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Effects of different energy sources on cell adhesion and bioleaching of a chalcopyrite concentrate by extremophilic archaeon *Acidianus copahuensis*

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ABSTRACT

Bioleaching is an alternative technology for recovering metals from mineral ores; thermophilic microorganisms instead of mesophiles can greatly improve metal solubilization, particularly from refractory mineral species. *Acidianus copahuensis* is a new species of thermophilic archaea, recently isolated in our laboratory. The effect of culture growth history on the attachment of *A. copahuensis* to a chalcopyrite concentrate was investigated in shake flasks at 65 °C. Cells adapted to growth with chalcopyrite as energy source showed higher attachment to the mineral concentrate. *A. copahuensis* cells reached 100% of copper extraction in the bioleaching of chalcopyrite concentrate carried out in shake flasks cultures incubated at 65 °C and pH 2.0. This high bioleaching yield was achieved even at that not so low initial pH value probably because this archaeal species is able to form sufficient amounts of ferric iron but keeping low redox potential (Eh) values. This work also describes the effect of the addition of other energy sources on the bioleaching activity. *A. copahuensis* achieved the best copper extraction without any addition but copper extraction decreased when alternative substrates were added.

1. Introduction

In the last decades there has been much interest in the development of biohydrometallurgical methods for metal extraction from sulfide minerals due to their advantages over pyrometallurgical and hydrometallurgical techniques (Johnson, 2014; Schippers et al., 2014). This stems from the capacity of biohydrometallurgy to be technically and economically employed for processing ore deposits of declining metal content (low-grade ores) and increasing complexity (refractory ores); these characteristics are realities in today's mining world as higher grade resources and easy-to-process ore deposits are being rapidly depleted (Brierley and Brierley, 2013). Metals for which this technique is mainly employed include copper, cobalt, nickel, zinc and uranium (Schippers et al., 2014).

Among metal sulfides that are oxidized by acidophilic microorganisms, chalcopyrite ($CuFeS_2$) has received special attention as it is one of the most abundant and wide-spread copper-bearing minerals, accounting for approximately 70% of the Earth's copper (Li et al., 2013). However, bioleaching of chalcopyrite on a commercial scale is still not fully developed due to its extremely slow kinetics and poor copper recovery (Li et al., 2013; Watling, 2013). Since it happens also in chemical leaching, passivation of chalcopyrite caused by the accumulation of intermediates such as copper polysulfide, sulfide, elemental sulfur, iron-hydroxy precipitates such as jarosite in ferric media and goethite and/or hematite in ferrous media could explain the stopping of the dissolution. Various methods have been proposed to enhance the bioleaching efficiency of chalcopyrite, spotlighting bioleaching at elevated temperatures using thermoacidophilic microorganisms which, in turn, results in faster reaction rates and less refractory character (Watling, 2006). At elevated temperatures, some archaeal genera including *Acidianus*, *Metallosphaera*, and *Sulfolobus*, all belonging to the order *Sulfolobales*, appear to be the most important bioleachers (Rodríguez et al., 2003a; Vilcáez et al., 2008).

However, although thermophiles have led copper extractions close to 100% in many lab studies, there are many other studies where lower copper extractions were obtained. Actually the precipitation of ferric ion as jarosite seems to be the cause of the passivation in the last cases; the formation of jarosite is inherent to the utilization of thermophiles since they oxidize sulfur to form sulfate which readily precipitates with ferric ion as jarosite in the range of 60–80 °C and pH values in the range of 1.5–2.5. Some researchers have proposed that the dissolution of chalcopyrite can be accelerated by controlling redox potential at a relatively low value (about 400 mV seems to be the critical maximum Eh) where passivation by jarosite is inhibited and allow higher chalcopyrite dissolution (Zhao et al., 2015). Vilcáez et al. (2009) have shown that leaching of chalcopyrite is efficient at an initial pH 1.0 and at thermophilic temperatures (65–80 °C) with small amount of ferric ion even in the absence of thermophiles, demonstrating that the abiotic sulfur oxidation and chalcopyrite dissolution at high temperatures is feasible. However, at higher pH values the activity of microorganisms seems to be indispensable. That is why, probably thermophiles with high sulfur

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oxidation and poor (but existent) capacity to form ferric ion (in order to generate not so high Eh values) could be more adequate for chalcopyrite dissolution.

Recently, we have isolated a new species of thermophilic archaea belonging to *Acidianus* genera from the geothermal Caviahue-Copahue system, located in the north-west of Neuquén Province, Argentina (Giaveno et al., 2013). *Acidianus copahuensis* has broad metabolic versatility. It grows on sulfur, tetrathionate, ferrous iron, and glucose under aerobic conditions; but it can also develop under anaerobic conditions. Furthermore, these cells can preferentially oxidize sulfur instead of iron which means Eh does not rise so fast in *A. copahuensis* cultures, and making them probably suitable for the bioleaching of chalcopyrite.

On the other hand, although several other factors affect the bioleaching process the attachment process of leaching microorganisms to the mineral surface —mediated by extracellular polymeric substances (EPS) surrounding the cells — is surely one of the most important factors. This conclusion has been mainly and almost clearly achieved for mesophilic bacteria (Vera et al., 2013) but there is little information about attachment and interfacial processes of archaea, and none for *A. copahuensis* has been reported until now.

The main aim of this work is to study the chance to use the new thermoacidophilic archaeon *A. copahuensis* to solubilize chalcopyrite taking advantage of its capacity of keeping low Eh values. The influence of the addition of other substrates is also studied. Finally it also aims at studying the attachment of cells previously grown on different energy sources.

2. Materials and methods

2.1. Mineral

The main chemical composition of the concentrate is (*w*/w): 24.20% Cu, 31.15% Fe, 0.76% Mo, 0.56% Zn, 0.26% Pb, 0.02% Ag, 0.002% Ni and 0.002% Au. X-ray diffraction (XRD) and Rietveld analysis show that the content of the mineral sample was 77.4% chalcopyrite ($CuFeS_2$) and 19.6% pyrite (FeS_2) as the major mineral phases, and 2% molybdenite (MoS_2), and <1% sphalerite (ZnS) as the minor phases. The fraction with particle size <62 μm was used in the experiments. The specific surface area of the mineral was 5.36 m^2/g (BET surface area).

2.2. Strain and culture conditions

A. copahuensis strain ALE1 DSM 29038 (Giaveno et al., 2013) was cultivated in flasks containing Mackintosh basal salt solution (MAC) (Mackintosh, 1978) at pH 2 supplemented with 10 g/L elemental sulfur (S°) powder, 3 g/L potassium tetrathionate ($K_2S_4O_6$), 6 g/L ferrous iron as $FeSO_4 \cdot 7H_2O$, 1% (*w*/*v*) chalcopyrite concentrate, 1 g/L glucose, and/or 1 g/L yeast extract as energy source. Flasks were incubated aerobically at 65 °C and 120 rpm. After growth, cultures were filtered and cells were harvested by filtration and centrifugation at 8000 rpm for 10 min. Cell pellets were washed twice with MAC solution in order to remove any trapped ions and then resuspended in fresh MAC solution.

2.3. Attachment experiments

Attachment experiments were performed in 100 mL flasks, each containing 50 mL MAC solution pH 2 with 5 g of mineral concentrate, and an initial cell number of 5×10^8 cells/mL. As inocula, cultures of *A. copahuensis* grown on different energy source (elemental sulfur, potassium tetrathionate, ferrous iron, and mineral concentrate in mixotrophic conditions with yeast extract additions; or in heterotrophic condition using just glucose) were used. Flasks were incubated at 65 °C with shaking at 120 rpm. Aliquots of 1 mL were taken at 0, 5, 10, 20, 30, 60, 90, 120, 180, 240, 300, 360, and 420 min. Cell number was determined in the liquid phase with a counting chamber by using a phase-contrast microscope. The amount of cells attached to the mineral surface was calculated as the difference between the number of initial cells inoculated and the number of planktonic cells. In order to test the attachment to the glass wall, some flasks were inoculated without mineral. Unspecific attachment values were discounted from the total attachment values to obtain the specific attachment to the mineral under study.

2.4. Bioleaching experiments

Cells used in bioleaching tests were previously transferred several times in chalcopyrite concentrate medium to adapt the microorganisms to the experimental conditions. Adaptation of *A. copahuensis* to grow on chalcopyrite concentrate as energy source was done by sub-culturing in MAC medium supplemented with 1% (*w*/*v*) chalcopyrite concentrate and 1 g/L yeast extract. Thermophiles were harvested by filtration and centrifugation (8000 rpm), washed twice with MAC solution (pH 2) and resuspended in fresh medium. Bioleaching experiments were carried out in sterile 250 mL narrow-neck Erlenmeyer flasks each containing 150 mL MAC medium at pH 2 supplemented with one or more energy sources (1 g/L elemental sulfur, 3 g/L potassium tetrathionate, 1 g/L ferrous iron, 1 g/L glucose and/or 1 g/L yeast extract), pulp density of 2% (*w*/*v*) and an initial cell population of 1×10^7 cells/mL. Flasks were incubated at 120 rpm on an orbital shaker in the dark at 65 °C. Each bioleaching condition was conducted in duplicate. Sterile controls were performed replacing the inoculum by the same volume of a thymol 2% (*w*/*v*) in methanol solution. Periodically, distilled water was added to the flasks in order to compensate for evaporation losses.

To determine leaching efficiency, sample solutions were routinely withdrawn from each flask to analyze Fe, Cu, pH, and redox potential (Eh) at regular intervals. The amounts of Cu and total soluble Fe released during bioleaching were determined on filtered samples by atomic absorption spectrophotometry (AAS). Ferrous iron concentrations were quantified by spectrophotometric *o*-phenanthroline method (Kolthoff et al., 1998).

Mineralogical analysis of the concentrate and leached residues were performed using XRD. Solids were retrieved by filtering leach solution samples through Whatman No. 42 cellulose filter paper. The residues were washed with distilled water (pH 2) and dried at 65 °C and then analyzed with a Philips 3020 X-ray diffractometer using CuKα radiation. The operational conditions were 50 kV and 80 mA. Steps scans were conducted from 5 to 70° in 0.02° increments using 1 s count time.

2.5. Scanning and transmission electron microscopy (SEM/TEM)

Leached residues were taken after 50 days of experiment to analyze mineral surface changes during leaching. Samples were fixed with glutaraldehyde 2.5% (pH 7.2) for 1 h at 4 °C, post-fixed with 2% osmium tetroxide and dehydrated in a graded series of alcohol (25–100%). Dehydrated samples were dried in a critical point chamber, coated with a thin layer of gold and visualized by a LEO EVO 40 XVP scanning electron microscope (SEM).

Microorganism ultra-structures and morphological characteristics of *A. copahuensis* were studied by transmission electron microscopy (TEM). Planktonic cells were fixed and post-fixed as described for SEM, and then embedded in Spur resin. Ultra-thin sections were stained with uranyl acetate and lead citrate and viewed in a JEOL CX II TEM. Basic visualization and measurement of cells were performed using sets of at least ten images of each sample with the software ImageJ.

3. Results and discussion

3.1. Attachment tests

Data from the attachment of *A. copahuensis,* cultured under different growth conditions, to a chalcopyrite concentrate are shown in Fig. 1 (outer graph indicates the attachment percentages while the inner graph shows a representative attachment kinetics). In all conditions,

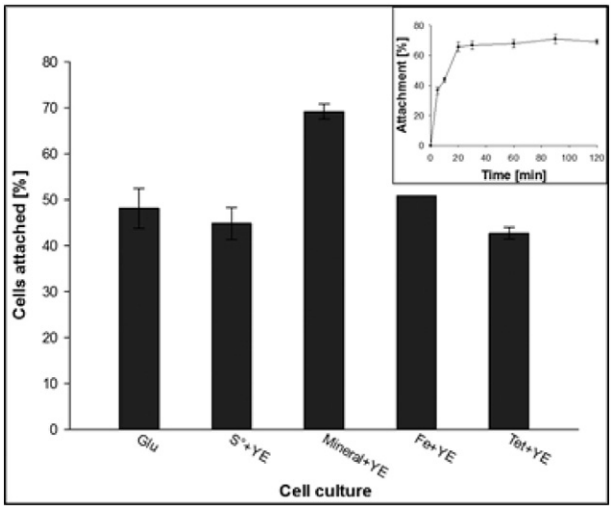

Fig. 1. Attachment of *A. copahuensis* cells to a chalcopyrite concentrate, cultured on five different growth conditions: glucose (Glu), elemental sulfur and yeast extract (S° + YE), chalcopyrite concentrate and yeast extract (Mineral + YE), ferrous iron and yeast extract (Fe + YE), potassium tetrathionate and yeast extract (Tet + YE). Top right graph shows the representative attachment kinetics of *A. copahuensis* cells to a chalcopyrite concentrate during the first 120 min. Error bars show standard deviation from three independent experiments (n=3).

the attachment to the glass wall was between 10–15%. The main attachment of cells to the mineral under study was noticeably high during the first 30 min of contact between the microorganisms and the mineral particles. In all experiments, the attachment rates for this mineral decreased significantly after 30 min, with the equilibrium phase being established 120–180 min after incubation. This trend of rapid attachment was reported by other researchers with different microorganisms and mineral substrates (Afzal Ghauri et al., 2007; Bromfield et al., 2011; Harneit et al., 2006; Rodríguez et al., 2003b).

The maximum level of attachment, determined once the equilibrium had been achieved, was comparable to when cells were pre-grown in mixotrophic conditions using sulfur and potassium tetrathionate as energy source, reaching approximately 45% of microbial adsorption. Cell attachment to the mineral was estimated to be 48% and 51% when cells were pre-grown with glucose and ferrous iron as electron donors, respectively. The largest attachment was 69% and was achieved with chalcopyrite adapted cells.

Previously, similar tests were performed to analyze the *A. copahuensis* attachment to pyrite (Castro et al., 2013). Although the exposed surface area of chalcopyrite concentrate in the current experiments was larger than that of pyrite in the previous experiments (unpublished data), in both cases the mineral surface area available for microbial attachment was in great excess of the maximum cell loading possible. Assuming that the available surface area was not a limiting factor, the highest *A. copahuensis* affinity for pyrite (43%) was less pronounced than that for chalcopyrite concentrate (69%). These results suggest that the attachment extension of *A. copahuensis* is mineral selective. Besides *A. copahuensis*, other thermophiles exhibited a preferential attachment to chalcopyrite over pyrite (Bromfield et al., 2011; Rodríguez et al., 2003a), which may be due to the different crystal structures (Zhu et al., 2008). Cell attachment to specific sites on the mineral surface is principally related to different attractants, most likely caused by charge imbalances on the surface; also hydrophobic interactions and covalent bonds play an important role mediating the microbial attachment to metal sulfide surfaces (Vera et al., 2013).

The growth medium used to cultivate *A. copahuensis* clearly affects its ability to attach to the chalcopyrite surface. A comparative overall pattern was shown by *A. copahuensis* in previous tests performed to analyze the attachment to pyrite (Castro et al., 2013). The results indicated an increased microbial attachment for inocula pre-adapted to the minerals; i.e., higher degrees of attachment to pyrite and chalcopyrite concentrate were achieved by *A. copahuensis* cultures pre-adapted to pyrite and chalcopyrite, respectively. Studies carried out with *A. copahuensis* showed that higher cell attachment to pyrite surface resulted in an enhanced pyrite leaching (Castro et al., 2013). In the same way, a direct relationship between cell attachment to the mineral surface and mineral dissolution rates has been reported for other acidophilic microorganisms (Gautier et al., 2008; Rodríguez et al., 2003c). In addition, acclimation of microorganisms to the leaching environments (i.e., high metal levels, temperature, pulp density, pH) is a well-known method for increasing microbial tolerance to such conditions, and many researchers have adopted this strategy to enhance bioleaching efficiency (Abdollahi et al., 2014; Vilcáez et al., 2008). These results suggest that it may be possible to influence *A. copahuensis* to attach to a particular mineral system through manipulation of culturing conditions, in order to enhance their bioleaching performance.

Some insights into the effects of substrates on cell morphology were observed in *A. copahuensis* through TEM. Fig. 2 shows representative views of thin sections of planktonic *A. copahuensis* cells in different growth conditions: autotrophic (elemental sulfur or potassium tetrathionate), mixotrophic (elemental sulfur or potassium tetrathionate, both supplemented with yeast extract), and heterotrophic (glucose) conditions. Most cells of *A. copahuensis* grown using different substrates were similar and displayed a pleomorphic shape with slightly variable dimensions; however, cells grown autotrophically on elemental sulfur were characterized by an oval morphology.

Also, it is possible to appreciate details of the archaeal cell envelope formed by a single membrane and covered by a paracrystalline glycoprotein layer (*S*-layer) (Giaveno et al., 2013). Capsules of *A. copahuensis* were observed covering the cells uniformly but differing in thickness and appearance. Capsules of cells growth in glucose, potassium tetrathionate (both autotrophic and mixotrophic) and mixotrophically on sulfur were similar in thickness (34–45 nm). There was a significant variation in the cell envelope observed for cells of *A. copahuensis* grown autotrophically on sulfur, with capsules extending 76 ± 8 nm away from the cell. They were compacted and appeared as a relatively dense mantle around the cells (Fig. 2a), that seems to contain a large amount of sulfur particles as it has been observed for other species growing on elemental sulfur.

Cell surface properties play an important role in the attachment process. EPS is well known to mediate the attachment of microorganisms to mineral surfaces. Previous studies in our laboratory have proved that *A. copahuensis* cells attach to diverse mineral surfaces and that these cells produce EPS (Fig. 3) (unpublished data). The EPS production greatly differs for different microbial species (Afzal Ghauri et al., 2007; Harneit et al., 2006) and also varies in composition according to the growth substrate (Liu et al., 2011; Tan and Chen, 2012), affecting the cells' surface properties. Previous reports have established that EPS production is enhanced during growth on solid substrates (Bellenberg et al., 2012; Gherke et al., 1998; Harneit et al., 2006). The fact that *A. copahuensis* adhesion is influenced by the cells' growth conditions might be due to variations in the composition and amount of their EPS. However, this assumption needs further biochemical evidence.

3.2. Bioleaching of a chalcopyrite concentrate

A. copahuensis was used to leach a chalcopyrite concentrate. The copper extraction (%), dissolved iron, Eh, and pH were monitored over a 120 days period at 65 °C. With the aim of investigating the effects of different energy sources on bioleaching of a chalcopyrite concentrate, a total of 14 bioleaching conditions were tested with *A. copahuensis*. To contrast chemical and microbial leaching, some tests were conducted without microorganisms; data were compared with the results obtained for inoculated systems.

In abiotic controls, the amount of Cu release from the mineral was very low and did not increase significantly over time (Fig. 4, inner graphs). At the end of the experiment, only 20% and 10% of Cu was

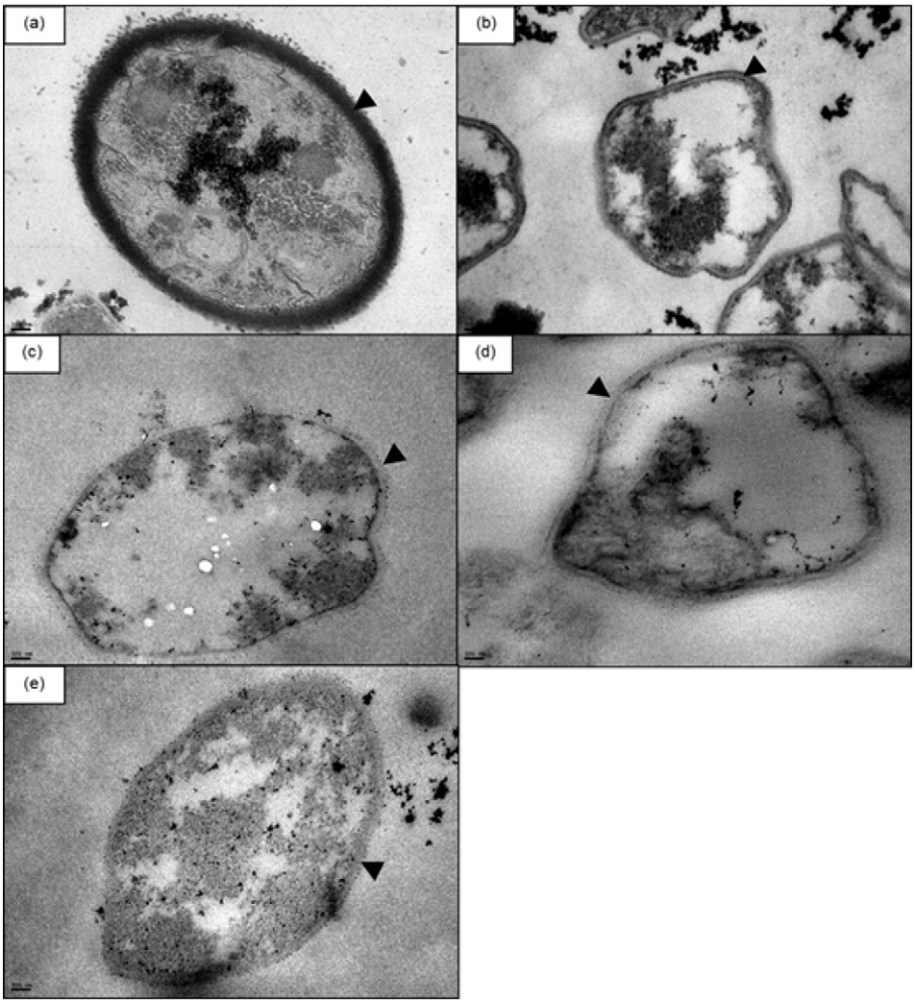

Fig. 2. Electron micrographs of ultrathin sections of *A. copahuensis* cells grown in different conditions: (a) elemental sulfur, (b) elemental sulfur and yeast extract, (c) potassium tetrathionate, (d) potassium tetrathionate and yeast extract, and (e) glucose. Arrows point cell envelopes. Size bars represent 100 nm.

abiotically leached from the non-inoculated controls with and without ferrous iron addition, respectively. The difference generated by the addition of ferrous iron is probably related to its slow abiotic oxidation and the consequent attack on the mineral. Galvanic effects caused by the direct contact between chalcopyrite and pyrite would be contributing on chalcopyrite leaching (Li et al., 2013).

Fig. 4 illustrates that *A. copahuensis* greatly enhanced copper extraction from chalcopyrite, reaching practically 100% of copper dissolution in tests without additional substrate; this first report for this species is in agreement with other researchers who reported similar results for other extreme thermophiles (Abdollahi et al., 2014; Konishi et al., 2001; Liang et al., 2014; Rodríguez et al., 2003a; Vilcáez et al., 2008;

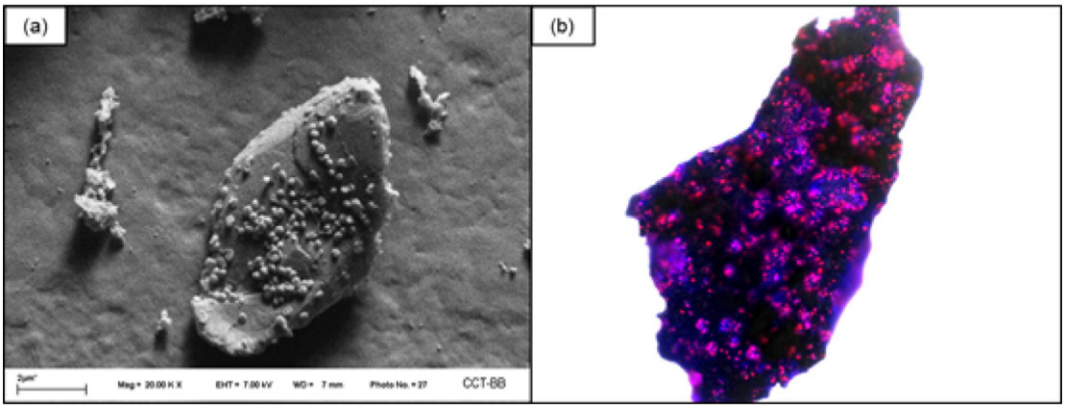

Fig. 3. Cells of *A. copahuensis* attached to mineral surfaces. (a) Cells attached to a low-grade zinc sulfide ore visualized by SEM; (b) Cells attached to a pyrite grain visualized by combined EFM, cells were stained with DAPI (blue) and TRITC-ConA (red) specifically bound to EPS.

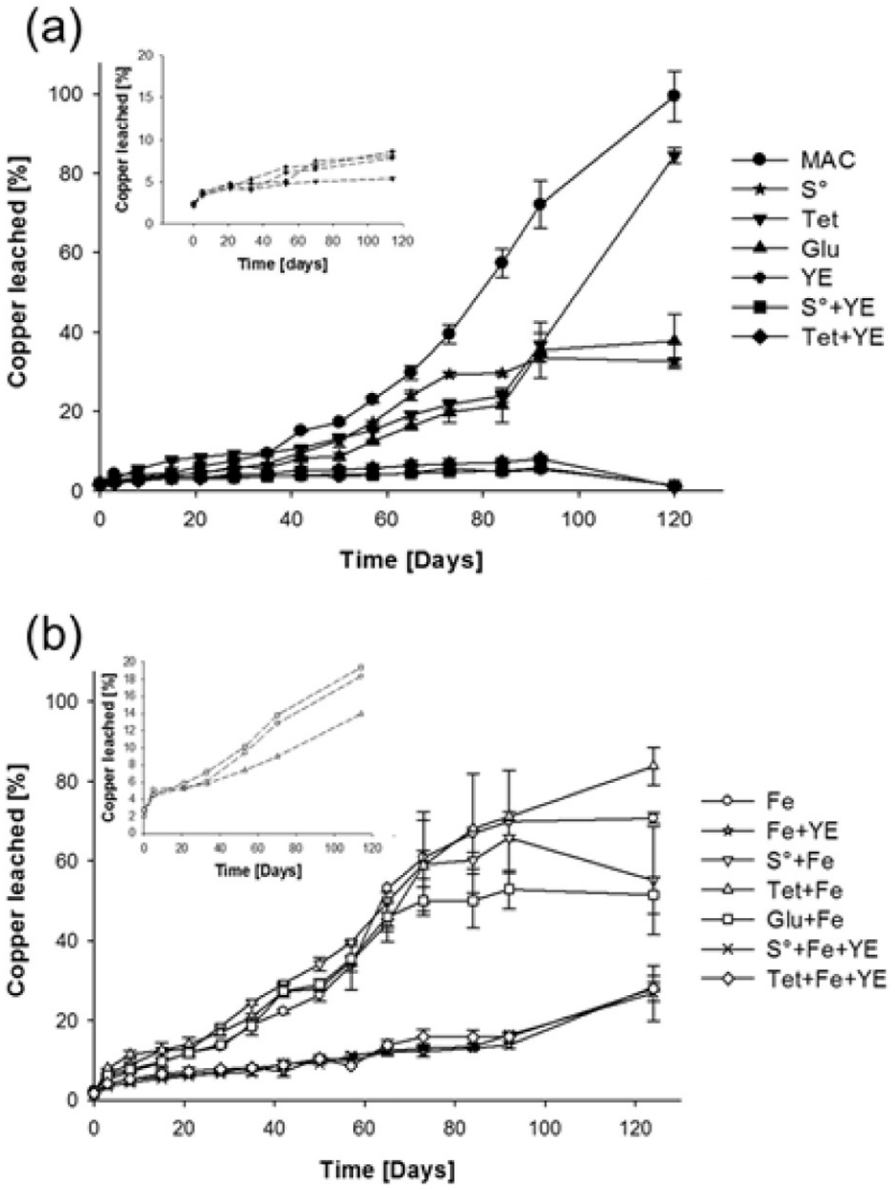

Fig. 4. Kinetics of copper solubilization from a chalcopyrite concentrate by *A. copahuensis*. (a) Media without ferrous iron supplementation; and (b) media with ferrous iron supplementation. Chalcopyrite dissolution was tested in shake flask cultures with 2% (*w*/*v*) pulp density incubated at 65 °C during 120 days. Small graphs show copper dissolution in abiotic controls (under autotrophic and heterotrophic conditions). Error bars show standard deviation from two independent experiments (n=2).

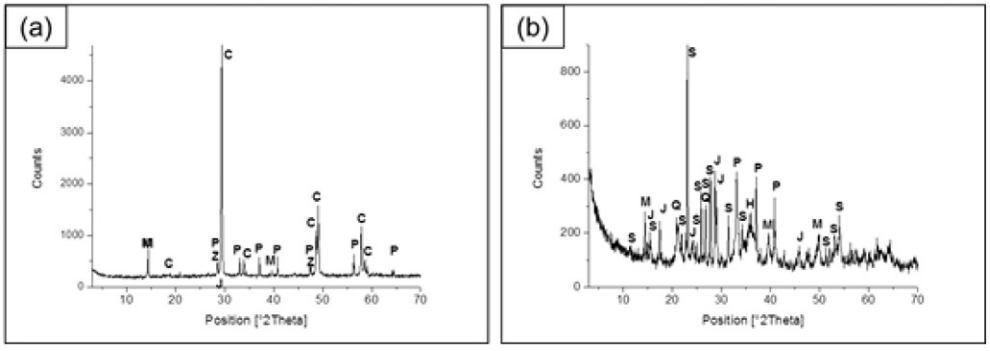

Fig. 5. X-ray diffractograms of: (a) the untreated mineral, and (b) a representative leach residue sample from a system with high copper recovery after the incubation with *A. copahuensis*. C, chalcopyrite; H, hematite; J, jarosite; M, molybdenite; P, pyrite; Q, quartz; S, sulfur, and Z, sphalerite.

Wang et al., 2014). Although in our case, this yield was reached at higher pulp density, not so low pH value and without any addition.

Mineralogical changes in leach residues were also evaluated by XRD (Fig. 5). The XRD patterns from systems with high copper recovery (Fig. 5b), indicated that compared with the untreated sample (Fig. 5a), its leached residues change significantly during the bioleaching with *A. copahuensis*. By comparison, the peaks corresponding to chalcopyrite disappear after bioleaching. This fact meant that chalcopyrite was dissolved after the bioleaching test, which was consistent with the copper solubilization data. The bioleached residues were still mainly composed of the undissolved pyrite and molybdenite in addition of the reaction products such as elemental sulfur, jarosite and hematite.

Corrosion of chalcopyrite surface during chemical leaching and bioleaching was examined by SEM of the leaching residues. The image of ore particles in the abiotic control after 50 days of incubation revealed very clean surfaces with very small amounts of precipitates (Fig. 6a). On the other hand, after interaction with *A. copahuensis*, the mineral surface suffered a strong attack (Fig. 6b). However, almost no cells were found on the mineral surface; probably the reason was that attached cells were covered by deposition of precipitates formed during the attack (Fig. 6c).

It is generally accepted that chalcopyrite dissolves under oxidation action of Fe(III) (Eq. (1)) and proton attack (Eq. (2)) via a polysulfide mechanism (Vera et al., 2013); it can be simplified and is represented by the following reactions:

$$CuFeS_2 + 4Fe^{3+} \rightarrow 5Fe^{2+} + Cu^{2+} + 2S^{\circ} \quad (1)$$

$$CuFeS_2 + 4H^{+} \rightarrow Fe^{2+} + Cu^{2+} + 2H_2S. \quad (2)$$

Hydrogen sulfide—liberated by acid dissolution— is oxidized to sulfur by Fe(III) (Eq. (3)).

$$H_2S + 2Fe^{+3} \rightarrow S^{\circ} + 2Fe^{+2} + 2H^{+} \quad (3)$$

In the presence of iron oxidizing microorganisms, Fe(II) is microbially oxidized to Fe(III) (Eq. (4)), thus regenerating the oxidant.

Sulfur produced by chalcopyrite dissolution is also metabolized to sulfuric acid as the final product (Eq. (5)), increasing the acidity of the solution and avoiding the diffusion barrier created by sulfur.

$$4Fe^{2+} + O2 + 4H^{+} \rightarrow 4Fe^{3+} + 2H_2O \quad (4)$$

$$2S^{\circ} + 3O_2 + 2H_2O \rightarrow 2SO_4{}^{2-} + 4H^{+} \quad (5)$$

Although these mechanisms are widely accepted for mesophilic microorganisms, they can also surely describe the process for thermophilic archaea as in this case.

Complete copper recovery (100%) – for our test without any other substrate – was accompanied by an increase of iron concentration due to mineral dissolution (Eqs. (1)–(2)); reaching ~900 ppm of total iron, mostly as ferrous iron (~600 ppm). Ferrous ions provided and/or released from the mineral dissolution was not completely oxidized to ferric ions by *A. copahuensis* mainly due to its low ability to catalyze Eq. (4). Previously, Giaveno et al. (2013) reported the capability of this strain to oxidize ferrous iron but at slow rates and low yields, as compared to growth on other substrates. Low ferric iron concentration in solution was reflected by a low redox potential level (below 400 mV) throughout the leaching experiment. Redox potential is an important factor in chalcopyrite leaching; under low values, chalcopyrite is known to dissolve at high rates (Córdoba et al., 2008a; Gericke et al., 2010; Li et al.,

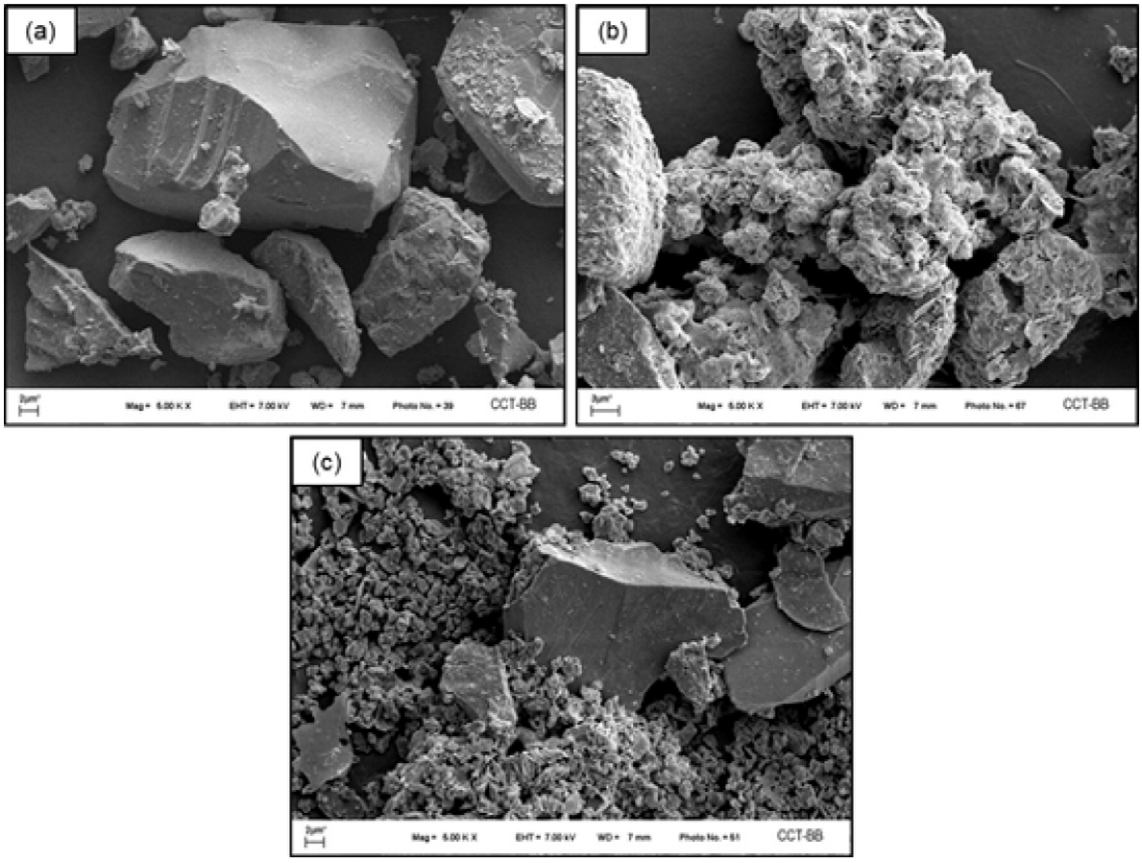

Fig. 6. SEM micrographs of ore particles after 50 days of incubation. (a) Chemically leached mineral in MAC medium; (b) after interaction with *A. copahuensis* in MAC medium; and (c) residues from tests supplemented with Fe(II).

2013; Watling, 2013). For chalcopyrite bioleaching, some researchers have proposed that its dissolution can be accelerated by controlling redox potential at relatively low values, and extremely high redox potentials at the initial stage of bioleaching can cause rapid passivation of chalcopyrite, thus inhibiting its further dissolution (Gericke et al., 2010; Petersen and Dixon, 2006; Zhao et al., 2015). Our experiments were carried out without external redox potential control, but the low (but not null) capability of *A. copahuensis* to oxidize ferrous iron contributes to keeping low redox potential conditions where chalcopyrite bioleaching is favored; that fact, added to the decrease of refractory behavior of chalcopyrite at higher temperature, seems to be an appropriate explanation for the high copper extraction achieved in this bioleaching test. The low iron-oxidizing activity of these cells suggests that, in the absence of iron supplementation, bioleaching would be driven through a contact mechanism where cells are attached to mineral surfaces and close to generation of other products more adequate for growth.

When comparing copper extractions from inoculated systems, it seems that substrate supplementations reduced bioleaching yields by *A. copahuensis* significantly (Fig. 4). Copper recoveries of 84.4%, 83.7%, and 70.6% were obtained in flasks supplemented with tetrathionate (Tet), tetrathionate and ferrous iron (Tet + Fe), and ferrous iron (Fe), respectively. Whereas yeast extract stood out as having a most negative effect on *A. copahuensis* bioleaching; copper dissolutions were low to 30% in flasks with yeast extract supply. A comparable copper recovery (~33%) was observed when cells were incubated autotrophically with sulfur as sole additional energy source. A slightly high yield was obtained in the presence of glucose (~38%). In both conditions, the addition of ferrous iron as a second substrate increased copper extraction to 55% and 51%, respectively. Negative effects in copper recoveries produced by addition of other sources suggest that cells were using them instead of oxidizing sulfur from the chalcopyrite. Cultures with yeast extract showed lower Eh values (most iron was ferrous) while the culture initially supplemented with iron reached the highest Eh (about 420 mV) which probably induced high precipitation of jarosite.

Many authors attribute slow kinetics on chalcopyrite bioleaching to the passivation layer formed during this process. There are various products that are suggested by different researches for different leaching conditions, including jarosite, chalcocite, covellite, and elemental sulfur (Li et al., 2013), though elemental sulfur and jarosite have been usually reported as responsible for the passivation of the chalcopyrite surface (Bevilaqua et al., 2002; Córdoba et al., 2008a; Vilcáez et al., 2008; Wang et al., 2014). Córdoba et al. (2008b) reported that iron supplementation caused a decline in bioleaching kinetics by mesophilic and thermophilic microorganisms. Such behavior has been associated to the nucleation and precipitation of jarosite. In our tests with iron addition, concentrations of total soluble iron at the end of the experiments were slightly higher or even lower than the initial amount. Taking in account that iron had to be solubilized at least together with copper from chalcopyrite; these data indicated strong iron precipitation. This was confirmed by XRD where jarosite peaks are increased in the residues coming from bioleaching systems (Fig. 5b). Also, SEM observations of the bioleaching residues confirmed abundant amounts of precipitates covering part of the chalcopyrite surface stopping sulfide attack (Fig. 6c). This is why the external addition of iron to the culture medium in tests with *A. copahuensis* contributed to the formation of passive barriers of jarosite on the mineral surface, preventing microbial access and limiting the diffusion of the leaching agent to the solid limiting copper recovery. This limited not only but mainly the contribution of contact mechanism to the bioleaching.

The addition of other energy sources (tetrathionate) did not generate such a serious decrease in copper recovery. As mentioned above, *A. copahuensis* prefers any energy source other than ferrous iron, which means that the rate of ferrous iron oxidation decreased, keeping low Eh and pH values with less jarosite precipitation and better copper recovery. In this sense, the best situation is culture without any addition, where iron is coming only from mineral dissolution; in those cultures the Eh was kept below 400 mV (low jarosite precipitation) but with small amounts of ferric iron which increases the dissolution of chalcopyrite.

Formation of jarosite was reported to be significantly reduced under low pH values (Pogliani and Donati, 2000). In most cultures pH tends to increase throughout these experiments with values over 1.8, indicating that jarosite formation was not completely inhibited. Even though in the culture with sulfur addition jarosite precipitation was almost null, copper recovery was low. That means that the presence of an additional surface where cells can be attached diminished the number of cells on the mineral, decreasing the contribution of contact mechanism. Bevilaqua et al. (2002) and Vilcáez et al. (2008) reported a negative effect of sulfur addition on bioleaching of chalcopyrite by *Acidithiobacillus thiooxidans* and *Sulfolobus metallicus*, respectively. These strains have shown to oxidize preferentially the additional elemental sulfur instead of the sulfur generated during the bioleaching process, which forms a passivation layer covering the mineral surface. In this case, the initial addition of iron increased the non contact mechanism and consequently copper recovery.

The addition of organic energy sources allowing heterotrophic growth drastically reduced copper recovery. The predominance of heterotrophic growth over autotrophic growth has also been previously reported (Giaveno et al., 2013), and our results indicate that this behavior strongly affects the efficiency of bioleaching.

4. Conclusions

Our results show that the adhesion properties of *A. copahuensis* cells were dependent from growth history. As expected, previous acclimation allows *A. copahuensis* cells to attach better to a chalcopyrite concentrate and this makes bioleaching more efficient.

This new species of *Acidianus* kept low Eh values, mainly in cultures without the addition of iron, allowing the highest copper recovery from chalcopyrite (close to 100%); this successful extraction was achieved at initial pH 2.0 (higher than the values used for other effective thermophilic microorganisms) where the contribution of chemical leaching at this temperature is less important. The addition of other substrates to the cultures decreased the dissolution of chalcopyrite being the effect more negative in cultures supplemented with organic energy sources.

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