. Introduction

The decline in high grade copper ores, suitable for extraction by conventional methods, has fuelled the development of technologies to extract copper from low grade ores as well as waste from current operations. Chalcopyrite, a primary, refractory copper sulphide is the most abundant copper-containing ore, but the economic viability of its extraction from low grade ores conventional methods is questionable (Watling, 2006). Heap bioleaching is considered a potential alternative as it has comparatively low operating costs and a reduced environmental impact (Watling, 2006; Pradhan *et al.*, 2008). The importance of this technology is likely to increase as demand drives the further exploitation of low-grade chalcopyrite ores.

There are still challenges associated with heap bioleaching to extract copper from chalcopyrite. At mesophilic temperatures, the extraction of copper is constrained by slow kinetics and the passivation of the surface. While there has been research devoted to understanding the mechanism in the mesophilic range (Yu *et al.*, 2008; Klauber, 2008), operation at above 60°C where these constraints are largely overcome is the favoured approach. In order to achieve these temperatures the presence of thermophilic iron and sulphur oxidisers within the heap is critical (Rodriguez *et al.*, 2003a; Gautier *et al.*, 2003).

Attachment of the microorganisms to the mineral surface has been shown to enhance the rate of mineral solubilisation during bioleaching (Gehrke et al., 1998; Fowler and Crundwell, 1999). van Loosdrecht *et al.* (1990) proposed a 4-step attachment mechanism that is widely accepted. The four attachment steps are: transportation, initial adhesion, firm attachment and colonisation. The initial adhesion of the cells is a physiochemical process, controlled primarily by hydrophobic and electrostatic forces (van Loosdrecht et al., 1990; Devasia et al., 1993; Harneit et al., 2005). It follows that the rate and extent of attachment is affected by the surface properties of the microorganism and the mineral substrate (Yee et al., 2000), which in turn are affected by solution chemistry, growth history, temperature and mineral composition.

Thermophiles have exhibited relatively poor attachment at ambient conditions (Africa, 2009). This has prompted the suggestion of a two step inoculation system, where the

thermophiles are introduced to the heap once it has already reached the maximum temperature obtainable with only mesophiles (approximately 45°C) (Zou *et al.*, 2006). To assess the validity of this suggestion the effect on attachment efficiency on raising the temperature to 45°C needs to be assessed. There have been numerous studies on the effect of temperature on the physiology of bioleaching organisms, but these have typically focused on iron and sulphur oxidising activity (Franzmann *et al.*, 2005 and Breed *et al.*, 1999).

Previous attachment studies have been done predominantly in well agitated shake flasks, where the mixing ensures suspension of the mineral ore particles and efficient transport of the cells to the mineral surface (Ghauri *et al.*, 2007; Rodriguez *et al.*, 2003b and Sampson *et al.*, 2000). In addition, the batch nature of the experiments ensures conservation of cells within the system so the equilibrium achieved is a function of the inoculum size. This type of experiment does not accurately represent the hydrodynamic conditions within a heap, where there is continuous flow of fluid through the system. The dynamic nature of the flow through system and deviations from perfect plug flow suggest that the equilibria between planktonic and weakly attached cells will differ from batch shake flask.

This study aims to address a number of the issues highlighted above. The effect of three temperatures, 25°C, 45°C and 65°C, on the attachment of *M. hakonensis* with different growth histories (elemental sulphur, ferrous iron, chalcopyrite and pyrite) to four mineral substrates (pyrite, chalcopyrite and a low grade ore) is investigated. The selected temperatures represent ambient, the maximum that can be maintained by mesophilic organisms (*Leptospirillum ferriphilum* and *Acidithiobacillus caldus*) and the typical operating temperature for thermophiles. Attachment in a batch agitated system and flow through column system is compared. In addition, the relationship between culture history and cell surface properties is investigated to support the attachment studies.

2. Experimental

2.1. Microorganisms and cultivation

Metallosphaera hakonensis was cultivated in Erlenmeyer flasks at 65°C, agitated at 180 rpm in an orbital shaker. Cultures were adapted to four different growth media: i) medium 88 (Deutsche Sammlung von Mikroorganismen und Zellkulturen) with no sulphur, but ferrous sulphate (50 mM) and yeast extract (0.02% w/v), ii) media 88 with elemental sulphur (0.05% w/v) adjusted to pH 2.0, iii) Norris media (Akcil *et al.*, 2006) with chalcopyrite concentrate (2% w/v) and iv) Norris media with pyrite concentrate (2% w/v). Cells were harvested by centrifugation (Beckman centrifuge, JA 20 rotor), initially at 2000 g for 3 min to remove fine ore particles and precipitate, then at 10000 g for 10 min to recover the cells. The cells were resuspended in 0K medium (Silverman and Lundgren, 1959) at pH 1.6 and cell concentration was determined by direct counting using a Thoma counting chamber.

Acidithiobacillus ferrooxidans was cultivated in an Erlenmeyer flask at 30°C in 9K medium (Silverman and Lundgren, 1959) agitated at 180 rpm on an orbital shaker.

2.2. Minerals

A chalcopyrite mineral concentrate (79% chalcopyrite), a pyrite mineral concentrate (92% pyrite) and a low grade copper sulphide ore (0.69% copper, primarily as chalcopyrite) were used in the experiments. The pyrite and low grade ore were wet-milled in a rod mill and then wet sieved and the 35-75 µm size fraction retained. The chalcopyrite concentrate was dry sieved to obtain the 35-75 µm size fraction. A portion of the low grade ore sample was floated to generate a low sulphide tailings. The tailings and quartz sand were used for control experiments.

The mineral substrates were conditioned to remove surface oxidation products by washing with 0.1 M HCl for 5 min, with acetone for 10 min, twice with distilled water and finally with acidified distilled water (pH 1.6). The minerals were placed in the oven (80°C) to dry before being used.

2.3. Attachment columns

Glass columns, with a diameter of 2.5 cm and a length of 19 cm, were constructed by Glasschem (Stellenbosch). The ends were closed with screw-cap lids with a rubber stopper and glass nipple. A peristaltic pump (Masterflex Console Drive, 7521-57) was connected to the glass nipple to allow solution to be pumped in from the bottom of the column, with collection of liquid out the top.

Glass wool and 100 glass beads (4 mm diameter, Lasec) were placed in the bottom of the column in order to ensure uniform distribution of liquid throughout the column. The columns were loaded with 300 mineral coated beads. Glass beads (6 mm diameter, Lasec) were coated in the desired mineral using clear glue (Bostik). The glue was shown to have a negligible effect on the activity of the microorganisms (data not shown). 0K media (pH 1.6) was pumped through the column for approximately 2 hours in order to remove any non-adhered mineral particles. In the case of the elevated temperature experiments, heating tape (MRC Heating tape, 200 W) was wrapped around the column to provide temperature control. Due to the slow flow rate used, heating the feed was not effective as the heat was lost from the feed before reaching the column.

2.4. Analytical methods

The pH measurements were performed using a Metrohm 713 pH meter with a Metrohm 6.0258.000 probe. Redox potential was determined using Metrohm 827 pH lab meter and a Metrohm 6.0451.100 probe. Conductivity readings were taken using an AZ 86555 pH/mV/Cond./TDS/Temp. meter with a SIN:9665110 probe. Cell concentration was determined microscopically using a Thoma counting chamber and an Olympus epifluorescent microscope (1500x magnification) using oil immersion and phase contrast optics. Dissolved oxygen was measured using a Mettler Toledo MT4304 K6/8 DO probe, connected to a Mettler Toledo $O_2$4100 meter. The ferrous iron concentration was determined using the 1-10 phenanthroline method (Muir and Anderson, 1977).

2.5. Cell surface characterisation

The initial attachment of the cells to the solid surface is a physicochemical process, with the surface properties of the cells playing an important role.

2.5.1. Zeta potential

Cells were prepared for zeta potential analysis by harvesting (previously described) and then re-suspending them in 0K media at pH 2, 3 and 4. Solutions were diluted such that the conductivity and attenuation of the samples were approximately the same. A refractive index of 1.33 was selected, based on published values for cells (Liang *et al* 2007, Wilson *et al* 2001 and Stramski 1999). The absorbency refers to the transparency of the sample and as the samples were dilute an absorbency of 1 could be used for the analysis. The zeta potential of the cells was determined using a Malvern Zeta-sizer and readings of each sample were repeated a minimum of 5 times per sample in order to assess reproducibility.

2.5.2. Hydrophobicity

The harvested cells were diluted until the OD_{400} was between 0.3 and 0.4. Cell suspension (3 ml) and hexadecane (0.8 ml) were placed in a test tube and vortexed for 1 min, before leaving them to stand for 30 min. The OD_{400} of the aqueous solution was then measured and this was used to determine the hydrophobicity of the solution (Natarajan *et al* 2003). These results were verified by repeating the experiment at an OD_{400} of 0.1 and performing cell counts at a 2x dilution.

2.6. Activity tests

To assess the possibility of an active component to the attachment mechanism, activity tests were conducted to determine ferrous oxidation and oxygen utilisation by the cells under the experimental conditions investigated in the attachment studies.

2.6.1. Iron oxidation assays

All experiments were performed in duplicate. An un-inoculated control flask, with the same iron concentration, was included to quantify abiotic iron oxidation at the temperatures tested. Erlenmeyer flasks (500 ml) were used, containing 300 ml Norris

media, adjusted to a pH of 1.6. These were autoclaved and 9 ml of filter sterilised ferrous solution was added as the energy source (final Fe^{2+} concentration 2.5 g/l). The flasks were then inoculated to achieve a total cell number of $1x10^8$ cells in the flasks. This concentration was chosen as it is just above the detection limit by direct microscopic counting and allowed for several cell replication cycles to occur before the substrate was exhausted. The flasks were covered and placed in an incubator at 25°C, 45°C and 65°C at 180 rpm. The liquid level was monitored and adjusted with distilled water to compensate for evaporation at 65°C.

The flasks were sampled (3 ml) twice daily and the following parameters measured: pH, redox potential and ferrous concentration.

A duplicate set of iron oxidation assays were performed using high cell inocula ($2x10^9$ cells) to replicate the cell loadings in the column experiments. The greater cell number would accelerate any observed iron oxidation.

2.6.2. Oxygen utilisation rate

A 250 ml Schott bottle fitted with an airtight rubber stopper, modified to accommodate the DO probe and inlet and outlet ports for nitrogen sparging, was used to determine oxygen utilisation. A magnetic stirrer bar was placed in the bottle to achieve mixing. Heating tape was wrapped around the bottle and set to 50°C in order to achieve a liquid temperature inside the bottle of 45°C.

The bottle was filled with 250 ml Norris medium and 22.5 ml ferrous sulphate solution (final Fe^{2+} concentration 2.5 g/l) and allowed to equilibrate for at least 1 h. Headspace was minimised in order to minimise oxygen diffusion out of solution. The probe was calibrated such that this equilibrium was set to 100% saturation. A microbial inoculum ($5x10^9$ cells) was added to the bottle. The cells were harvested as described above and then re-suspended in Norris medium. DO readings were then taken over a 3 h period or until the percentage saturation reached zero. Experiments were repeated to assess reproducibility. An abiotic control was run in order to quantify oxygen utilisation due to chemical iron oxidation or diffusion out of the system. A second, positive control using *Acidithiobacillus ferrooxidans* was run at 30°C to confirm the experimental setup was working.

2.7. Shake flask attachment experiments

Duplicate experiments were carried out in 250 ml Erlenmeyer flasks with a total volume of 100 ml, comprising 0K medium (pH 1.6), a 2% (w/v) solids loading and a total cell inoculum of $2x10^9$ cells.

The flasks were placed in orbital shaking incubators at 180 rpm and set to the desired temperature. Samples (1 ml) were taken over a 2 h period at the following intervals: 1, 5, 10, 20, 30, 60 and 120 min and the cell concentration of the samples determined by direct counting. The matrix for the shake flask experiments is detailed in Table 1. The matrix was repeated for 25°C, 45°C and 65°C resulting in 36 unique experimental conditions.

Table 1

Experimental matrix used to assess the effect of temperature and growth history on attachment.

	Mineral concentrate		
Growth History	Pyrite	Chalcopyritee	Low grade ore
Ferrous iron	X	X	X
Elemental sulphur	X	X	X
Pyrite adapted	X	X	X
Chalcopyrite adapted	X	X	X

Control experiments using the flotation tailings and quartz sand were conducted at 45°C and 65°C with the sulphur grown culture in order to assess the preferential attachment to sulphide minerals.

2.8. Column experiments

Duplicate columns were loaded with 300 beads coated with the appropriate mineral. The inoculum (10 ml of approximately $1x10^8$ cells/ml) was fed into the bottom of the column as a pulse. The inoculum was pumped into the column at a rate of 1 ml/min and thereafter 0K medium (pH 1.6) was pumped in at the same flow rate. Effluent fractions (15 ml) were collected continuously over 15 min periods for three hours, yielding a total of 12 samples. A control column, loaded with uncoated glass beads, was run in order to assess potential retention of unattached cells within the bead matrix.

The following analyses were performed on each sample: pH, redox potential, conductivity and cell counts. The experimental matrix used for the column experiments is detailed in Table 2.

Table 2
Experimental matrix used to assess the effect of temperature and growth history on attachment to mineral coated glass beads in column experiments.

Growth history	Chalcopyrite concentrate			Low grade ore		
	25°C	45°C	65°C	25°C	45°C	65°C
Ferrous iron	X	X	X			X
Elemental sulphur	X	X	X	X	X	X
Chalcopyrite concentrate	X	X	X			X

3. Results and discussion

3.1. Shake flask experiments

The attachment of *M. hakonensis*, cultured on elemental sulfur, to a chalcopyrite concentrate at 65°C is presented in Figure 1. Attachment was rapid during the first 10 minutes, with an equilibrium established within approximately 20 minutes.

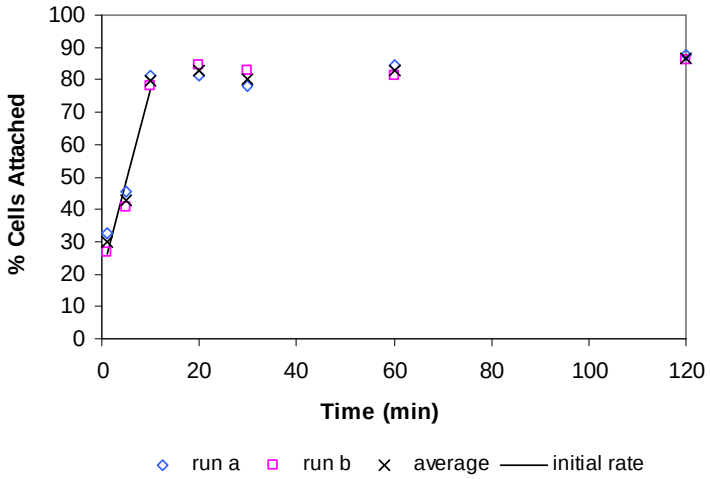

Fig. 1. Attachment of a sulphur grown *M. hakonensis* culture ($2x10^9$ cells total) to a chalcopyrite concentrate (2 % wt vol^{-1}) at 65°C (pH 1.6) in an Erlenmeyer shake flask over a 2 hour time period. Results are representative of duplicate experiments represented by (a) and (b) on the graph. Initial rate of attachment was calculated from the straight line indicated in the figure.

This trend of rapid attachment was observed for the attachment of *M. hakonensis* to all mineral types (chalcopyrite, pyrite and low-grade ore mineral systems) and was not affected by the growth history of the culture.

To assess the microbe-mineral affinity during the initial attachment stage quantitatively, the initial rate of attachment to all mineral systems under investigation was calculated. The initial rate of attachment was calculated from the slope of the curve (Figure 1) during the first 10 minutes and these are summarized in Table 3.

Table 3

Maximum rate of attachment ($x10^{8}$ cells.min^{-1}) and the rate constant (k ($x10^{-11}$.min^{-1})) for the attachment of *M. hakonensis* to different mineral substrates, in batch flasks, as a function of temperature. Results represent the mean of duplicate experiments.

	Culture growth history							
Mineral	Sulphur		Ferrous iron		Pyrite		Chalcopyrite	
Substrate	rate	*k*	rate	*k*	rate	*k*	rate	*k*
Chalcopyrite								
25°C	0.454	1.135	0.292	0.730	0.400	1.000	0.242	0.605
45°C	1.142	2.855	0.668	1.670	0.766	1.915	0.516	1.290
65°C	1.126	2.815	0.580	1.450	0.938	2.345	0.588	1.470
Pyrite								
25°C	0.334	0.835	0.188	0.470	0.586	1.465	0.340	0.850
45°C	0.874	2.185	0.506	1.265	0.846	2.115	1.144	2.860
65°C	1.078	2.695	0.606	1.515	1.066	2.665	1.264	3.160
Low grade ore								
25°C	0.516	1.290	0.396	0.990	0.308	0.770	0.626	1.565
45°C	1.320	3.300	0.432	1.080	1.084	2.710	0.886	2.215
65°C	2.344	5.860	0.980	2.450	1.028	2.570	1.330	3.325

The initial rate of attachment increased with increasing temperature for all mineral substrates. The trend was consistent for all growth histories. However, the extent to which the rate increased varied with growth culture history, with the highest attachment rates observed for sulfur grown cells. This trend was consistent across all mineral systems, with the rate of initial attachment more than doubling when the temperature was increased from 25°C to 45°C and a further increase of up to 50% observed when it was increased from 45°C to 65°C.

The maximum level of attachment, determined once the equilibrium had been reached, to three mineral substrates is presented in Figure 2.

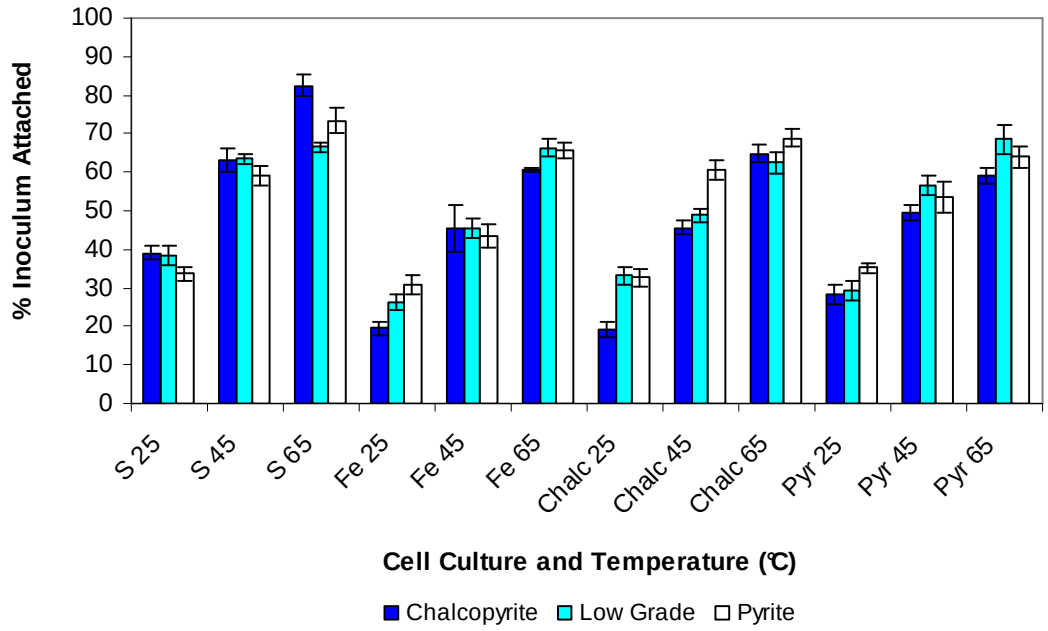

Fig. 2. Attachment of *M. hakonensis*, cultured on four different growth media: ferrous iron (Fe), elemental sulphur (S), pyrite (Pyr) and chalcopyrite (Chalc) to three mineral substrates (chalcopyrite concentrate, pyrite concentrate and low grade ore. Experiments were conducted for two hours at 25°C, 45°C and 65°C.

In all cases, the initial rates of attachment correlate well with overall extents of attachment, which is consistent with the system reaching equilibrium within 20 minutes. The relationship between increased temperature and extent of attachment was similar to that between temperature and attachment rate, with the greater impact observed between 25°C and 45°C.

The cells cultured on elemental sulphur exhibited the greatest levels of attachment to the chalcopyrite concentrate at all three temperatures investigated. An overall attachment of 39%, 63% and 83% was achieved at 25°C, 45°C and 65°C respectively. Slightly lower levels of attachment were observed with the other cell cultures. The maximum extent of attachment to chalcopyrite was obtained at 65°C for all cultures. The extent of attachment to chalcopyrite achieved at 65°C was 61%, 65% and 59% with cells cultured on ferrous iron, chalcopyrite concentrate and pyrite concentrate respectively. Similar levels of attachment were observed for all mineral substrates with sulphur grown cells exhibiting

the greatest levels. Overall, the greatest extent of attachment was 83% and was achieved with the sulphur cultured cells on chalcopyrite concentrate at 65°C.

A similar study conducted by Rodriguez and co-workers (2003b) showed attachment of a mineral adapted mixed thermophilic culture to be greater than 90% to chalcopyrite and pyrite at 68°C. However, their study used a pulp density of 5% and a 10 ml inoculum of exponential phase (> 10^8 cells/ml) culture. The inoculum size was similar to this study, but the greater solids loading likely contributed to the increased attachment. Etzel *et al.* (2008) exposed *Metallosphaera sedula* to synthetic pyrite crystals and showed levels of attachment of 60% after 16 days of exposure. The synthetic crystals were significantly larger (± 2 mm) than the material used in the current study. The direct comparison of results with other published studies is complicated by differences in inoculum concentration, solids loading, particle size and the expression of results as % attachment rather than cells attached per unit surface area. Despite these differences, the shake flask experimental protocol followed in most studies (Rodrigues *et al*., 2003b; Ohmura *et al*., 1993 and Sampson *et al*., 2000) is similar.

Previous studies (Harneit *et al.,* 2006; Yu *et al.,* 2008; Porro *et al.,* 1997 and Devasia *et al.,* 1993) have established that the growth medium used to cultivate the cells clearly affects the ability of the cells to attach to the mineral surfaces. The growth media affects the composition of the extracellular polymeric substance (EPS) secreted by the microorganisms. The EPS composition affects the surface properties, which influence the microorganisms' ability to attach to sulphide mineral surfaces.

Kinzler and co-workers (2003) conducted studies to determine the composition of the EPS of *Acidithiobacillus ferrooxidans*. The study concluded that ferric iron complexed with the EPS significantly improved the rate and extent of attachment to pyrite. The extent of attachment was comparable for the mineral adapted and Fe grown cells, which is consistent with the current study.

The enhanced attachment achieved with sulphur grown cells is similar to results obtained by Sampson *et al*. (2000) for two species of *Sulfobacillus*.

The attachment at each set of conditions did not vary greatly with mineral substrate, indicating that *M. hakonensis* did not attach preferentially to the different mineral sulphides. This has been observed in previous shake flask studies with archae (Rodríguez

et al, 2003b). However, the relatively high level of attachment to the low grade ore is not consistent with previous studies using mesophilic organisms (Mikkelsen *et al*, 2007; Rodriguez *et al.*, 2003 and Harneit *et al.*, 2006) where a clear preference between sulphide minerals and gangue was observed.

To further investigate this, control experiments were conducted assessing the attachment of sulfur grown *M. hakonensis* to a flotation tailings and quartz sand. The tailings sample was prepared by floating the low-grade ore, which reduced the sulphide mineral content from 5.2% to below 0.5%. Quartz sand was used as quartz was the predominant (44.8 wt %) gangue mineral contained in the low-grade ore sample. Attachment levels to the tailings were slightly lower than those observed with the mineral sulphides. Equilibrium attachment levels of 49% and 61% were achieved for the tailings at 45°C and 65°C respectively. However, attachment of the cells to the quartz sand at 65°C over the 2 h period was negligible. These results indicate that *M. hakonensis* does not attach preferentially to the copper sulphides in the substrates, but attaches to non-quartz components in the gangue material as well.

The relatively high level of attachment to the low grade ore and specifically to the gangue material of the low-grade ore suggests that the surface properties of *M. hakonensis* differ significantly from the mesophilic cultures studied. This difference influences the physicochemical interactions between the microorganism and the mineral, influencing the initial adhesion that is observed (van Loosdrecht *et al.*, 1990 and 1987).

There are distinct differences between the cell walls of gram positive and gram negative bacteria as well as archae, so it cannot be assumed that thermophiles attach to solid substrata in the same way as mesophiles. Gram positive bacterial walls consist primarily of a single type of molecule, peptidoglycan, and are relatively thick. The cell wall is negatively charged and is partly responsible for the overall negative surface charge of the microorganism. Gram negative bacterial walls are multilayered and relatively complex. Unlike bacterial cell walls, archael cell walls do not contain peptidoglycan, but rather a variety of related and unrelated polysaccharides. The most common type of archael cell wall is the paracrystalline surface layer (S-layer) which consists of protein or glycoprotein and is generally of hexagonal symmetry.

3.2. Column experiments

The column experiments were designed to better approximate conditions within a heap, while retaining a greater degree of control and reproducibility than could be achieved by packing columns with agglomerated ore. The columns were packed with an equal number of similarly sized, evenly coated beads to ensure a relatively consistent surface area for attachment. Solution was pumped in from the bottom so the columns were fully saturated to minimise channelling. Data are presented as % cells retained within the column, rather than % attached, as the planktonic cells have a certain residence time within the column and perfect plug flow cannot be assumed. However, once the retention has stabilised (Figure 3), the remaining cells are most likely attached to the mineral. A control experiment, using uncoated glass beads, was conducted and confirmed that the cells were not retained in the column due to hydraulic restrictions or attachment to the column walls or glass beads. The results are consistent with the negligible attachment to quartz in the shake flask experiments.

A typical profile obtained in the column experiments is presented in Figure 3. The final value for the cells retained in the column was determined as the mean value from the time the retention line stabilised (75 min in Figure 3). The results obtained across the experimental matrix are summarised in Figure 4.

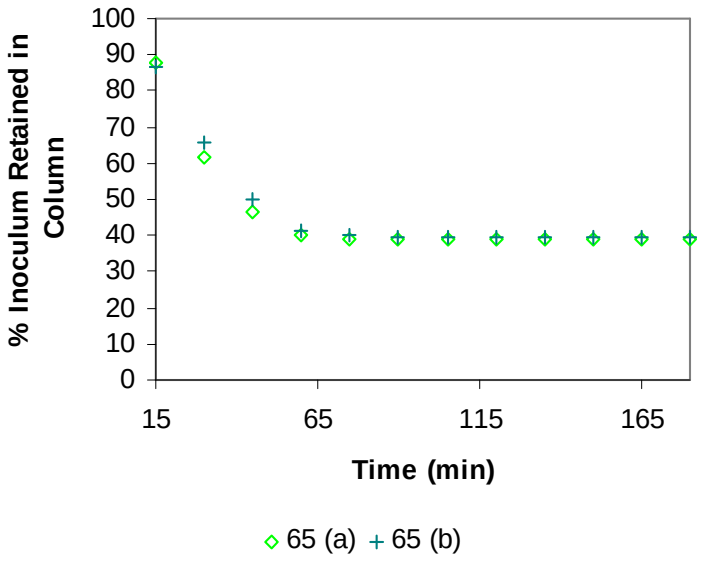

Fig. 3. Retention of sulphur grown *M. hakonesis* ($1x10^9$ cells total) in the packed-column reactor with chalcopyrite concentrate coated beads operated at 65°C. The results are representative of duplicate experiments, indicated by (a) and (b) on the graph.

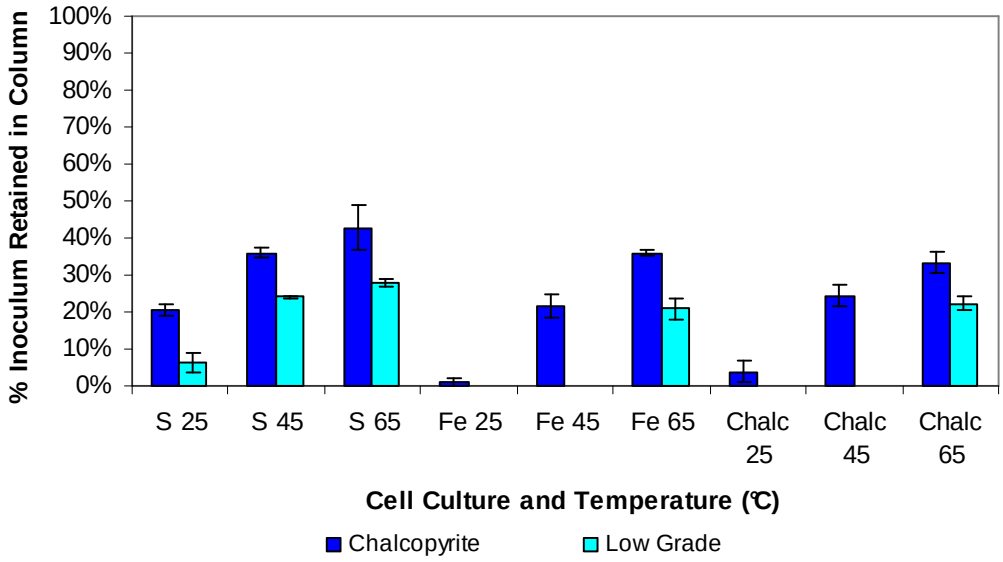

Fig. 4. Final percentage of *M. hakonensis* retained in columns loaded with chalcopyrite and low-grade ore coated beads as a function of temperature and culture history. Experiments with low-grade ore coated beads at 25°C and 45°C were only conducted with sulphur grown cells.

The trends observed in the column experiments were similar to the shake flasks, with the retention of cells in the column increasing at higher temperatures. Similarly, the sulphur grown cells were retained to a greater degree. However, the level of retention (Figure 4) was significantly lower than the attachment observed in shake flasks (Figure 2). Previous studies have shown that microbial attachment can be modelled using the Langmuir adsorption model. A basis of this model is that the extent of adsorption is a function of the number of open sites on the adsorbent and the concentration of adsorbing species. In the shake flasks there is conservation of cells within the system, while in the columns the planktonic cells move through the column and are eventually washed out. This is an important finding and validates the development of the experimental system.

The attachment to the low-grade ore followed the same trend as to the chalcopyrite concentrate, but the levels of attachment were lower. This is different to the trend observed with the shake flask studies where there was no obvious difference between attachment to the different substrates. This may be a result of the differences in the experimental systems, but addition research is required to provide conclusive evidence.

No cell retention was observed in the control column filled with uncoated glass beads. This confirmed that the retention observed in the columns loaded with coated beads was due to attachment to the mineral substrates and not due to hydraulic effects or attachment to the column walls.

These results indicate that while shake flask experiments are sufficient in identifying attachment trends, they overestimate the level of attachment likely to occur in packed beds (heaps). The columns that were used in these experiments were only 20 cm in length and a greater level of attachment can therefore be expected with an increase in this length. However, the data from the column experiments may be better suited for validating attachment models in heap systems.

3.3. Metabolic activity

The level of attachment of *M. hakonensis* was higher with an increase in temperature, peaking at the temperature at which the cells would show optimal metabolic activity. In view of this correlation, activity tests were conducted to investigate whether initial attachment could be influenced by metabolic activity. Ferrous iron oxidation and oxygen utilisation tests were selected for this purpose. For the iron oxidation tests the length of the lag phase and subsequent iron oxidation rate were used to assess activity. The lag phase was shortest for cells that had been adapted to ferrous iron (24.5 h) and longest for cells adapted to grow on sulphur (79.3 h), with chalcopyrite adapted cells showing an intermediate lag (46.3 h). These results are consistent with the growth history, but indicated that negligible iron oxidation would have occurred over the three hour duration of the column experiments.

The rate of ferrous iron oxidation increased with an increase in temperature, but was negligible over the attachment study time period of 3 hours (data not shown). A ferrous iron oxidation test was carried out with the same cell concentration as the shake flask attachment studies ($2x10^8$ cells/ml) confirmed negligible iron oxidation activity over a 3 hour period. The experiment was conducted at 65°C using the cells cultured on elemental sulphur as they had shown the greatest extent of attachment in the shake flask studies conducted.

The oxygen utilisation rate (OUR) for sulphur grown cells at 45°C was also determined to be negligible over the 3 hour period. The OUR was determined at 45°C and not 65°C due to the constraint of the temperature limit of the dissolved oxygen (DO) probe. *Acidithiobacillus ferrooxidans* was used in the positive control, confirming the validity of the assay. The results indicated insignificant metabolic activity of *M. hakonensis* at 45°C over the 3 h period.

The very low metabolic activity of the cells observed over the experimental period suggests that the attachment process is independent of the metabolic activity of the cells. It is possible that *M. hakonensis* behaves in the same way as *Leptospirillum* and requires an elevated redox potential before iron oxidation can take place, resulting in the extended lag period observed. The Brownian motion of the cells will increase with an increase in temperature and this could increase the number of collisions between the cells and mineral substrate. The effect of temperature on solution chemistry and surface properties of the cells and minerals needs to be investigated further to determine the role that they could play in the observed increase in attachment.

3.4. Cell surface characterisation

As the metabolic activity was shown to have a negligible effect on the initial attachment of the cells, it can be assumed that the initial attachment of the cells is a purely physicochemical process. The cells' surface properties play an important role and understanding these is important in analysing the attachment results. Cell surface properties influence the hydrophobic and electrostatic interactions, which can be complementary or one can dominate the other.

Previous studies have shown that the growth history of microorganisms affects the extracellular polymeric substances (EPS) present (Kinzler *et al.*, 2003; Harneit *et al.*, 2006). The composition of the EPS affects the surface properties of the cells. Uronic acids and the complexing of Fe^{3+} ions in the EPS has been shown to result in a net positive charge in *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* cells, which would aid the attachment to negatively charged substrates (Harneit *et al.*, 2006).

The relative hydrophobicity of *M. hakonensis* cultured on elemental sulphur, ferrous iron and chalcopyrite, was determined using 2-phase partitioning with hexadecane. The

results indicated that chalcopyrite-adapted cells were the most hydrophobic, with an average of 14.3% of the cells partitioning into the organic phase, compared to 5.9% and 5.0% for iron- and sulphur-grown cultures respectively. These results are of a similar magnitude to those obtained by Nataranjan and Das (2003) for *Acidithiobacillus ferrooxidans*, although their study showed sulphur-grown cells to be more hydrophobic. Nataranjan and Das (2003) showed that hydrophobicity of both iron- and sulphur-grown cells was substantially increased in the presence of increasing concentrations of potassium phosphate. A number of ionic species have been shown to have a similar effect, attributed to the dehydration of proteins, which increases the surface area of their hydrophobic domains. The reproducibility of the assays in this study was relatively poor so while chalcopyrite-adapted cells appeared most hydrophobic, the difference between iron- and sulphur-grown cells was not statistically significant. Contact angle measurements may be a more effective way of assessing cell hydrophobicity.

Copper sulphide minerals have been reported to be hydrophobic under the experimental conditions used in this study. This suggests that if hydrophobic interactions were dominant in the initial attachment of *M. hakonensis* to the substrates, chalcopyrite-adapted cells should have exhibited the greatest degree of attachment. This was not the case, with sulphur-grown cells showing significantly greater attachment in all cases. The data cannot be assessed based on hydrophobic interactions alone as the surface charge also plays an important role in the initial attachment process and could possibly be the dominant factor under the experimental conditions investigated.

The zeta potential of *M. hakonensis* cultured on elemental sulphur, ferrous iron and chalcopyrite, was determined using a Malvern Zetasizer Nano Series. Cells were re-suspended in 0k medium at pH 2 and pH 3 to remain consistent with the pH and solution chemistry of the attachment studies as well as to mimic the conditions encountered in an industrial bioheap. The measured potentials are presented in Table 4.

Table 1
Zeta potential of the three cultures of *M. hakonensis* (elemental sulphur, ferrous iron and chalcopyrite adapted), at a pH of 2 and 3, measured using a Malvern Zetasizer. The conductivity of all samples tested was kept approximately constant in order to ensure that the results obtained were comparable.

	Cell growth history		
	Sulphur	Ferrous iron	Chalcopyrite
pH	Zeta (mV)	Zeta (mV)	Zeta (mV)
2	-2.115	-1.077	-2.758
3	-2.436	-4.826	-8.642

The zeta potential of the sulphur-grown cells remained relatively constant and only decreased by 0.321 mV when the pH was increased from 2 to 3, whereas the ferrous-grown and chalcopyrite-adapted cultures showed a much greater change in the zeta potential when the pH was increased to 3 (Table 4). This suggests that the composition of the cell surface components differed.

The addition of acid during the initial stage of heap bioleaching results in the dissolution of some of the acid consuming gangue material. This causes the pH within the heap to increase and pH profiles are created throughout the heap. A similar increase in pH was observed in the attachment experiments when the acidified medium was first contacted with the coated beads. The pH in the columns increased to above pH 2. At the low pH values, it has been determined that the mineral substrates investigated typically exhibit a negative charge (Poortinga *et al.*, 2002). The zeta potential of the cells cultured on elemental sulphur remained relatively constant at the increased pH while the ferrous iron- and chalcopyrite-adapted cells became more negative. This would reduce the repulsive force between the cells and the negatively charged minerals. This explanation is consistent with work on bacteria that concluded that electrostatic interactions play an important role in solutions with low ionic strength and when hydrophobic interactions were not prevalent. These studies suggest that the electrostatic forces between the mineral and the cell are the fundamental factors in the initial attachment of *At. ferrooxidans* to mineral substrates and that the attachment of the cells decreases with increasing electrostatic repulsion (Rodriguez *et al.*, 2003b; Devasia *et al.*, 1993).

4. Conclusion

Both the shake flask and column attachment studies showed a clear trend of increased levels of attachment as the experimental temperature was increased from 25°C to 65°C. The extent of attachment was lower in the column experiments due to the flow through nature of the experiments. The shake flask results may overestimate the extent of attachment obtained due to the increased microbe-mineral interaction within the system.

The metabolic activity of the cells was shown to be limited under the experimental conditions and time period, confirming that the initial attachment was a physicochemical phenomenon.

The sulphur grown cells exhibited the greatest levels of attachment to all mineral substrates. The investigation of the surface properties of the cells suggested the surface charge played a more important role in attachment than hydrophobic interactions.

In contrast to previous work with mesophiles the cells did not show a clear affinity for sulphide minerals and significant attachment to the non-quartz fraction of the gangue was observed. The cell wall and membrane composition of archae differ significantly from bacteria. These differences most likely contribute to the differences observed.

The study confirms that attachment of thermophilic archae is suppressed at mesophilic temperatures. The data suggest that a secondary inoculation of thermophiles once the heap has reached 40 to 45°C may enhance their retention and improve subsequent colonisation.

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