Available online at www.sciencedirect.com

SCIENCE DIRECT®

Bioelectrochemistry 64 (2004) 79–84

Bioelectrochemistry

www.elsevier.com/locate/bioelechem

Oxidative dissolution of chalcopyrite by *Acidithiobacillus ferrooxidans* analyzed by electrochemical impedance spectroscopy and atomic force microscopy

D. Bevilaqua[a], I. Diéz-Perez[b], C.S. Fugivara[c], F. Sanz[b], A.V. Benedetti[c], O. Garcia Jr.[a,*]

[a] *Department of Biochemistry and Chemical Technology, Institute of Chemistry, São Paulo State University, P.O. Box 355, 14801-970, Araraquara, SP, Brazil*
[b] *Center for Bioelectronics and Nanobioscience and Department of Physical Chemistry, University of Barcelona, Marti i Franqués 1, 08028-Barcelona, Spain*
[c] *Department of Physical Chemistry, Institute of Chemistry, São Paulo State University, P.O. Box 355, 14801-970, Araraquara, SP, Brazil*

Received 26 November 2003; received in revised form 13 January 2004; accepted 19 January 2004
Available online 18 May 2004

Abstract

The microbiological leaching of chalcopyrite ($CuFeS_2$) is of great interest because of its potential application to many $CuFeS_2$-rich ore materials. However, the efficiency of the microbiological process is very limited because this mineral is one of the most refractory to bacterial attack. Knowledge of bacterial role during chalcopyrite oxidation is very important in order to improve the efficiency of bioleaching operation. The oxidative dissolution of a massive chalcopyrite electrode by *Acidithiobacillus ferrooxidans* was evaluated by electrochemical impedance spectroscopy (EIS) and atomic force microscopy (AFM). A massive chalcopyrite electrode was utilized in a Tait-type electrochemical cell in acid medium for different immersion times in the presence or absence of bacterium. The differences observed in the impedance diagrams were correlated with the adhesion process of bacteria on the mineral surface.

Keywords: *Acidithiobacillus ferrooxidans*; Electrochemical impedance spectroscopy (EIS); Atomic force microscopy (AFM); Chalcopyrite

1. Introduction

Acidithiobacillus ferrooxidans, formerly *Thiobacillus ferrooxidans* [1], is an acidophilic chemolithoautrophic bacterium that utilise ferrous iron (Fe^{2+}) or reduced sulphur compounds, including mineral sulphides, as the sole energy sources for its growth. Due to its capacity to oxidise metal sulphides, this microorganism is one of the most important utilised in bioleaching industrial operations to recover metals, such as copper, uranium and gold.

Among the metal sulphides that are oxidised by *A. ferrooxidans*, chalcopyrite ($CuFeS_2$) has deserved special attention since it is the principal mineral source of the 10 million tons of copper produced annually worldwide and at same time the most refractory to bacterial or chemical attack [2].

The biological mechanisms and the reactions that come into play during the oxidative dissolution of metal sulphides are controversial and still poorly understood [3]. Two mechanisms are known to be responsible for the bioleaching of sulphides, namely indirect and direct mechanisms. The indirect mechanism operates by the chemical action of acidic ferric sulphate solutions produced by bacterial oxidation of ferrous iron or pyrite (FeS_2), normally present in these environments. In this mechanism, adhesion of the bacteria to the mineral surface is supposed to be not required. The direct mechanism, on the other hand, involves the enzymatic attack of the mineral by the bacterium, for which intimate contact and, hence, adhesion are required. Several publications have been devoted to explain the role of cell adhesion during the early steps of bacterial sulphide oxidation [4–6]. These authors have shown that sulphur-grown cells of *A. ferrooxidans* present more hydrophobicity than those grown in ferrous iron medium. However, this condition could not be associated with more or less adhesion of the cells. More recently, other studies suggested that exopolymeric substances (EPS) present in cell envelope [7,8] might play very important role in the initial stages of adhesion.

The analysis of the electrochemical impedance spectroscopy (EIS) data can provided information about physical, chemical and biochemical process occurring in the elec-

* Corresponding author. Tel.: +55-16-201-6677; fax: +55-16-222-7932.
E-mail address: oswaldo@iq.unesp.br (O. Garcia).

1567-5394/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bioelechem.2004.01.006

trode/electrolyte (sulphide/culture medium) interface in the presence and absence of *A. ferrooxidans*, as recently demonstrated by Bevilaqua et al. [9] for bornite (Cu_5FeS_4).

In recent years, atomic force microscopy (AFM) has opened exciting new potentialities in microbiology and biophysics for probing microbial organisms. As reported by van der Aa and Dufrêne [10], AFM presents capabilities to visualize the surface ultra-structure of microbial cells; it also allows estimation of the width and height of bacterial cells, and the thickness of exopolymeric substances, as well as measuring local physical properties such as adhesion forces and elasticity. AFM has been used to follow cell adhesion as one of the most effective on-line techniques for biomaterial imaging due to the optimal high resolution attained either ex situ and also in situ, in vivo, single bacterium cell imaging on a well-defined surface [11]. Besides, characterization of substratum roughness, including measurements depth and diameter of individual corrosion pits is also possible, making AFM and important partner tool for EIS studies.

As mentioned before, the bacterial oxidation of chalcopyrite has been studied extensively under different conditions, particularly in acidic aqueous conditions; however, the electrochemical studies involving microorganisms for bioleaching purposes is still incipient. Because of the complexity and variability of the interaction between chalcopyrite and the oxidizing environment, including bacteria, there are many aspects that are not understood.

This work's aim is to improve our knowledge on the aspects governing the action of *A. ferrooxidans* during chalcopyrite bacterial oxidative dissolution utilizing EIS and AFM techniques.

2. Material and methods

2.1. Bacterial strain and growth conditions

A. ferrooxidans strain LR was used in this work [12]. The culture was grown in a T&K mineral salts medium [13] at pH 1.8 plus ferrous sulfate as energy source. The cells for electrochemical analysis and adhesion experiments were obtained after growth for 48 h in a shaker (150 rpm and 30 °C) by successive washing and centrifugation ($5000 \times g$) to eliminate residual ferric ion from the medium. The washed suspension was centrifuged at $12\,000 \times g$ for 25 min, washed twice in a Milli-Q water of 18 MΩ cm and finally suspended in 10 ml of the mineral salts medium, using pro-analysis purity grade chemicals and Milli-Q water. The cell suspension was standardized by the modified Lowry protein determination method [14].

2.2. Mineral samples preparation

Research-grade $CuFeS_2$ used in this study was obtained from Ward's Natural Science Establishment (Rochester, NY). Samples of chalcopyrite were cut in pieces of approximately 1 cm^2 using a diamond saw. One face was hand-polished through four grit sizes of silicon carbide paper with a final polishing using alumina suspension of 0.3 μm particle size. To eliminate impurities, the samples were sonicated with acetone, ethanol and Milli-Q water (15 min each one), and then were dried with pure argon and stored in desiccators before using.

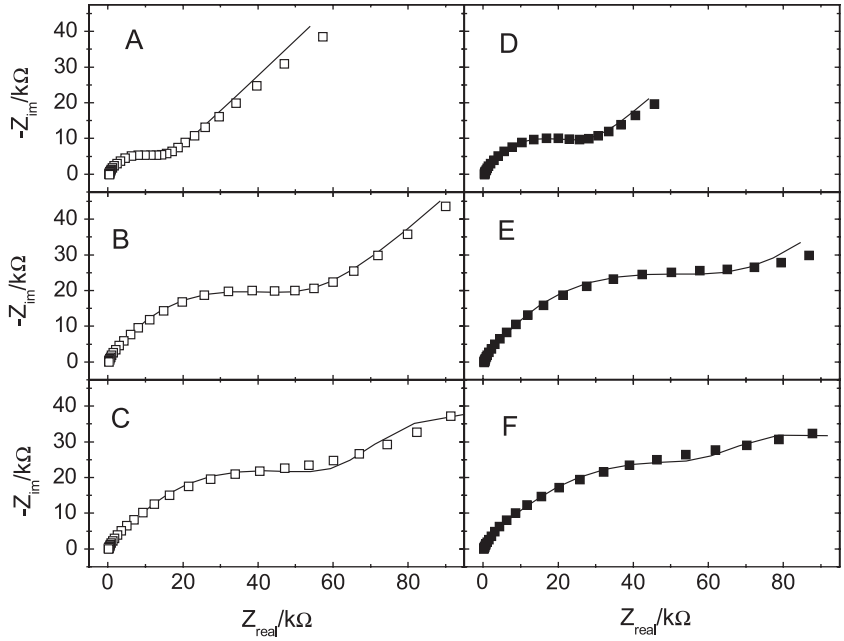

Fig. 1. Experimental (symbol) and simulated (solid line) Nyquist plots of (A and D) 7 h, (B and E) 71 h and (C and F) 288 h of immersion. Open symbols: impedance diagram without *A. ferrooxidans*-LR; closed symbols: impedance diagram with *A. ferrooxidans*-LR.

Table 1
Parameters' values using the circuit $R_s(Q(R_1W))$ for different incubation times of chalcopyrite electrodes in the presence or absence of *A. ferrooxidans*-LR

Sample	R_s/Ω	$Y_{0,1}/10^{-6}$ S s^n	n_1	$R_1/10^4$ Ω	$W/10^{-5}$ S s^n	$\chi^2/10^{-3}$
Control 7 h	373	8.71	0.76	2.60	13.9	2.33
Control 24 h	384	9.04	0.75	4.51	11.9	2.91
Inoculated 7 h	354	3.08	0.79	1.33	8.59	2.64

2.3. Electrochemical measurements

A Tait-type electrochemical cell that allows the use of working electrodes of different size and shape, including a sheet geometry, was used in this work. This cell type eliminates the need for machining or special mechanical preparation of the samples and facilitates the microscopic observation after electrochemical studies. In this cell, the working electrode (chalcopyrite) was fixed at the bottom of the cell with an O-Ring, and the other electrodes were fixed at the cell top. The exposed area of the sulfide was 0.28 cm^2. The electrochemical cell was provided with a jacket connected to a thermostatic bath and a recirculation pump to maintain constant the temperature during the test, which was fixed at 30 °C. An $Ag/AgCl/KCl_{sat}$ electrode connected to the solution through a Luggin capillary was used as reference. A Pt wire with large area was used as auxiliary electrode. A Pt disc electrode connected to the reference by a 10-μF capacitor was used to minimize the noise at low frequency and the phase shift at high frequency [15]. All immersion tests were carried out using 10 ml of aerated iron-free mineral salts solution of T&K medium (pH 1.8), ionic strength 0.08 mol l^{-1}. In the inoculated tests, the concentration of the cells of *A. ferrooxidans*-LR was $\sim 5 \times 10^9$ cells ml^{-1}. Control experiments were carried out utilizing the same work solution without inoculum.

Open-circuit potential (E_{OC} vs. time) and electrochemical impedance measurements of the chalcopyrite electrode were performed using an electrochemical system from EG&G PARC, model 283 and a Frequency Response Analyzer EG&G PARC, model 1025, monitored by a microcomputer using the M398 software. After the attainment of the steady state, the electrode was submitted to impedance electrochemical measurement. The impedance spectra were obtained by applying on the open-circuit potential a small amplitude sine wave voltage (10 mV rms) with a frequency range from 1×10^5 to 1×10^{-2} Hz and 10 points per decade. All the imaginary and real impedances are given on a geometric area (0.28 cm^2) basis, and the data were analyzed using the Equivalent Circuit program developed by Boukamp [16]. The impedance diagrams were recorded in different incubation times (7, 24, 71, 120, 288 h) in the absence and presence of the bacteria into the electrolyte. For all conditions studied, the E_{OC} values before and after the impedance measurement were the same within 2 mV.

2.4. Adhesion experiments

Imaging of a biological sample with AFM requires a near atomically flat surface in order to discriminate topographical features. Initial values of roughness for chalcopyrite surfaces were typically around 4 and 5 nm, respectively. To eliminate impurities the samples were rinsed with acetone, ethanol and water purified to 18 MΩ cm (15 min each one in an ultrasonic bath), and then were blown dry with pure argon and kept in desiccators before using. The mineral samples were incubated without shaking at 30 °C in 1 ml of iron-free mineral salts solution of T&K medium (pH 1.8) in water purified to 18 MΩ cm to containing a cell suspension ($\sim 5 \times 10^{10}$ cells). The samples were taken out from the solution after different times of incubation, rinsed exhaustively with water purified to 18 MΩ cm to remove any remaining unattached bacteria and dried in purified argon. A Nanoscope III Extended Multimode Atomic Force Microscope (Digital Instruments, Santa Barbara, CA, USA) operating in a tapping mode in air was used to image cells. The Digital Nanoscope software (version 4.42r8) was used to analyse the topographic images.

At the end of each experiment, the solution was withdraw from the cell and filtered for Cu and Fe analysis by inductively coupled plasma (ICP) emission spectroscopy.

3. Results and discussion

Fig. 1 shows the experimental and simulated impedance diagrams for chalcopyrite electrode recorded after 7, 71 and 288 h of incubation in the absence and presence of *A. ferrooxidans*-LR. Diagrams for 24 and 120 h are not shown, because they are very similar to the other incubation times. All spectra showed an incomplete semicircle in the high

Table 2
Parameters' values using the circuit $R_s(R_1C)(Q(R_2W))$ for different incubation times of chalcopyrite electrodes in the presence or absence of *A. ferrooxidans*-LR

Sample	R_s/Ω	R_1/Ω	$C/10^{-6}$ F	$Y_{0,1}/10^{-6}$ S s^n	n_1	$R_2/10^4$ Ω	$W/10^{-5}$S s^n	$\chi^2/10^{-3}$
Control 71 h	266	2124	2.34	4.77	0.78	4.78	6.50	3.73
Control 120 h	284	2179	2.46	5.42	0.75	6.0	5.99	3.53
Inoculated 24 h	389	1308	13.4	10.3	0.75	4.7	13.2	1.96
Inoculated 71 h	320	2269	7.68	10.2	0.75	6.35	9.99	3.15
Inoculated 120 h	370	2427	8.36	11.0	0.73	6.85	9.43	2.62

Table 3
Parameters' values using the circuit $R_s(R_1Q)(Q(R_2O))$ for different incubation times of chalcopyrite electrodes in the presence or absence of *A. ferrooxidans*-LR

Sample	R_s/Ω	R_1/Ω	$Y_{0,1}/10^{-6}$ S s^n (n)	$Y_{0,2}/10^{-6}$ S s^n (n)	$R_2/10^3$ Ω	$Y_{0,1}/10^{-5}$ S s^n (B/$s^{0.5}$)	$\chi^2/10^{-3}$
Control 288 h	175	45,320	2.34 (0.76)	3.69 (0.86)	4.62	7.52 (6.43)	6.73
Inoculated 288 h	212	43,750	19.4 (0.76)	7.92 (0.81)	9.67	8.90 (5.85)	5.01

frequency region followed by a Warburg characteristic impedance for lower incubation times. The semicircle increased and the Warburg component shifted to lower frequencies or lost influence for higher incubation times. Since the electrode is a natural mineral, its surface is heterogeneous, and when attacked by the electrolyte, its resistance increases probably due to a sulphur coating formation [17,18]. In fact, sulphur was detected as a new crystalline phase during chalcopyrite oxidation by *A. ferrooxidans* [19]. At the same time, the semi-infinite linear diffusion diminishes in importance since the reaction is partially blocked. The impedance data obtained in the non-containing bacteria electrolyte for 7 and 24 h of incubation times and 7 h in the presence of bacteria were well fitted using the equivalent circuit $R_s(Q(R_1W))$. R_s is the solution resistance and $Q(R_1W)$ is connected to the active region of the electrode. The values for the parameters of the circuit are in Table 1. The table indicates that χ^2, which represents the sum of quadratic deviations between experimental and calculated data, has a low value. R_1 clearly increased with time immersion indicating the increase in the film and charge transfer resistance. The constant phase element (CPE) is related with the capacitance of the electrode/electrolyte interface. The n values were about 0.75 independent of the incubation times and can be attributed to the surface heterogeneities and roughness, since a $0.5 < n < 1$ value is associated to the electrode heterogeneity and roughness [20,21]. The Y_0 values increased in the presence of the bacterial cells, which were attached but EPS were not excreted yet, as discussed latter.

For incubation times of 71 and 120 h in the absence of bacteria and 24, 71 and 120 h in presence of bacteria, the equivalent circuit $R_s(R_1C)(Q(R_2W))$ represented a good fit of the impedance data (Table 2). This circuit was proposed considering that a part of the surface is covered by an adherent layer with certain porosity, probably composed by sulfur, hydroxides, biomolecules and cells, represented by R_1C element, where R_1 is related to the film resistance and C with its capacitance. Q represents the constant phase element for the active region of the electrode associated with the capacitance of this part of electrode/electrolyte interface, with an n about 0.75, associated with a roughness and heterogeneous surface. The R_2 is the charge transfer resistance, W is the Warburg impedance and the serial R_2W association represents the faradaic current branch.

For the immersion time of 288 h in the absence and presence of bacteria, the equivalent circuit $R_s(R_1Q_1)(Q_2(R_2O))$ fitted well the impedance data (Table 3). In this circuit, R_1Q_1 association represents the biological or sulphur film described above and R_2 has the same meaning as described before. The O represents a finite diffusion element that considers the electrode surface to be almost totally covered by sulphur, biomolecules and cells, and then, supposes that the diffusion through this film limits the mass transport process. The serial R_2O association also represents the faradaic current branch.

Fig. 2 depicts a set of images corresponding to *A. ferrooxidans*-LR cells attached on chalcopyrite at different incubation times. Cell attachment could be clearly observed for lower incubation time (30–60 min) even after exhaustive washing before the image was taking out (data not shown). Comparing the surface of the inoculated samples (Fig. 2A and B, at 3 and 7 days, respectively) with that surface of a non-inoculated (Fig. 2C, at 28 days), the presence of a film is evident. EPS could be related with this film. As considered before, the EPS present in the cell envelope might play a very important role in the adhesion. However, this film was not observed in early incubation times, so this work could not relate the film to cell adhesion. The EPS formed remains around the cell and may increase

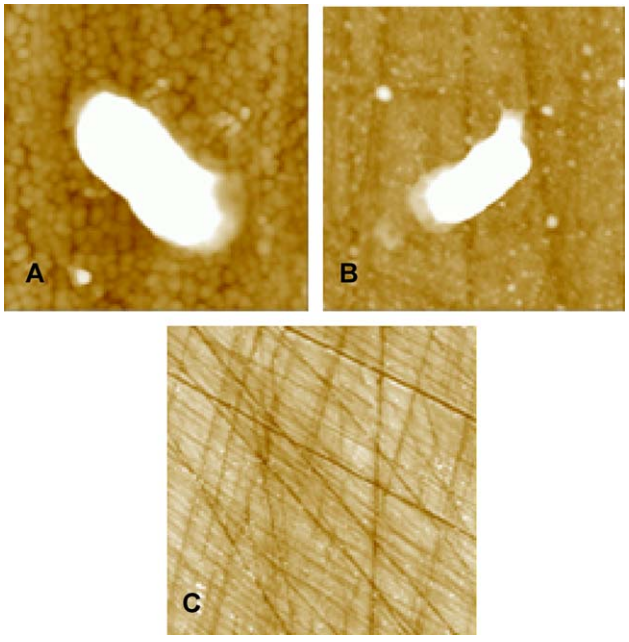

Fig. 2. AFM images of chalcopyrite surface after different incubation times in the cell suspension: (A) 3 days; (B) 7 days. The images are real zoom: (2 × 2) μm^2 area and 400 nm Z range for (A) and (3 × 3) μm^2 and 400 nm Z range for (B); (20 × 20) μm^2 (C) shows the blank mineral surface after 28 days immersion on the suspension media without the presence of the bacteria (400 nm Z range).

Table 4
Solubilization of copper and iron from chalcopyrite electrode after electrochemical measurements in absence or presence of *A. ferrooxidans*-LR

	Cu (mg l^{-1})	Fe (mg l^{-1})
Control	0.58	1.40
Inoculated	0.71	9.00

The copper and iron contents in the initial electrolyte solution were <0.05 and 0.10 mg l^{-1}, respectively.

the contact interaction area between cells and the mineral substrate.

Table 4 shows copper and iron dissolution of chalcopyrite electrode after electrochemical measurement (288 h) in order to verify the influence of the *A. ferrooxidans*-LR in the solubilization of these ions. It can be seen that the solubilization of iron was enhanced by almost sevenfold in the presence of bacteria compare to control; however, the copper solubilization was negligible in both conditions. This result is very interesting since the refractory behaviour of chalcopyrite is specifically regarding copper oxidative dissolution by bacteria but not for iron. It has been reported that the presence of ferric sulphate in a leaching system, decreases copper solubilization from chalcopyrite even in these oxidant conditions [22]. As mentioned before, a passivating layer formed during the (bio)leaching of chalcopyrite prevents the diffusion of ions to and from the mineral surface, decreasing copper release [2]. The nature of this layer has been not elucidating yet.

4. Conclusions

Microorganisms like all living beings behave in a complex way regarding their physiology and metabolism during growth while utilizing organic or inorganic substrates. To understand the physiological characteristics of *A. ferrooxidans* during its utilization of mineral sulphides as an energy source and solubility mechanism for valuable metals, it is necessary to combine electrochemical and surface analysis techniques to obtain more detailed information about how the bacteria interact with minerals.

In this study, the adhesion of bacteria on the mineral surface was detected early (30–60 min) of testing, and it could be associated with the difference observed in the impedance diagrams during first hours of test. After about 71 h of incubation, the composition of adsorption layer included cells, biomolecules and sulphur. Collectively, the layer was formed on the chalcopyrite surface and functions as a capacitor, which makes the diffusion of molecule and ions to and from the electrode surface difficult. In the test solution that did not contain bacteria, similar behaviour was observed after 71 h. This time differential is probably due to the sulphur coat formation at the electrode surface. The demonstrated recalcitrant behaviour of chalcopyrite, with the respect to copper dissolution from the mineral but not from iron, also reinforces the existence of this passivating coating. The AFM images also confirm these coating features and behavior expectations.

Acknowledgements

Authors thank the financial support from Conselho Nacional de Pesquisa (CNPq), Brazil, and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Brazil. The facilities offered by Servèis Científico-Tècnics of Universitat de Barcelona, Spain, is also acknowledged.

References

[1] D.P. Kelly, A.P. Wood, Reclassification of some species of *Thiobacillus* the newly designated genera *Acidithiobacillus* gen. nov. *Halothiobacillus* gen. nov. and *Thermithiobacillus* gen. nov., Int. J. Syst. Evol. Micrbiol. 50 (2000) 511–515.
[2] K.A. Third, R. Cord-Ruwisch, H.R. Watling, The role of iron-oxidizing bacteria in stimulation or inhibition of chalcopyrite bioleaching, Hydrometallurgy 55 (2000) 225–233.
[3] M. Boon, The mechanism of 'direct' and 'indirect' bacterial oxidation of sulphide minerals, Hydrometallurgy 62 (2001) 67–70.
[4] M.C.M. van Loosdrecht, J. Lyklema, W. Norde, G. Schraa, A.J.B. Zehnder, Eletrophoretic mobility and hydrophobicity as a measure to predict the initial steps of bacterial adhesion, Appl. Environ. Microbiol. 53 (1987) 1898–1901.
[5] G. Curutchet, E. Donati, Iron-oxidizing and leaching activities of sulphur-grown *Thiobacillus ferrooxidans* cell on other substrates: effect of culture pH, J. Biosci. Bioeng. 90 (2000) 54–58.
[6] M.I. Sampson, C.V. Phillips, R.C. Blake II, Influence of the attachment of acidophilic bacteria during the oxidation of mineral sulfides, Miner. Eng. 13 (2000) 373–389.
[7] K. Kinzler, T. Gehrke, J. Telegdi, W. Sand, Bioleaching—a result of interfacial processes caused by extracellular polymeric substances (EPS), in: V.S.T. Ciminelli, O. Garcia Jr. (Eds.), Biohydrometallurgy: Fundamentals, Technology and Sustainable Development: Part A. Bioleaching, Microbiology and Molecular Biology, Elsevier, Amsterdam, 2001, pp. 191–197.
[8] T. Gehrke, J. Telegdi, D. Thierry, W. Sand, Importance of extracellular polymeric substances from *Thiobacillus ferrooxidans* for bioleaching, Appl. Environ. Microbiol. 64 (1998) 2743–2747.
[9] D. Bevilaqua, I. Diéz-Perez, C.S. Fugivara, F. Sanz, A.V. Benedetti, O. Garcia Jr., Characterization of bornite (Cu_5FeS_4) electrodes in the presence of the bacterium *Acidithiobacillus ferrooxidans*, J. Braz. Chem. Soc. 14 (2003) 637–644.
[10] B.C. van der Aa, Y.F. Dufrêne, In situ characterization of bacterial extrqacellular polymeric substances by AFM, Colloid Surf., B 23 (2002) 173–182.
[11] H.H.P. Fang, C. Kwong-Yu, X. Li-Chong, Quantification of bacterial adhesion forces using atomic force microscopy (AFM), J. Microbiol. Methods 40 (2000) 89–97.
[12] O. Garcia Jr., Isolation and purification of *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* from some coal and uranium mines of Brazil, Rev. Microbiol. 20 (1991) 1–6.
[13] O.H. Tuovinen, D.P. Kelly, Use of membrane filters and ferrous iron agar to determine viable number and comparison $^{14}CO_2$ _ fixation and iron oxidation as measures of growth, Arch. Microbiol. 88 (1973) 285–298.
[14] E.F. Hartree, Determination of proteins: a modification of the Lowry method that gives a linear photometric response, Anal. Biochem. 48 (1972) 422–427.

[15] F. Mansfeld, S. Lin, Y.C. Chen, H. Shih, Minimization of high-frequency phase-shifts in impedance measurements, J. Electrochem. Soc. 135 (1988) 906–907.
[16] B.A. Boukamp, A nonlinear least-squares fit procedure for analysis of immittance data of electrochemical systems, Solid State Ionics 20 (1986) 31–44.
[17] R.P. Hackl, D.B. Dreisinger, E. Peters, J.A. King, Passivation of chalcopyrite during oxidative leaching in sulfate media, Hydrometallurgy 39 (1995) 25–48.
[18] I. Lázaro, N. Martínez-Medina, I. Rodríguez, E. Arce, I. González, The use of carbon paste electrodes with non-conducting binder for the study of minerals: chalcopyrite, Hydrometallurgy 38 (1995) 277–287.
[19] D. Bevilaqua, A.L.L.C. Leite, O. Garcia Jr., O. Tuovinen, Oxidation of chalcopyrite by *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans* in shake flasks, Process Biochem. 38 (2000) 587–592.
[20] R. Levie, On the impedance of electrodes with rough interfaces, J. Electroanal. Chem. 261 (1989) 1–9.
[21] M. Cai, S.M. Park, Oxidation of zinc in alkaline solutions by electrochemical impedance spectroscopy, J. Electrochem. Soc. 143 (1996) 3895–3902.
[22] N. Hiroyoshi, H. Miki, T. Hirajima, M. Tsunekawa, Enhancement of chalcopyrite leaching by ferrous ions in acidic ferric sulphate solutions, Hydrometallurgy 60 (2001) 185–197.