SILVER EFFECT ON THE CHALCOPYRITE-, IRON- AND SULPHUR- OXIDISING CAPACITY OF A MIXED CULTURE OF MODERATELY THERMOPHILIC MICROORGANISMS

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ABSTRACT
The use of moderately thermophilic microorganisms is an attractive alternative to the silver-catalysed bioleaching of chalcopyrite. Firstly, chalcopyrite dissolution kinetics improves with raising temperature, which represents an advantage compared to mesophilic microorganisms. Besides, moderate thermophiles maintain a pH lower than mesophiles favouring mineral dissolution. Secondly, currently thermophilic microorganisms are less resistant to silver and to high pulp densities than moderately thermophilic microorganisms.

The effect of silver on bioleaching cultures has scarcely been treated in the literature. Several authors consider that silver inhibits Fe(II) oxidation by Ac. ferrooxidans, while several others point out that at optimum silver concentrations the growth of many iron-oxidising bacteria is inhibited.

This work is concerned with the influence of silver on the chalcopyrite-, iron- and sulphur-oxidising capacity of a moderately thermophilic culture. Tests were carried out with chalcopyrite, sulphur powder and Fe(II) as the sole energy source. The study of different parameters has revealed that the presence of silver favours the S-oxidising capacity of the culture and inhibits its Fe-oxidising one. Microorganisms observed by optical and both electronic scanning and transmission microscopy showed morphological differences between the different cultures; bacteria of longer length were detected in tests with sulphur with increasing amounts of silver.

Key words: bioleaching, chalcopyrite, moderate thermophiles, silver, bacterial selection.

INTRODUCTION
Chalcopyrite is resilient to oxidation in acid medium and, thus, its bioleaching kinetics are very slow. Unlike chalcopyrite, chemical and biological processes have shown good copper extractions from secondary sulphides (chalcopyrite, covellite, bonnئتite and enargite) [1]. The use of mesophilic microorganisms of the type Ac. ferrooxidans, in stirred tanks and heaps, has not been very effective in the treatment of chalcopyrite up to date. On the other hand, processes developed in stirred tanks with extremely thermophilic microorganisms have produced faster chalcopyrite dissolution rates than mesophiles or moderate thermophiles but with the drawback that they are only effective at very low pulp densities, possibly due to the low solubility of oxygen at high temperatures [2].

In the literature there is a description of different acidophilic microorganisms [3-4], among them: Arthrobacter caldus [5], Lepotrichia thermophila [6], Caldocellum saccharolyticum [7] and species of the genus Sulfolobus [8]. All of them with an optimum range of temperature between 45 and 50°C, with a maximum of 60°C and all species with good resistance, being able to tolerate high levels of toxic metals and to grow at low pH [9].

In most years, moderate thermophiles have gained acceptance. New microorganisms have been isolated, such as those found in pyritic coal deposits in Australia [10] and in mine waters in Rio Tinto (Huelva, Spain) [11], which have been used in chalcopyrite bioleaching processes catalysed with silver [12] and in the treatment of additional raw minerals [13,14,15]. Recently, a process in two steps has been proposed for the bi-oxidation of gold refractory concentrates using moderately thermophilic microorganisms in the initial step, as a result of its high resistance to As (III) [16].

The use of these microorganisms in the bioleaching of copper ores in the presence of silver is now an attractive alternative. One of the advantages over mesophilic microorganisms is that chalcopyrite dissolution kinetics improve with temperature. In addition, since the oxidation reactions of biocatalysing
processes are exothermic, the temperature of continuous systems could exceed the optimum temperature for monopiles; therefore, with moderate thermophiles less cooling would be required than with monopiles. Besides, moderately thermophilic microorganisms maintain a lower pH than monopiles, creating better conditions for mineral dissolution [3]. On the other hand, extremely thermophilic microorganisms are less resistant to silver and to high pulp densities than moderate thermophiles [17].

There are not many bibliographic references of the effect of silver on microbacterial cultures used in bioleaching. Several authors consider that silver inhibits Fe (II) microbiological oxidation by *At. ferrooxidans* through a probably mixed mechanism where the active sites of bacterial enzymes can be occupied or deactivated by the silver ion (Ag⁺) [18]. Norris and Kelly have studied the toxic effect of silver on the growth of a great number of iron-oxidizing bacteria and have found that the microorganisms' growth was inhibited in the presence of 0.10 mg/l Ag⁺ [19]. Nevertheless, the resistance of each culture varies as a function of its previous adaptation and exposure to different amounts of silver.

Besides the effect of silver on the oxidizing capacity of the culture, it is also fundamental to know if the catalyst favours the selection between the different species present in the culture. These phenomena can considerably decrease mineral dissolution since they can modify the mixed culture condition. Generally, the mutual action of two or more acidophilic microorganisms can be more effective in bioleaching than that derived from each microorganism separately. This synergy has been described many times, mainly referred to the improvement of mineral oxidation. It has been demonstrated that mixed cultures of *Ferromicrobium acidophilus* and *At. thiooxidans* (or the mixotrophic *Acidithiobacillus acidophilum*) can oxidize pyrite whereas the dissolution of this mineral has not been observed with pure cultures of these acidophilic microorganisms [20].

The synergistic effect between Fe-oxidizing moderately thermophilic bacteria of the genus *Sulfobacillus* spp. and *At. ferrooxidans* results in mixed cultures with a rapid oxidation capacity of ferrous ion. This avoids the addition of external organic compounds or the necessity of increasing the levels of carbon dioxide, such as it happens with pure cultures [8]. This was accounted for *At. ferrooxidans*, which has a slower rate of iron oxidation but an inducible, high-affinity mechanism for carbon to the more efficient iron-oxidizer *Sulfobacillus thermosulfidooxidans*, which has a limited ability to scavenge carbon dioxide from air.

This work collects information about the bioleaching of a chalcopyrite concentrate with moderately thermophilic microorganisms in the presence of silver ion (Ag⁺) and the influence of such ions on the Fe- and S-oxidizing capacity of the culture. Evidences of microorganisms selection are also presented and the morphological changes produced by the presence of silver.

**MATERIALS AND METHODS**

**Mineral concentrate**
The chalcopyrite concentrate used, named RT, was supplied by Rio Tinto Mines (Huelva, Spain) with a chemical composition: 22.44% Cu; 31.30% Fe; 2.70% Zn; 0.02% Pb and 38.79% S. The main mineral phases were chalcopyrite and pyrite, with small amounts of sphalerite and silica. The particle size was 90% less than 74 μm.

**Bacterial cultures**
The bacterial culture of moderately thermophilic microorganisms used, named TMRT, was started from mine waters of Rio Tinto, Huelva (Spain), at 45°C and pH 1.5, in modified Norris medium, without iron and chlorides, with the following chemical composition: 0.4 g/l MgSO4·7H2O; 0.2 g/l (NH4)2SO4 and 0.1 g/l K2HPO4 [5]. The cultures were grown with the chalcopyrite concentrate named RT as energy source. Initially, this mixed culture consisted of sulphur- and iron-oxidizing bacteria [12]. In order to carry out the bioleaching tests, two different types of inocula were prepared from this culture.

- **Silver non-adapted culture** (RT Ag non-adapted inoculum).
- **Silver-adapted culture** obtained by subsequent growth in the presence of different silver concentrations: 0.3 g Ag per kg of concentrate (RT 0.3 Ag adapted inoculum) and 2 g Ag per kg of concentrate (RT 2 Ag adapted inoculum).

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Biototoxic test

Biototoxic experiments were performed with the chloroprocyn concentrate in an orbital shaker at 150 rpm, with 1 % (v/v) pulp density and at 4°C. Sterile tests were also carried out using 2% thymol in sterile alcohol as bactericide. The initial pH in all the experiments was 5.5 and was kept below this value by addition of diluted Na2SO3 periodically.

In the biototoxic tests with silver, the adequate amount of silver was firstly added to the Noris strain medium as Ag2SO4 (0.3 and 2 g Ag/kg of concentrate respectively) and then the bacterial inoculum (5 % v/v). The following tests were performed:

1. RT 0.3 g Ag NA: RT concentrate with 0.3 g de Ag per kg of concentrate and silver non-adapted inoculum.
2. RT 2 g Ag NA: RT concentrate with 2 g of Ag per kg of concentrate and silver non-adapted inoculum.
3. RT 2 g Ag Al: RT concentrate with 2 g of Ag per kg of concentrate and inoculum adapted to the same amount of silver.
4. RT 0.3 g Ag Al: RT concentrate with 0.3 g de Ag per kg of concentrate and inoculum adapted to the same amount of silver.
5. RT Ag NA: RT concentrate without Ag and silver non-adapted inoculum (control).
6. RT sterile: RT concentrate sterilized without Ag and inoculum.
7. RT 2 g Ag steril: RT concentrate sterilized with 2 g Ag per kg of concentrate but without inoculum.

The tests were periodically analyzed by measuring pH, redox potential, Fe (II) concentration and bacterial population. Total iron and copper were determined by atomic absorption spectrophotometry (ZAB). Fe (II) was analyzed spectrophotometrically using the o-phenanthroline method [21]. The cell number was determined in the liquid phase with a Thoma counting chamber (depth, 0.1 mm) by using an Olympus BX40 microscope with phase-contrast.

Samples (mineral and bacteria) from these tests were visually analyzed by scanning electron microscopy (SEM) in a JSM-6400 JEOL microscope. These samples were prepared following the procedure of the critical point in order that dehydration of microorganisms was slow enough to keep their original shape and size.

Fe (II) and S oxidation tests

These experiments were carried out under similar conditions to biototoxic ones but using 1 g of FeSO4·7H2O and 1 g of alkaloid powder as substrate, in 100 ml of Norris nutrient medium at pH 1.5.

Tests were inoculated with different cultures grown from the biotoxic tests, prior centrifugation and washing with the nutrient medium in order to remove mineral particles. The experiments performed are reflected in Table 1. The oxidation of substrates by the medium was also studied in non-inoculated tests (control test).

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Cultures were followed identically to biototoxic test. The great variety of microorganisms in the cultures was determined by transmission electron microscopy (TEM) in a JEOL 1010 microscope with TEM operating at 100 kV. The preparation procedure of biological samples for their observation that was used previously consisted in 2 % w/v osmium tetroxide.

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RESULTS AND DISCUSSION

Effect of silver on chalcopyrite bioleaching at 45°C

Figure 1 clearly shows the silver catalytic effect on the chalcopyrite dissolution with moderately thermophilic microorganisms at 45°C. In the presence of silver, the dissolution rate increased and the percentage of dissolved copper is twice the amount obtained in the bioleaching test without silver. Sterile tests only extracted 5% of copper. With inocula from silver non-adapted cultures (RT 0.3Ag NAI, RT 2Ag NAI) there was a little improvement of dissolution on increasing silver concentration. This effect was negligible in the bioleaching tests with silver-adapted cultures.

Figure 1 also shows the percentage of Fe (II) versus total iron during bioleaching tests. The cultures with the higher content of Fe (II) were those catalysed with the higher amount of silver. Besides, the percentage of Fe (II) was higher for the tests inoculated with silver-adapted bacteria, which would suggest an inhibition of the iron-oxidising capacity of the culture by the silver effect.

Figure 1: Bioleaching test of RT concentrate with moderately thermophilic microorganisms (45°C). Evolution of copper and Fe (II) percentage.

SEM visual analysis of these cultures (mineral and bacteria) revealed significant differences on the bacterial morphology in tests with and without silver (Figures 2A and 2B). In tests without silver, microorganisms were rod-shaped bacilli of approximately 1 μm of length (Figure 2A). In contrast to cultures with silver, where the bacterial population was more abundant and the predominant bacilli had larger length, generally longer than 2 μm (Figure 2B). This seems to confirm that the differences found in the iron- and sulphur-oxidising capacity are directly related to microorganisms' morphology.

Figure 2: SEM micrographs of the RT concentrate cultures with moderately thermophilic microorganisms (45°C): A) Without silver. B) With silver.

In order to determine whether or not in the silver-adapted cultures there was a bacterial selection that would have affected the Fe (II) oxidation capacity to Fe (III), growth tests were carried out on S and Fe (II) from the bioleaching tests cultures.
Effect of sulfur on the capacity of S and Fe (II) oxidation of bacterial culture

The evolution of cultures grown on elemental sulfur is shown in Figure 3. In all cases, the bacterial action was clear since all the cultures presented lower pH values than the control test without inoculation. For this substrate, pH was lower as silver concentration increased. This trend would indicate that cultures grow in the presence of silver have a higher S-oxidizing capacity, and thus produce a slight increase of the amounts of sulphuric acid from the oxidation of sulfur.

With respect to the bacterial growth in the test with sulfur, in general, the number of microorganisms was very similar for all cultures (Figure 3).

The iron-oxidizing capacity of microorganisms is directly related to the evolution of ferric ion tod, therefore, to the oxidizing potential of the medium. The potential evolution is shown in Figure 4. In all cases, there was a progressive increase of the potential with two tendencies clearly differentiated: the culture without silver had the highest potential values; while cultures adapted to different silver concentrations reached lower values. In the latter case, the highest potential values corresponded to cultures adapted to the lowest silver concentration. The potential values of tests with silver are close to those of the control test (without inoculation), which is a clear evidence of inhibition to Fe (II) oxidation by the culture, such as that observed by De et al. for At. ferrooxidans [18]. These results, therefore, point out a decrease of the ferrous oxidation capacity in the silver-adapted cultures with respect to those grown in the absence of silver. Besides, the Fe-oxidation activity is lower with increasing silver concentration. Cultures grown on Fe (II) practically maintained a constant bacterial population along the experiment (Figure 4) but this abundant than in tests grown on S.

Figure 5 represents the percentage of Fe (II) versus total iron for cultures with Fe (II) as the sole substrate. These results show the same trend that the potential evolution in Figure 4. Therefore, cultures with inocula adapted to higher amounts of silver oxidize lower amounts of Fe (II).

These results are similar to those of biotransforming tests, where silver-adapted cultures oxidized a smaller
proportion of Fe (II). The bacterial selection produced by silver could be responsible for the higher copper extraction obtained with silver-adsorbed cultures. The product formed during silver-catalyzed chalcoprite dissolution is elemental sulphur. Therefore, cultures grown continuously in the presence of silver have a higher sulphur-oxidizing ability and dissolve, in a more efficient way, the sulphur layer formed decreasing its passivating effect (Equations (1), (2) and (3)). Besides, it is necessary to consider the negative effect produced by the decrease of the iron-oxidizing ability in silver-adsorbed cultures since, as it is shown in the dissolution mechanism mentioned previously, Fe(III) repartitions Ag(I) and a decrease of Fe(III) reduces the amount of silver deposited on the chalcoprite surface.

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\begin{align*}
CuFeS_2 + 4 Ag^{+} & \rightarrow 2 Ag_2S + Fe^{2+} + Cu^{2+} & (1) \\
2 FeS_2 + 0.5O_2 + H_2S_2 & \rightarrow FeS_2O_4 + H_2O & (2) \\
Ag_2S + 2 Fe^{3+} & \rightarrow 2 Ag^{+} + 2 Fe^{2+} + S^0 & (3)
\end{align*}
\]

It is significant that in the bacterial growth curve of cultures with Fe (II), silver non-adsorbed cultures present a lower number of bacteria. However, Fe (II) oxidation to Fe (III) was faster and complete (Figures 4 and 5). From the overall analysis of the results, silver clearly influences Fe (II) oxidation, capacity of microorganisms, favoring the activity of S-oxidizing microorganisms and decreasing Fe-oxidizing capacity.

![Bacterial growth on ferrocyanide ion of moderatly thermophilic microorganisms (45°C) from bioleaching tests. Percentage of Fe (II) with respect to Fe (total).](image)

**Effect of silver on the morphology of bacterial culture**

Optical microscopy of microorganisms grown on S and Fe (II) revealed significant morphological differences between each other. Cultures grown on Fe (II) were bacilli with a similar morphology and size for the different tests. However, in cultures grown on sulphur, microorganisms were bacilli of longer length, and the larger the amount of silver in the inoculum, the longer was its length.

Cultures grown on Fe (II) and elemental sulphur were observed by transmission electron microscopy in order to elucidate the predominant types of bacteria with each substrate and depending on the amount of silver used.

Figure 6A shows the most abundant bacteria in the inoculum adapted to RT concentrate without silver and with Fe (II), which could correspond morphologically to the genus Sulphobacter. Figure 6B shows another type of bacteria with a more homogeneous internal density, found in less proportion in the culture. Figures 6C and 6D show the two kinds of bacteria of the silver-adapted culture. In the case, both were found in identical proportion and were similar to those in the silver non-adapted culture. Cultures grown on sulphur, in general, contained longer bacilli than those found in cultures with Fe (II), in agreement with the optical microscopic observations. Figure 7 shows several micrographs of inocula adapted to RT concentrate under different conditions.

As can be seen in the micrographs, the presence of silver increased the length and number of bacteria (Figures 7A, 7B, 7C and 7D). The silver non-adapted culture grown on S had mainly bacilli with a size around 3 μm. However, silver-adapted cultures grown on S showed a higher bacterial population number.
with a size around 5 μm. In the silver non-adapted culture, the predominant type of bacteria had a high internal density, possibly of the genus *Shewanella* (Figure 7 B). On the contrary, the presence of silver favoured the growth of a new type of bacteria with rod-shaped morphology, large length and low internal density, and constituting about half of the population in the culture (Figure 7 D).

Figure 6: SEM micrographs of moderately thermophilic cultures (45°C) adapted to RT concentrate on Fe (II): A and B) without silver adaptation. C and D) adapted to 7 g Ag/kg concentrate.

Figure 7: TEM micrographs of moderately thermophilic cultures (45°C) adapted to RT concentrate on elemental sulphur: A and B) without silver adaptation. C and D) adapted to 2 g Ag/kg concentrate.
The microscopy study of the different cultures revealed that shorter cells can be associated to microorganisms with Fe-oxidizing capacity while the longer cells have S-oxidizing capacity. Within this latter, the presence of silver favours the growth of certain bacteria, longer or length than bacterial in cultures without silver.

Norris stated that *L. ferrooxidans* presents lower sensitivity to several metals than *A. ferrooxidans*. In our moderately thermophilic bacteria cultures, an enrichment phenomenon of some species could have occurred since the most abundant and homogenous cultures, with respect to the species contained, were those with silver. This could be related to the fact that silver can act as inhibiting agent for the growth of some bacteria, which would favour the growth of sulphur-oxidating ones. The inhibition would imply the suppression of the competance among the different types of microorganisms regarding the energy source and others compounds in the medium, facilitating the growth of species not affected by the analyte.

On the other hand, recent studies [23] on *A. ferrooxidans* showed that its Fe-oxidizing capacity is more sensitive to the presence of certain ions than its S-oxidizing capacity. The presence of chlorides, phosphates and nitrates at low concentrations inhibits the iron-oxidizing capacity of *A. ferrooxidans* without affecting its sulphur-oxidizing capacity. However, sulphur oxidation decreases by the high osmotic pressure. This study points out the higher sensitivity of the iron-oxidizing capacity of *A. ferrooxidans* versus different toxic substances.

CONCLUSIONS

Both chloroppytite dissolution and copper extraction considerably increased in the presence of silver, but also proved the selection of microorganisms initially present in the mixed culture, which decreased in effectiveness. Chloroppytite dissolution with moderately thermophilic microorganisms increased remarkable in the presence of silver. The presence of silver favored the sulphur-oxidizing activity of the culture and decreased its Fe(II) oxidation capacity. The effect of the selectivity that the culture exerted on the microbial populations of the mixed culture can decrease the passivating effect of the sulphur layer formed during the chloroppytite dissolution by favoring its sulphur-oxidizing capacity. On the other hand, a decrease of the iron-oxidizing capacity of the culture suppressed the regeneration of Ag(I), since Fe(III) is responsible for such regeneration.

Besides, silver affected moderately thermophilic microorganisms morphology by favoring the growth of bacillus with longer length, unseen in the original culture. In order to avoid this selection, it is recommendable to make an exhaustive control during culture growth to detect any selection between microorganisms, which would unbalance the original population decreasing its effectiveness and losing its condition of 'mixed' culture. Additionally, it is advisable to renew periodically the silver-catalyzed biotechning cultures by those that have not been in contact with the catalyst.

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