Hydrometallurgy 157 (2015) 107–115

Contents lists available at ScienceDirect

Hydrometallurgy

journal homepage: www.elsevier.com/locate/hydromet

Copper resistance, motility and the mineral dissolution behavior were assessed as novel factors involved in bacterial adhesion in bioleaching

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ARTICLE INFO

Article history:
Received 8 April 2015
Received in revised form 6 July 2015
Accepted 28 July 2015
Available online 5 August 2015

Keywords:
Adhesion
Bioleaching
Chalcocite
Mineral surface
Detachment

ABSTRACT

A study was carried out on the adhesion to sulfide minerals of chemolithoautotrophic acidophilic bacteria obtained from industrial copper bioleaching operation. For this purpose, a mixed culture obtained from an industrial process and two metabolically different pure strains of *Acidithiobacillus*: *A. ferrooxidans* and *A. ferridurans* were used. These microorganisms showed significant differences in adhesion with respect to pyrite and chalcocite in terms of the temporal dynamics patterns and preference. A complex dynamics that involve cycles of attachment and detachment can only be explained by considering both, the intrinsic characteristics of the microorganisms, such as hydrophobicity, resistance and motility, and the properties of each mineral like their hydrophobicity, dissolution behavior and ionic contribution.

1. Introduction

Copper sulfide bioleaching makes it possible to process low-grade ores at low cost and with low environmental impact. Bioleaching involves chemical and biological processes in which microorganisms have a fundamental role. The interaction between these microorganisms and minerals is one of the principal factors driving the efficiency of the copper recovery. This interaction has been thoroughly studied (e.g., reviews by Watling, 2006 and Schippers et al., 2013), and has been designated as an "indirect contact mechanism", a process that depends on the contact between microorganisms and the mineral substrate and in which the extracellular polymeric substances (EPS) secreted by the cells play a fundamental role (García-Meza et al., 2013; Kinzler et al., 2003; Yu et al., 2008; Zeng et al., 2011). Attached microorganisms are capable of catalyzing chemical reactions that lead to a build-up of Fe(III) in the EPS, which causes mineral oxidation and the subsequent release of metal ions (Kinzler et al., 2003; Schippers et al., 2013).

In the biofilm development, the mineral–cell interaction is a complex process that involves a cycle with three stages — attachment, growth and detachment of the cells (Davey and O'Toole, 2000) — and bacterial adhesion to a given substrate depends on selective preference (Rodríguez et al., 2003). Previous to the attachment process, there is a searching stage, in which the cells look for available substrata. This phase is thought to be governed by physical and chemical interactions between the planktonic cells and the mineral (Hermansson, 1999). Particularly, hydrophobicity (Liu et al., 2004b; Pakshirajan, 2007; Takeuchi and Suzuki, 1997; Zhu et al., 2012), ionic chemical interactions (Devasia et al., 1993; Gehrke et al., 1998; Hermansson, 1999; Sand and Gehrke, 2006) and physical factors (Africa et al., 2010; Edwards and Rutenberg, 2001; Zhu et al., 2012) are relevant in this stage. In addition, stability in the next stages of the long-term community on the solid substrate is hardly influenced by biological factors, such as the production of polymeric substances (Sand and Gehrke, 2006; Zhu et al., 2012), resistance to metals (Alvarez and Jerez, 2004; Dopson et al., 2003), microbial differential metabolic traits (Dopson et al., 2003), microbial interactions (Florian et al., 2011) and Quorum Sensing (González et al., 2012). In bioleaching, most of the factors just mentioned have been studied considering macroscopically measurable variables. However, the characteristics of the mineral–bacterium interstitial zone differ from those of the surrounding area, and it is at this microscopic level that the above mentioned factors actually determine the result. At present, there is only an incipient understanding of the interactions at ionic and molecular level as well as of bacterial communication and the importance of the dissolved-metal concentration gradient resulting from bioleaching. In addition, it is well known that each microorganism present in bioleaching has distinctive characteristics and the composition of the communities impacts the operative results (Demergasso et al., 2010; Florian et al., 2011). Current knowledge on the matter has been derived from adhesion studies with bioleaching bacteria involving pure cultures as well as mixed cultures. However, the medium-term temporal dynamics of attached populations and communities have not been completely understood up to now. These dynamics are complex and there are still no mathematical models capable of predicting with any precision temporal fluctuations in the community

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http://dx.doi.org/10.1016/j.hydromet.2015.07.018
0304-386X/

Table 1
Brief description of the microorganisms used in this investigation.

Bacterial strains	Phylogenetic affiliation	Resistance to $CuSO_4$ (mM)	Fe(II) oxidation rate ($mg\ h^{-1}$)	S° oxidation rate ($mg\ h^{-1}$)
Acidithiobacillus ferridurans (*Af*D2)	FOA/Group II	325	64	10.6
Acidithiobacillus ferrooxidans IESL32 (*Af*DM)	FOA/Group I	100	74	11.7
Leptospirillum ferriphilum	*Leptospirillum*/Group II	100	–	–

FOA = Fe(II)-oxidizing acidithiobacilli (Amouric et al., 2011). Resistance to CuSO4 and oxidation rates were obtained from Araya, 2012.

(Demergasso et al., 2010). It has been reported that highest copper extraction occurs in the first stages of the industrial processes (Demergasso et al., 2010), when *Acidithiobacillus ferrooxidans*, *Acidithiobacillus thiooxidans* and *Leptospirillum ferriphilum* are the most important microorganisms (Demergasso et al., 2005b; Olson et al., 2003; Rawlings et al., 2003) and early-stage adhesion phenomena are determinant (Kinzler et al., 2003). This means that, within a few hours, microorganisms must be capable of choosing a suitable mineral substrate and adhering to its surface so as to obtain the energy they need for their metabolism. This stage is critical to the process and, since industrial progress depends on scientific understanding, this topic has become extremely important in biotechnology. Therefore, the present report aims at studying adhesion phenomena at microscopic level and to follow them over some time in order to gain valuable information to better understand these bioleaching systems.

Chalcocite and pyrite are two commonly found minerals in most of the industrial bioleaching processes. Chalcocite is the one yielding the greatest amount of copper in these processes (Demergasso et al., 2010; Gentina and Acevedo, 2013). It is well known that chemical chalcocite dissolution and copper release occur in stages (Adewale-Bolorunduro, 1999; Leahy et al., 2007; Thomas et al., 1967), and require the presence of iron oxidizing microorganisms (Leahy et al., 2007; Ruan et al., 2010). However, presently, only a partial description of the dynamics of microorganism adhesion on chalcocite exists (Echeverría and Demergasso, 2009). Pyrite (FeS_2) is also very common in these processes and its relation with oxidizing microorganisms has been thoroughly studied (Norris and Kelly, 1978; Rodríguez et al., 2003; Ruan et al., 2010; Schippers et al., 2000, 2013). Both minerals are used by oxidizing bacteria as sources of energy, but they differ in their chemical composition, which has a significant effect on substrate selection by microorganisms (Ruan et al., 2010).

By means of microscopy techniques, we were able to follow the adhesion dynamics directly over the mineral surface. In this way, we saw the effect of the mineral composition and of the surrounding media in pure and mixed bacterial cultures.

2. Materials and methods

2.1. Microorganisms

Adhesion tests were performed on a mixed culture of microorganisms obtained from an industrial bioleaching process. The microorganisms were characterized by means of Real Time PCR using specific probes for *A. ferrooxidans*, *A. thiooxidans* and *Leptospirillum*. The cells present were identified as *A. ferrooxidans*, 7.2×10^6 cells mL^{-1}, *A. thiooxidans*, 1.0×10^7 cells mL^{-1}, and *L. ferriphilum*, 2.9×10^4 cells mL^{-1}. In addition, two pure strains isolated from industrial processes were used in adhesion dynamics and inhibition assays: *Acidithiobacillus ferridurans* D2 (*Af*D2 — formerly Group II *A. ferrooxidans*) and *A. ferrooxidans* IESL32 (*Af*DM). The strains *Af*D2 and *Af*DM were chosen because they are predominant in an industrial bioleaching process, and present over 99% similarity with the type strain *A. ferridurans* ATCC 33020 and *A. ferrooxidans* ATCC 23270, respectively. The characteristics of the microorganisms used in this investigation are listed in Table 1.

2.2. Mineral substrata

The minerals used in this study are greater than 99% pure chalcocite and pyrite (from Museo Mineralógico, Universidad de Atacama, Copiapó, Chile). They were ground to 3 mm fragments and suspended in an epoxy resin solution (Epoxy Cure 2 Epoxy System, Buehler, USA), so that one side could be polished to a shiny finish (Fig. 1). The fragments were then removed from the resin, pyrite was washed with boiling 6-N hydrochloric acid to eliminate iron ions, and then both minerals were passed through an acetone wash to remove any remaining sulfur, as specified in the protocol described (Schippers et al., 1999).

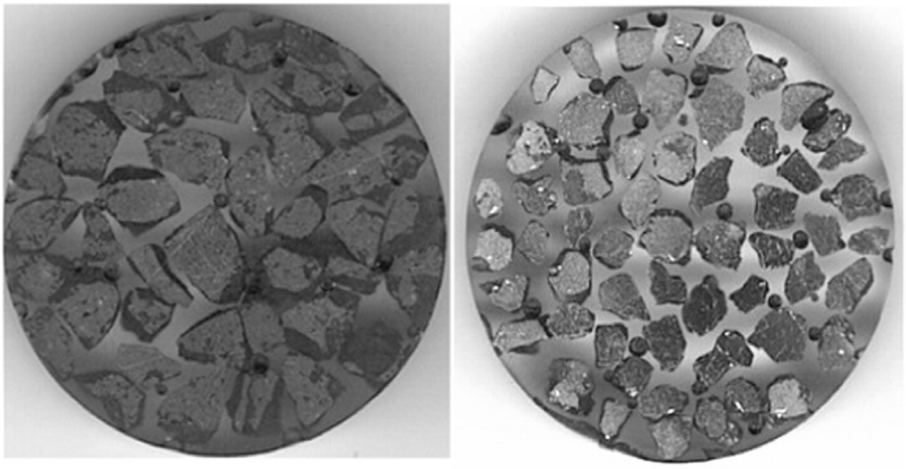

Fig. 1. Polished mineral fragments. On the left, chalcocite; on the right, pyrite. Diameter of the plates: 3 cm.

Before starting the tests, the mineral fragments were sterilized using ethanol 70% and dried.

2.3. Bacterial adhesion test

Prior to inoculation, 100 mL of the mixed culture described above was grown for 48 h, filtered in a 0.22 μm nitrocellulose filter and washed by filtering 100 mL of the same medium to eliminate iron residues. Afterward, the cells were resuspended putting the filter in an Erlenmeyer flask with 50 mL of TK (Tuovinen–Kelly) saline medium (Tuovinen and Kelly, 1973) at pH 1.8. The cells were then counted and the volume of medium was adjusted to obtain 5.0×10^6 cells mL^{-1} total cells concentration.

2.3.1. Mounting

Three tubes containing 10 mL with 5.0×10^6 cells mL^{-1} of the microorganisms suspended in TK medium with a mineral fragment, but without addition of iron or sulfur, were incubated for 4 days at room temperature while gently shaken. Another test tube was set up using glass fragments of similar size as a control. Tests without bacteria were performed to measure copper dispersion on the filter.

2.3.2. Sample preparation for analysis

For the purpose of the analysis, a tube scheduled for a given day was completely emptied and the solution and mineral fragment were examined separately.

The mineral fragments obtained from the tubes were gently washed three times using TK saline medium and paraformaldehyde was added until a 4% concentration was reached. The fragments were fixed at room temperature for a period of 1 h before counting. In parallel, the cells in the solution were collected using a 0.22 μm polycarbonate filter (Millipore, Isopore). The filters were left to dry and then cut into pieces which were labeled with permanent ink pen; they were dehydrated using ethanol and left again to dry in the same manner as the minerals. Filters incubated without cells were dried for further analysis by SEM–XRD.

2.4. Cell count

Total cell counts were obtained using DAPI (4′,6 diamino-2-phenylindole, Sigma), as described by Pernthaler et al. (2002). Initial taxon-specific cell counts in suspension were obtained using Real Time PCR with specific probes for *A. ferrooxidans*, *L. ferriphilum* and *A. thiooxidans* (see above). A slightly modified CARD-FISH technique (Catalyzed Reporter Deposition Fluorescent in Situ Hybridization) (Demergasso et al., 2005a; Pernthaler et al., 2002) was used to determine the number and type of cells attached to the minerals at different stages of incubation. Cells were counted using an OLYMPUS IX-81 epifluorescence microscope, fitted with a 100 × 1.4 N.A. plan-apochromatic oil immersion objective. Scanned images were taken by means of confocal microscopy (OLYMPUS CLSM FV-1000 Spectral) and for microphotographs a Leica DMLS fluorescence microscope, equipped with a Q-imager Micropublisher camera was used.

2.4.1. CARD-FISH

To prevent non-specific adhesion, the mineral samples obtained from the tests were left to stand in 300 μL of hybridization buffer (Pernthaler et al., 2002) for 15 min prior to analysis. Then the samples were rinsed and placed in a tube containing the buffer with a suitable probe. The tube was sealed and the samples were incubated in a dark moist box for 3 h. Three probes were used for this test: TF359 for *A. ferrooxidans* (Schrenk et al., 1998), LF581 for *Leptospirillum* Groups I and II (Schrenk et al., 1998) and ATT0223 for *A. thiooxidans* (Hallberg et al., 2005). Washing and mounting steps were performed according to the protocol described by Pernthaler et al. (2002).

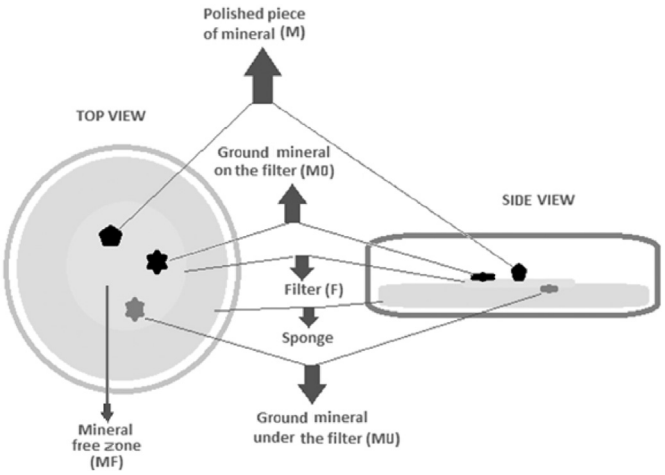

Fig. 2. Diagram describing the inhibition test on filter supported by sponge.

2.5. Measurement of the area available for substrate adhesion

A pyrite fragment surface was polished on one side to a mirror-like surface and washed in the same way as described above. The polished mineral fragment was placed in a glass tube with 1 mL of culture medium and 1×10^7 cells mL^{-1} and was left to stand for 2 h. The mineral fragment was carefully removed without touching the polished side and it was gently washed using sterile culture medium. The cells on the polished surface were counted using DAPI as described above. All steps were done in triplicate. The cells in the residual solution were also counted by filtering and DAPI staining. If the initial and final number of cells in a suspension is known, the number of total cells attached to the mineral fragment can be estimated by subtraction. The BET technique based on nitrogen adsorption (Brunauer et al., 1938) was also used to calculate the surface area of the pyrite fragments to corroborate

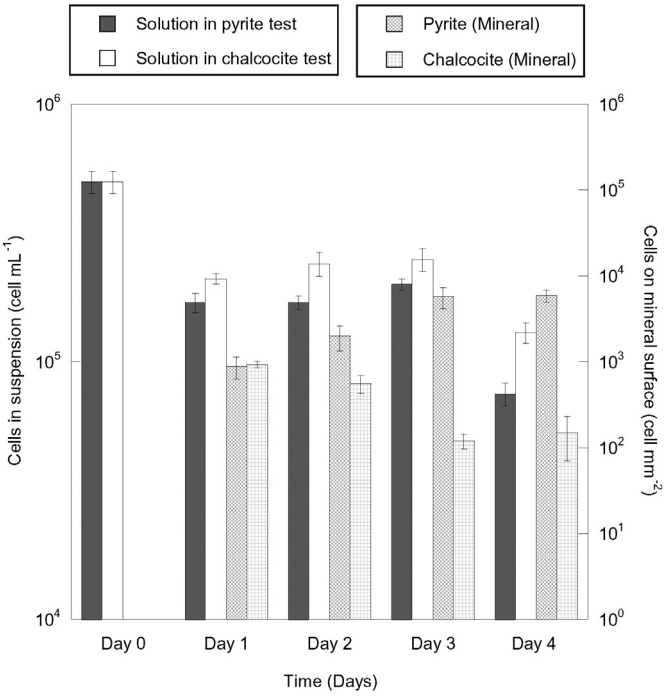

Fig. 3. Total cell counts by DAPI staining during the adhesion test: cells in the suspension (without iron added), on the left "y" axis, and attached to the minerals (chalcocite or pyrite), on the right "y" axis.

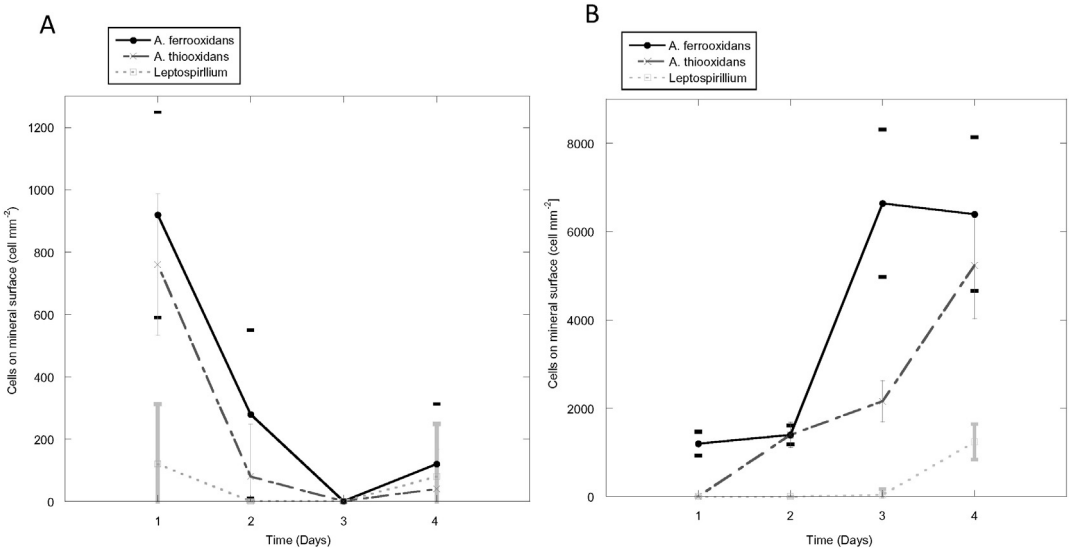

Fig. 4. Attached cell counts determined by CARD-FISH using specific probes for *A. ferrooxidans*, *A. thiooxidans* and *Leptospirillum* (Groups I and II). (A) Chalcocite; (B) Pyrite. No iron was added. Standard deviation bars are showed: No line (caps only), *Af*; black line, *At*; gray line, *Lf*.

the results. It is also possible to estimate the number of total cells attached using the number of cells per unit of area counted on the polished side of the fragment and the surface area of the pyrite fragment.

2.6. Measurement of Gibbs energy in cell adhesion

Strains of two closely related species of *Acidithiobacillus* were used: *Af*D2, belonging to *A. ferridurans* (formerly Group II of *A. ferrooxidans*), capable of resisting up to 325 mM of copper and *Af*DM belonging to *A. ferrooxidans* (formerly Group I), capable of resisting up to 100 mM of copper (Table 1). The Gibbs energy was calculated for each of the mineral–bacterial cells interactions. The methodology was based on the technique for measuring the contact angle of a water or culture medium droplet on the surface of the mineral (Ohmura et al., 1993). A stereoscopic microscope and a photographic camera mounted horizontally were used to take photographs of 3 μL droplets (n = 34) placed on the surface of both minerals using a micropipette, following the described methodology. Free energy was calculated using modified Young's equation (Li and Neumann, 1992).

$$\gamma_{lv}\cos\theta_Y = \gamma_{sv} - \gamma_{sl} \tag{1}$$

$$\cos\theta_Y = -1 + 2\sqrt{\frac{\gamma_{sv}}{\gamma_{lv}}}\cdot e^{-\beta(\gamma_{lv}-\gamma_{sv})^2} \tag{2}$$

$$\Delta G_{ADHESION} = \gamma_{mb} - \gamma_{ml} - \gamma_{bl} \tag{3}$$

where θ represents the angle between the solid–liquid interface and the liquid–vapor interface, and γ represents the surface tension between: lv = liquid–vapor, for water the reported value is 72.7 and for TK medium was 76.5; sv = solid–vapor and sl = solid–liquid (solid refers to bacteria and the mineral; liquid refers to water and culture medium). To be precise, β should be obtained empirically by means of substitutions and equation adjustments. However, this value has little effect on the final result; for comparative purposes, it may be considered constant and equal to 0.0001247 $(m^2\ mJ^{-1})^2$, which is the average of the tests carried out by Li and Neumann (1992) on different materials. To calculate Gibbs energy (Eq. (3)), the following surface tensions (γ) were considered: mb = mineral–bacterium; ml = mineral–liquid and bl = bacterium–liquid, as obtained using Eqs. (1) and (2).

To calculate Gibbs energy of cell adhesion between bacteria of the same type (colony formation), Eq. (3) would read as follows:

$$\Delta G_{ADHESION} = \gamma_{bb} - \gamma_{bl} - \gamma_{bl} \tag{4}$$

where γ_{bb} represents bacterium–bacterium tension and when Eqs. (1) and (2) calculations are used, the result is 0. Therefore, ΔG for this interaction would be:

$$\Delta G_{ADHESION} = -2\gamma_{bl}. \tag{5}$$

2.7. Effect of the substrate on microbial dynamics. Filter cultures

A volume containing a total of 10^8 cells of each strain was taken from a liquid culture medium. The strains *Af*D2 and *Af*DM were then separately filtered using 0.2 μm polycarbonate filters (2.2 cm diameter) which were placed in a Petri dish, on a synthetic plastic sponge (2.7 cm diameter, previously autoclaved) soaked in TK saline medium containing 2 g L^{-1} of Fe(II) (Fig. 2). The filters were arranged on the sponge so that the side containing the cells faced upward to ensure bacterial growth (Bellenberg et al., 2012; Echeverría and Demergasso,

Table 2
Comparison of the number of microorganisms per unit of area of pyrite predicted by means of BET against direct counts of free-cell using three different pyrite fragments. The BET area-mass value used for calculations was 83 ± 25 ($cm^2\ g^{-1}$)

Pyrite fragment	Mineral fragment weight (g)	Total area available for adhesion as estimated using BET (cm^2)	Total cells on mineral estimated by subtraction of counts in suspension (cells)	Cells attached to the mineral surface as predicted by BET (cells cm^{-2})	Direct cell count on the surface of the mineral (cells cm^{-2})
1	0.11	9.2	1.2E + 07	1.3E + 06	1.4E + 06
2	0.10	8.4	1.5E + 07	1.8E + 06	1.4E + 06
3	0.091	7.6	1.2E + 07	1.6E + 06	1.5E + 06

2011). For each of the nine days of the test, three replicate Petri dishes were set up. Each filter was divided into 4 sections. In one section, the filter surface (MO) was covered in finely ground mineral to assess inhibition by direct contact of cells with the mineral. In the second section, the ground mineral was placed between the filter and the sponge (MU) to determine if ions released by the mineral prevented bacterial growth even if there was no direct contact of the cells with the mineral. A 3 mm diameter mineral fragment (M), polished to a mirror-like surface (using 0.2 μm particle polishing paste) was placed on the third section to assess bacterial adhesion. The fourth section (F), without any mineral, was used as a control for bacterial growth. To verify the effect of Fe(II), three additional plates were set up without Fe(II) for each of the nine days of the test with chalcocite as the mineral substrate; this test was inoculated with *Af*DM. For this test, the filter surface was not covered in ground mineral; consequently, the filter was divided into three sections: MU, F and M.

The cells present in each of these sections were counted by means of DAPI staining and epifluorescence microscopy.

2.8. SEM–XRD analysis

Electron microscope with X-ray diffraction was used to map the copper distribution over polycarbonate filter. A filter with 1 mm spot of ground chalcocite on it edge was placed on a sponge soaked with liquid medium with Fe(III) (2 g L^{-1}) in a Petri dish. After 10 days of incubation, the filter was let dry and were mounted on an epoxy resin brick (Epoxy Cure® 2 Epoxy System, Buehler, USA) and coated with carbon by sublimation. Copper distribution was mapped on the whole filter to determine differences in copper concentration by distance from spot.

3. Results and discussion

3.1. Attached-cells count in iron free medium by means of DAPI staining

The highest percentage of total cell adhesion occurred during the first day: a total of about 1000 cells mm^{-2} was observed representing 66% of the total cells originally in suspension for pyrite and 58% for chalcocite. In the glass controls, the number of microorganisms in suspension varied less than 2% during the first day (data not shown). The number of suspended cell does not show significant variation during the following days (Fig. 3).

3.2. Specific count of attached cells in iron free medium by means of CARD-FISH

The ratios of *A. ferrooxidans*, *A. thiooxidans* and *L. ferriphilum* in the communities attached to chalcocite without iron II supplement varied throughout the test from the initial inoculation, which shows that there is differential adhesion to the same mineral as a result of microorganism selection (Fig. 4). All three organisms can be detected from the first day; however, the numbers of identifiable microorganisms decreased with time until they disappeared completely on about the third day. On the fourth day, all the three members of the bacterial community were again attached to the surface but were widely scattered. Recolonization by free or detached cells after the first stage of copper dissolution could explain this feature.

In the case of pyrite, there was a steady increase of attached bacteria during the four days of the experiment; the numbers were an order of magnitude greater than the numbers observed for chalcocite. These results suggest that chalcocite dissolution has an impact on bacterial adhesion for the three species studied, though two of them initially attached better to chalcocite than to pyrite.

The decrease of cells in solution noted on the first day of the test coincides with the appearance of cells attached to both minerals (Fig. 3), which means that the cells are gradually becoming attached to the mineral surface. The increase of attached cells during the following days did not coincide with a significant decrease of the cells in the solution, which would indicate cell division on the surface of the minerals. A decrease in the number of attached cells would result from cell detachment.

Table 3
Average values for the hydrophobicity of minerals and cells determined empirically and expressed in terms of the contact angle measured and the solid–vapor surface tension calculated.

Mineral	Contact angle (θ)		γ_{sv} (mJ m^{-2})	
	Water	TK medium	Water	TK medium
Pyrite	52.1 ± 4.5	74.6 ± 2.3	52.1	38.7
Chalcocite	80.0 ± 4.8	95.5 ± 4.2	34.8	25.5
*Af*D2	30.0 ± 3.7	31.0 ± 4.2	64.4	63.9
*Af*DM	43.3 ± 2.4	44.9 ± 3.7	57.4	56.6

3.3. Total attached cell estimation

The cell counts on polished pyrite showed practically no variation between replicates. The value obtained for the three mineral fragments using the BET technique was 83 cm^2 g^{-1}, with an error of 25 cm^2 g^{-1} due to the fact that the solids were slightly porous and fell close to the detection limits of the equipment used. The number of cells per unit of area present on the mineral surface was estimated using the 83 cm^2 g^{-1} ± 25 cm^2 g^{-1} value and the difference between the initial and final numbers of the cells in solution (Table 2). The number of attached cells calculated for each of the three tests was quite close to the number obtained by direct cell counts on the surface. This confirms the assumption that the decrease in the cell number in the solution corresponds to the cells attached to the mineral. However, according to the descriptions of Edwards and Rutenberg (2001), bacteria adhere preferably to rougher surfaces of the mineral. For this reason, by counting cells on polished surfaces, an underestimation of the total cell count should be expected.

3.4. Hydrophobicity calculation

According to the solid–vapor tension calculations, based on the contact angle, chalcocite is lower than pyrite hydrophobicity and *Af*DM has the lowest hydrophobicity value of the two bacteria (Table 3). This means that, in an aqueous medium, chalcocite and *Af*DM would tend to associate. Bacterial adhesion thermodynamics indicate that when ΔG is negative for all interactions, the lower values would represent the greater capacity to adhere to the mineral or to form cell groups (Liu et al., 2004a; Liu et al., 2004b). According to the values calculated in Tables 3 and 4, chalcocite would be the most hydrophobic mineral and the adhesion between both minerals and *Af*DM is the most thermodynamically favored.

The number of *Af*DM attached cells is significantly higher on chalcocite than on pyrite in the first day of the experiment (Fig. 5C vs D). However, there is not a significant difference between the numbers of *Af*D2 attached cells on both minerals in the same time (Fig. 5C vs D). Those results are predicted by hydrophobicity calculation (Tables 3 and 4).

Table 4
Adhesion free energy calculated for the different bacterium–mineral combinations used in this study.

$\Delta G_{ADHESION}$ (mJ m^{-2})	*Af*D2		*Af*DM	
	Water	TK medium	Water	TK medium
Pyrite	−6.0	−6.0	−11.4	−15.4
Chalcocite	−9.2	−8.9	−18.3	−20.4
*Af*D2	−2.9	3.4	–	–
*Af*DM	–	–	−8.8	−4.7

Data for this table were calculated using information from Table 3.

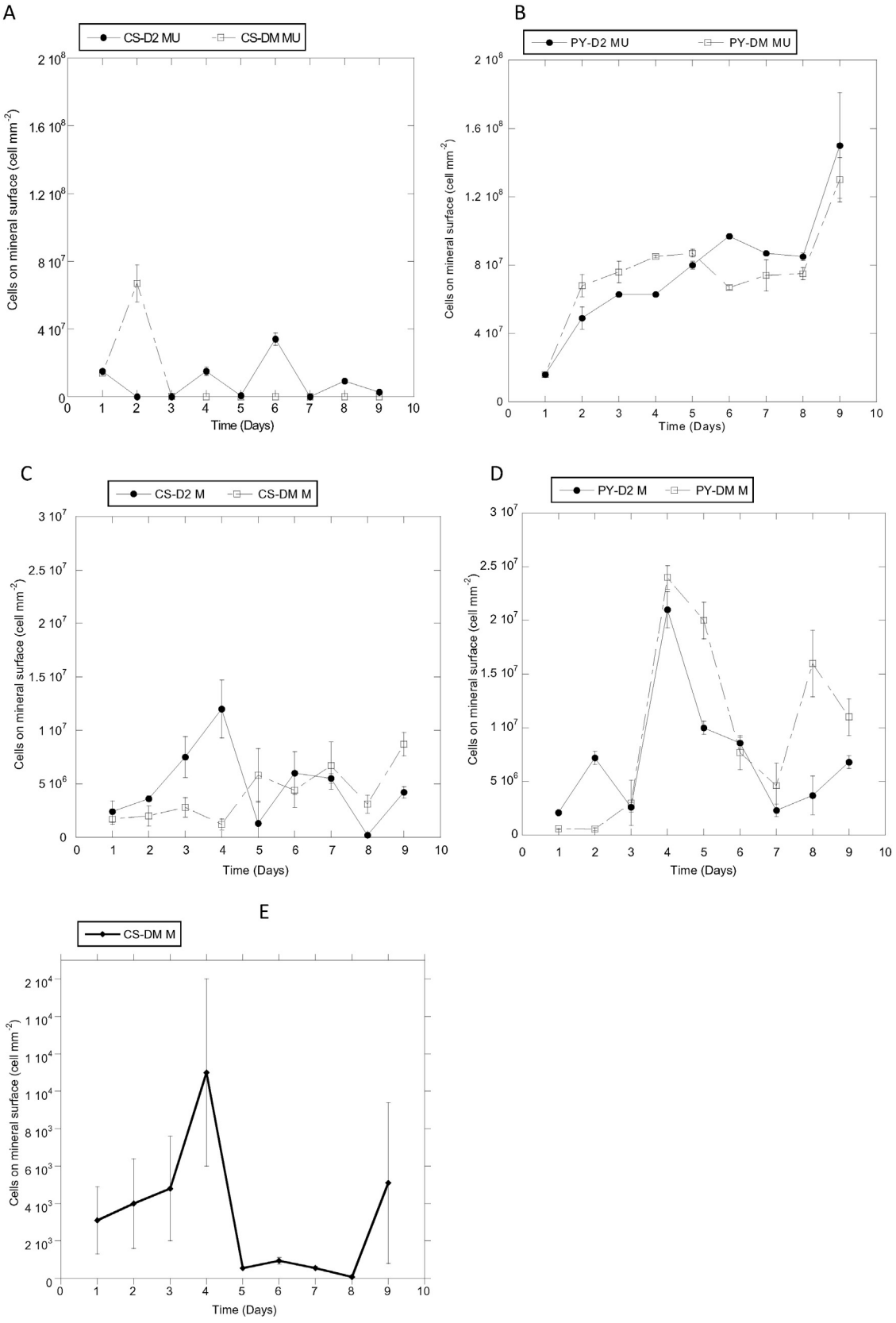

Fig. 5. Adhesion test and mineral resistance in the bacterial growth test on floating filters. CS = chalcocite, PY = pyrite; D2 = *Af*D2, DM = *Af*DM strains; MU = ground mineral under the filter; M = mineral fragment. (A) Bacteria on filter with ground chalcocite under filter MU. (B) Bacteria on filter with ground pyrite under filter MU. (C) Bacteria on chalcocite mineral with iron II added. (D) Bacteria on pyrite mineral with iron II added, (E) Bacteria on chalcocite mineral without iron II added. Bars are standard deviations.

However, there is not a significant difference between the numbers of *Af*D2 attached cells on both minerals. The existence of dense cellular colonies separated by void spaces has been reported previously for *A. ferrooxidans* (Florian et al., 2011) and explains the high standard deviation value for cell counts on the chalcocite surface. In addition, those results suggested that hydrophobic interactions are not the only responsible factor for the adhesion of *A. ferrooxidans* to particular mineral surfaces as previously reported (Sampson et al., 2000). The occurrence of colonies is an indication of growth and cell division on the surface of the mineral.

3.5. Inhibition tests on filter

Control filters without cells were used to determine dispersion of soluble copper when chalcocite was exposed to acidic ferric sulfate solution. Mapping of the filters surface with SEM–XRD shows a decreasing concentration of copper salts with distance, having the highest levels where chalcocite spots were placed. These results are in accordance with those predicted by the Fick's Law (salt concentration is inverse to the distance from the origin); they also show that solutions are not homogeneous and have big variations on copper concentration depending on distance from the source.

The number of cells of both strains (*Af*DM and *Af*D2) with Fe(II) added increased on the filter with ground pyrite, while it remained almost constant with ground chalcocite (Fig. 5A and B).

The cells directly attached to the pyrite surface reached higher number than the cells attached to the chalcocite fragment (Fig. 5C and D). Nevertheless, the difference of attached cells of both strains onto the mineral surface (Fig. 5C versus D) is lower than the one observed on the top of the filter (Fig. 5A and B).

Those results are consistent with a toxicity response of bacteria against Cu (Alvarez and Jerez, 2004; Dopson et al., 2003), which consider that there is less mineral surface area exposed on the mineral fragment than on the ground mineral and then less Cu is released. The effect observed is also related to the level of Cu tolerance that is higher for *Af*D2 (Fig. 5A and C). In addition, according to the data, pyrite would not inhibit the growth of these two strains attached to the adjacent filter section with no direct contact.

In general, adhesion dynamics to pyrite follow a similar pattern for both strains (Table 1). Adhesion on pyrite fragment on the filter reached a maximum on the fourth day for *Af*D2 and *Af*DM, slowly decreasing in both cases and increasing again after the seventh day (Fig. 5D).

The number of cells on the chalcocite surface shows similar stages as those presented on pyrite fragments.

The results confirmed the occurrence of cell detachment through the quantitative reduction in the number of cells on the mineral surface. Cell detachment from pyrite surface has been suggested through microscopic observations (cell numbers were not available on the surface of the mineral) and has been explained because of the increase in the Fe(II) concentration due to mineral oxidation (Milic et al., 2012). Then free cells oxidize the Fe(II) in the solution. The reduction in the number of attached cells was more pronounced in chalcocite (Fig. 5C). That feature could be explained due to the fact that the slime formed on the surface of the chalcocite is non-adherent and it is breaking away from the mineral fragment, as has been previously described (Adewale-Bolorunduro, 1999) and was confirmed through our experiments (Fig. 6). In addition, the gradually increase in the Cu concentration during chalcocite leaching and a chemotactic response of *At.f.* would be also what causes the difference observed between *At.f.* detachment from pyrite and chalcocite (Fig. 5C and D).

Cell growth onto the mineral surface was also confirmed by cell number determination and by colony formation (Fig. 5C, D and E). *Af*D2 microorganisms tend to form smaller colonies (Fig. 7). This is *A. ferridurans* (formerly belonging to Group II of *A. ferrooxidans*), which have a polar flagellum and the cell-to-cell adhesion energy (ΔG) is positive in the culture medium used (Table 4). These features mean that they do not tend to stay gathered and can react faster than *Af*DM to unfavorable micro-environmental conditions. Consequently, toward the end of the test, *Af*DM with the highest measured hydrophobicity (Liu et al., 2004b) (Table 4) and the most negative cell-to-cell adhesion (ΔG) calculated forms larger and more stable colonies than *Af*D2. The attachment–detachment–attachment cycle (Fig. 5C, D and E) fits well with the observed dissolution pattern of chalcocite explained by the shrinking core and particle model (Ruan et al., 2010) proposed by Braun et al. (1974).

In the presence of chalcocite, the effect was completely different. Chalcocite lixiviation depends largely on the presence of catalytic microorganisms attached to the mineral surface (Ruan et al., 2010). Nevertheless, strains are strongly inhibited by this mineral, even when there is no direct contact of the organisms with chalcocite (Fig. 5A). Variations in the number and ratio of the microorganisms present in the solution (observed prior to exposure by CARD-FISH), and variations in cell counts over time (Figs. 3, 4 and 7) occurred in response to the copper released into the solution (Cheng and Lawson, 1991; Leahy et al., 2007; Ruan et al., 2010). In industrial processes, this phenomenon may be associated with the ionic release and the appearance of bacteria in the leach solution (Rodríguez et al., 2003). This diffusion increases copper concentrations adjacent to the microbe–mineral contact zone. This would generate a toxic microenvironment that exceeds the levels tolerated by the studied strains (Table 1). This in turn affects the adhesion capacity and growth of these microorganisms and would probably trigger cell detachment. Chemotaxis responses allow motile microorganisms to rapidly move toward a microenvironment optimal for their growth and survival. The cyclic behavior may be caused by structural changes in the outer layers of chalcocite (Adewale-Bolorunduro, 1999; Leahy et al., 2007). A thin solid crust was formed on the filter and over the mineral surface, between the third and fourth day of the test. This crust came off on the fifth day (Fig. 6), coinciding with the dissolution model for chalcocite (Cheng and Lawson, 1991; Leahy et al., 2007; Ruan et al., 2010). Non-motile microorganisms with high ΔG for adhesion are strongly adhered to mineral surface, thus highly affected by toxic microenvironment. Detachment of these microorganisms is proposed as a passive mechanism depending on the layer release of the mineral. The growth dynamics seen during the adhesion on the filter test without Fe(II) are similar to that observed during the liquid medium adhesion test, in which a small number of microorganisms were grouped in isolated colonies (Fig. 7). No cell growth was seen on the surface of the filter; no cells were counted after the second day (graphs not shown). Our results showed that iron is fundamental to forming *Acidithiobacillus* biofilms on chalcocite (Fig. 5). The presence of ferrous iron has also

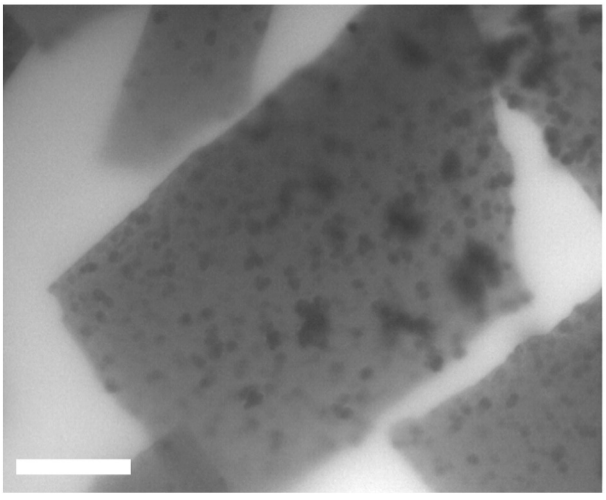

Fig. 6. Crust detachment on the fifth day of chalcocite test. Black dots were related to bacterial attachment sites. Bar 10 μm.

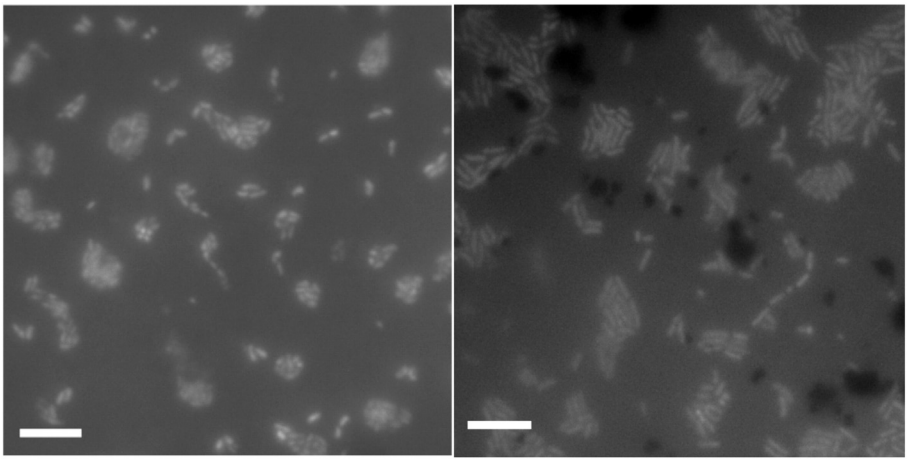

Fig. 7. Cell and colony morphological differences for *Af*D2, on the left, as observed on the surface of the filter, on the right, and *Af*DM. Bar 5 μm.

been reported as necessary for the initial formation of *A. ferrooxidans* biofilms (Dong et al., 2013). Previous reports have also suggested that ferrous iron at high levels would inhibit adhesion of *A. ferrooxidans* to iron containing minerals (Dong et al., 2013; Sand et al., 2001; Third et al., 2000). The present study showed that pyrite stimulates bacterial adhesion and increases the number of attached cells without generating toxicity; cell detachment is less and likely dependent on the behavior of the biofilm monolayers formed by the cells (Liu et al., 2011).

More studies are necessary to determine the effects in copper recovery using mixed industrial minerals, considering mineral–microorganism affinity and reactivity.

4. Conclusions

The behavior of bioleaching microorganisms and their interaction with each of the minerals present in an ore are extremely important, but have hardly been studied. Using various approaches to understand the factors that affect leaching processes at microscopic level will help to establish the fundamental knowledge for studying the phenomena observed at the industrial level. This investigation has assessed the effect of intrinsic factors of microorganisms and properties of minerals. It is necessary to consider the combined effect of both kinds of factors, since none of them could explain the phenomena separately.

- From the mineral point of view, hydrophobicity was found to be one important factor in the early stages of the bacteria–mineral interaction because it determines the free energy of the adhesion process. After this stage, the fluctuations and differences observed between bacterial adhesion to pyrite and to chalcocite are related with differences in the ionic contribution of each mineral and, in the case of chalcocite, the dissolution kinetics and release of the oxidation products.
- From a biological point of view, bacterial properties such as chemotaxis, motility and copper resistance, determine the grade and the stability of the attachment.

Acknowledgments

This work has been supported by Biotecnor Ltda., grant number BTL012015AE. We thank Dr. Michael Schlöemann and Dr. Corale Brierley for critical reading of the manuscript. We thank Mr. Luis Campos from the Mineralogical Museum, Universidad de Atacama, Chile, for his generous gifts of chalcocite samples used in this study.

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