Available online at www.sciencedirect.com
SCIENCE DIRECT®
Enzyme and Microbial Technology 36 (2005) 301–306
ENZYME and MICROBIAL TECHNOLOGY
www.elsevier.com/locate/enzmictec

Kinetic study of ferrous sulphate oxidation of *Acidithiobacillus ferrooxidans* in the presence of heavy metal ions

G. Cabrera*, J.M. Gómez, D. Cantero

Department of Chemical Engineering, Food Technology and Environmental Technologies, University of Cadiz, Puerto Real 11510, Spain

Received 24 December 2003; accepted 7 September 2004

Abstract

Acidophilic microorganisms such as *Acidithiobacillus ferrooxidans* have the capability to carry out processes of bioleaching, biosorption and bioprecipitation of heavy metal ions, which have important environmental applications. *At. ferrooxidans* derives the energy for their metabolism from ferrous iron oxidation, process, which can be affected by the presence of heavy metals in the medium. Moreover, organic matter produces an inhibitory effect over the ferrous iron oxidation of *At. ferrooxidans*. In this work, heterotrophic bacterium *Acidiphilium* sp. was added when the medium is supplemented with organic matter to reduce this negative effect. The purpose of this work is the kinetic study of ferrous sulphate oxidation by *At. ferrooxidans* in the presence of different concentrations of several heavy metal ions (Cr(III), Cu(II), Cd(II), Zn(II) and Ni(II)) and compare this kinetic behaviour with a mixed culture with *Acidiphilium* sp.

The obtained results show a non-competitive inhibition of heavy metals over bacterial oxidation of ferrous sulphate. In accordance with this kind of inhibition, a kinetic equation has been proposed to predict the behaviour of *At. ferrooxidans* in the presence of heavy metals in the range of concentrations studied.

Keywords: *Acidiphilium* sp.; *Acidithiobacillus ferrooxidans*; Ferrous sulphate oxidation; Heavy metal; Kinetics

1. Introduction

The accumulation of heavy metals in water, air or soils is an important environmental problem. In recent years, several technologies have been developed with the aim of reducing or removing the presence of heavy metals in contaminated media. Among these technologies, those based on the use of microorganisms are of particular interest. A number of microorganisms have the capacity to solubilize heavy metals present in aqueous solution (bioleaching) [1], are able to adsorb heavy metals through their cellular structures (biosorption) [2] or can precipitate heavy metals in solution to facilitate removal of the contaminant (bioprecipitation) [3].

Acidithiobacillus ferrooxidans and *Acidiphilium* sp. are acidophilic bacteria usually found in acid mine effluents. *At. ferrooxidans* is an autotroph, its carbon source is carbon dioxide and oxidises ferrous iron or reduced sulphur compounds in order to grow [4]. *Acidiphilium* sp. is a heterotroph and uses organic matter as carbon source [5]. Both microorganisms show a particular tolerance to several heavy metals [6–10]. Due to those tolerance characteristics, and their ability to transform metal ions, *At. ferrooxidans* and *Acidiphilium* sp. can be used to solve certain environmental problems caused by heavy metals.

At. ferrooxidans have the ability to oxidise ferrous sulphate to ferric sulphate under aerobic acidic conditions, according to the following equation [4]:

$$4FeSO_4 + 2H_2SO_4 + O_2 \xrightarrow{\text{Bacteria}} 2Fe_2(SO_4)_3 + 2H_2O \quad (1)$$

The recovery of ferric iron by this acidophilic microorganism is an interesting process since the ferric iron is

* Corresponding author. Tel.: +34 956016382; fax: +34 956016411.
E-mail address: gema.cabrera@uca.es (G. Cabrera).

0141-0229/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.enzmictec.2004.09.008

one of the most useful reagents in hydrometallurgy due to its oxidizing property. Several processes in which high concentrations of ferric iron are used (such as coal desulphurization or the leaching of arsenic, nickel, uranium and copper in acidic conditions) have been developed [4]. The recovering of the reactant (ferric iron) for its following reusing has a relevant importance in this kind of processes. The wide applicability of ferrous iron oxidation has promoted numerous works aimed at studying kinetics in solution.

First studies on the kinetic behaviour of *At. ferrooxidans* in ferrous iron oxidation appeared in 1970 by MacDonald and Clark [11] and Lacey and Lawson [12]. They presented that experimental growth rates can be adjusted to the Monod equation. In spite of its simplicity, the application of this expression presents several disadvantages, as it is only adjusted to initial rates and it does not consider inhibitory effects. Later on, Liu et al. [13] proposed an expression that took into account the effect of competitive inhibition for ferric iron ion, and Suzuki et al. [14] incorporated terms about inhibition due to cellular concentration. Kumar and Gandhi [15] described the growth of *At. ferrooxidans* with a kinetic model that considered ferrous iron oxidation, cellular death, optimum pH and the presence of jarosites. This model was suitable to predict the evolution of ferrous iron concentration but not the evolutions of both ferric iron concentration and pH; however, it includes a high number of parameters. Pagella et al. [16] proposed an expression relating bacterial growth rates to several inhibitory effects and the ferrous iron uptake rate. This model is excessively complicated and it is not useful to design biological reactors. Gómez et al. [17] proposed a simplification of Liu's equation, which considered a kinetics with competitive inhibition by the product. This model is based on direct measurement of microbial population by a simple count technique. All these methods do not consider the influence of metal ion concentration on biological oxidation of ferrous iron.

Kupka and Kupsáková [18] studied kinetics of ferrous iron sulphate oxidation by resting cells suspensions of *At. ferrooxidans* as a function of the substrate concentration. The study examined the effect of nickel(II) and cupric(II) on the ferrous iron oxidation kinetics. The experimental data were treated according to the Monod kinetic equation and proposed a non-competitive inhibition due to the presence of both heavy metal ions.

The purpose of this work is to develop a kinetic equation for ferrous iron oxidation by *At. ferrooxidans*, in pure or mixed culture with *Acidiphilium* sp., in aqueous ferrous sulphate solution in the presence of several heavy metal ions. The experiments were performed with and without addition of organic matter to the synthetic medium in order to be similar to a natural medium. This kind of compounds produces an inhibitory effect over ferrous iron oxidation of *At. ferrooxidans* [19]. Then, when the medium was supplemented with organic matter, heterotrophic bacterium *Acidiphilium* sp. was added to reduce this negative effect.

2. Materials and methods

2.1. Microorganisms

The bacterial strains used in this work were *At. ferrooxidans* and *Acidiphilium* sp. (a mixed culture of *Acidiphilium facilis* and *Acidiphilium organovorum)* isolated from Rio Tinto mines (Huelva, Spain) and kindly made available by the Biohydrometallurgy Group of The University of Seville.

2.2. Media

The medium used to grow and maintain *At. ferrooxidans* was that proposed by Silverman and Lundgren [20], 9K medium ($(NH_4)_2SO_4$ 3.0; $MgSO_4$ 0.5; K_2HPO_4 0.5; KCl 0.1; $Ca(NO_3)_2$ 0.01 g/l), and a ferrous sulphate solution (10 g Fe(II)/l). Mixed culture was cultivated in SMS salt medium [10] ($(NH_4)_2SO_4$ 0.2; $MgSO_4$ 0.4; K_2HPO_4 0.1; KCl 0.1 g/l) containing yeast extract (0.25 g/l) and a ferrous sulphate solution (5 g Fe(II)/l).

2.3. Experimental conditions

The 500-ml erlenmeyer flasks were used, containing 200 ml of medium and 10% (v/v) of inoculum. The initial pH was adjusted, to avoid the excessive precipitation of ferric iron products, to 2.0 in the case of *At. ferrooxidans*, and to 3.0 for mixed culture (5% of each inoculum). Flasks were incubated at 30 °C and 200 rpm in a rotary shaker. Experiments were finished when ferrous iron concentration decreased to less than 20 mg/l.

To study the influence of heavy metal ions on the cultures growth, these were exposed to different concentrations of each ion: Cr(III), Cu(II), Cd(II), Zn(II) and Ni(II). The media were supplemented with metal sulphate solution to a final volume of 200 ml. The salts used were: $Cr_2(SO_4)_3$, $CuSO_4{\cdot}5H_2O$, $CdSO_4{\cdot}8/3H_2O$, $ZnSO_4{\cdot}7H_2O$ and $NiSO_4{\cdot}6H_2O$.

2.4. Analytical methods

The oxidation of ferrous iron sulphate was monitored by determining its residual concentration in the medium, using the 1,10-phenantroline method [21]. This method is based on the complexation reaction between ferrous iron and 1,10-phenantroline; the orange complex is measured by spectroscopy (HP8453) at 515 nm. In order to measure the concentration of total iron in solution, the ferric iron was reduced to ferrous iron using hydroxylamine as the reducing agent. The measurement of total ferrous iron was performed using the aforementioned method. The concentration of ferric iron in solution was taken as the difference between the ferrous and total iron concentration.

The bacterial concentration was determined by counting in a Neubauer chamber in conjunction with anoptical micro-

Table 1
Maximum tolerated concentration (MTC) for pure and mixed cultures of *Acidithiobacillus ferrooxidans* in the presence of heavy metal studied (g/l)

	MTC (g/l)				
	Cr(III)	Cu(II)	Cd(II)	Zn(II)	Ni(II)
Pure culture	0.4	10	10	30	30
Mixed culture	0.4	4	15	40	20

scope (Olympus BH-2) according to the method described by Gómez and Cantero [22].

The concentration of heavy metal ions present in the medium was determined by atomic absorption spectroscopy (UNICAM939 and PU7000 Philips). Samples were maintained at pH lower than 2.0 with nitric acid (60%).

3. Results and discussion

The study of metal influence over bacterial ferrous iron oxidation was carried out by performing several series of experiments with different concentrations of each metal in tandem with a control of a pure or mixed culture.

The tolerance limits for each metal and culture were obtained, in a previous work [23], studying the evolution of bacterial growth (Mcel/ml) and ferrous sulphate concentration (mg/l) as a function of metal concentration. These values were called maximum tolerated concentrations (MTC), defined as the maximum concentration at which bacterial growth is observed. Data in Table 1 for both cultures have shown a high tolerance to Zn(II) and Ni(II), tolerance limits higher than 20 g/l, and a very low tolerance for Cr(III), bacterial growth was not observed for concentrations higher than 0.4 g Cr(III)/l. The procedure carried out to obtain the kinetic equation of ferrous sulphate oxidation in aqueous solution by *At. ferrooxidans*, in pure or mixed culture with *Acidiphilium* sp., in the presence of metallic ions started with the calculation of specific growth rates. They were obtained from experimental data, bacterial concentration, X_i (Mcel/ml), at each time point, t_i (s), values corresponding to exponential growth phase. A numerical differentiation procedure was followed in accordance with the following calculation algorithm, where μ_i is the specific growth rate in each moment:

$$\mu_i = \frac{1}{X_i}\frac{\mathrm{d}X}{\mathrm{d}t} \tag{2}$$

The results obtained represent the set of values of specific growth rate for different ferrous iron concentrations measured at each time point (t_i) and for each metal concentration tested. Fig. 1a and b show two representations of the specific growth rate values evolution as a function of the substrate concentration in the exponential growth phase for pure and mixed culture in presence of Zn(II). In order to obtain a kinetic equation that adjust the experimental data, a Monod expression, proposed by Kupka and Kupsáková [18] for ferrous sulphate

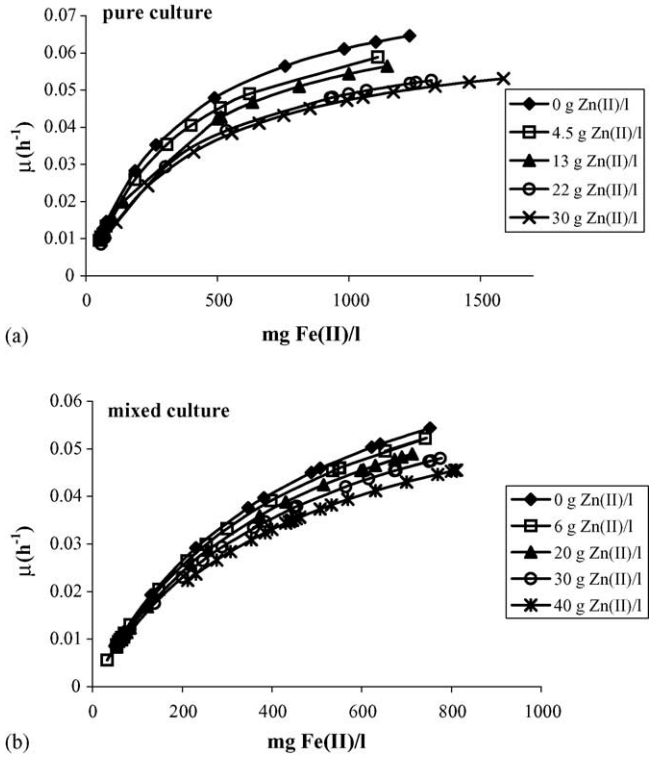

Fig. 1. Representation of experimental specific growth rate vs. substrate concentration for *At. ferrooxidans* in the presence of zinc(II) (a) pure culture and (b) mixed culture with *Acidiphilium* sp.

oxidation in presence of copper(II) and nickel(II), was considered:

$$\mu = \frac{\mu^* S}{K_S^* + S} \tag{3}$$

A statistical analysis program was used to carry out the mathematical fitting of characteristic parameters of kinetic equation. This program operates by non-linear regression based on the Marquardt algorithm [24]. This algorithm requires initial values to carry out the fitting of coefficients; the values proposed by Gómez et al. [3] were considered: $\mu_{max} = 0.14\,h^{-1}$ and $K_S = 0.94$ g/l. For each experience several values of maximum specific growth rate, μ^*_{max} (h^{-1}), and constant of saturation, K_S^* (mg/l)) as a function of metal concentration were obtained, results are shown in Tables 2 and 3.

These values reveal that the presence of heavy metal in the medium exerts a significant influence on kinetic parameters calculated. In relation to specific growth rate, a non-competitive inhibition can be observed, because the maximum specific growth rate changes considerably when inhibitor concentration increases in the medium while constant of saturation does not suffer a significant variation. These assumptions can be confirmed with the representation of reciprocal specific growth rate versus reciprocal substrate concentration (Fig. 2a and b). So, we can conclude this type of inhibition. Therefore, the reaction mechanism for this kind

Table 2
Kinetic parameters obtained as a function of metal concentration for pure culture of *Acidithiobacillus ferrooxidans*

Ion	Concentration	μ^*_{max} (h^{-1})	K^*_S (mg/l)
Chromium(III) (g Cr/l)	0	0.082	372.525
	0.1	0.055	390.22
	0.2	0.053	398.34
	0.3	0.051	405.75
	0.4	0.048	414.31
Copper(II) (g Cu/l)	0	0.086	366.474
	2	0.068	380.562
	6	0.064	400.123
	10	0.061	407.521
Cadmium(II) (g Cd/l)	0	0.085	373.34
	3	0.081	380.75
	4.5	0.079	385.65
	6	0.076	390.65
	7.5	0.069	394.14
	10	0.067	400.63
Zinc(II) (g Zn/l)	0	0.084	368.45
	4.4	0.079	380.12
	13.2	0.076	398.23
	22.05	0.069	408.52
	30	0.067	414.75
Nickel(II) (g Ni/l)	0	0.084	365.13
	8.8	0.076	374.22
	13.2	0.074	385.75
	15.4	0.071	390.32
	30	0.063	395.28

of inhibition could be represented as follows:

$$\begin{array}{ccccc} E + S & \leftrightarrow & ES & \rightarrow & E + P \\ + & & + & & \\ I & & I & & \\ \updownarrow & & \updownarrow & & \\ EI + S & \leftrightarrow & ESI & & \end{array} \quad (4)$$

A fitting by non-linear regression for maximum apparent specific growth rate expression was performed according to the characteristic expression for non-competitive inhibition:

$$\mu^*_{max} = \frac{\mu_{max} K_I}{1 + K_I} \quad (5)$$

The constants of inhibition obtained for each species and each heavy metal are showed in Table 4. Fig. 3a and b show representations of apparent maximum specific growth rate versus inhibitor concentration for same cases.

The results present a concordance with previous data about the tolerance of these species to the heavy metal ions studied (Table 1); in that way, the highest values of constant of inhibition (Table 4) belong to pure or mixed cultures which presented the uppermost maximum tolerated concentrations (MTC) such as nickel and zinc ions experiments. The comparison between pure or mixed culture of MTC and K_I values (Tables 1 and 4) for each metal shows that cultures which present a high tolerance to the metal present a high inhibition, minor K_I. In Cu(II) and Ni(II) cases, *At. ferrooxidans*

Table 3
Kinetic parameters obtained as a function of metal concentration for mixed culture of *At. ferrooxidans* and *Acidiphilium* sp.

Ion	Concentration	μ^*_{max} (h^{-1})	K^*_S (mg/l)
Chromium(III) (g Cr/l)	0	0.081	391.23
	0.2	0.054	394.23
	0.4	0.047	398.27
Copper(II) (g Cu/l)	0	0.087	442.37
	2	0.084	445.25
	4	0.081	446.72
Cadmium(II) (g Cd/l)	0	0.086	385.42
	6	0.078	390.55
	15	0.069	391.75
Zinc(II) (g Zn/l)	0	0.088	465.12
	6	0.085	467.38
	20	0.081	468.29
	30	0.077	469.42
	40	0.072	471.62
Nickel(II) (g Ni/l)	0	0.086	475.13
	4	0.083	480.23
	10	0.081	482.74
	15	0.073	484.65
	20	0.071	486.32

inhibition increases in the presence of heterotrophic strain, however, this presence seems to improve the tolerance to Cd(II) and Zn(II) ions. In presence of Cr(III) the behaviour of pure and mixed culture is very similar.

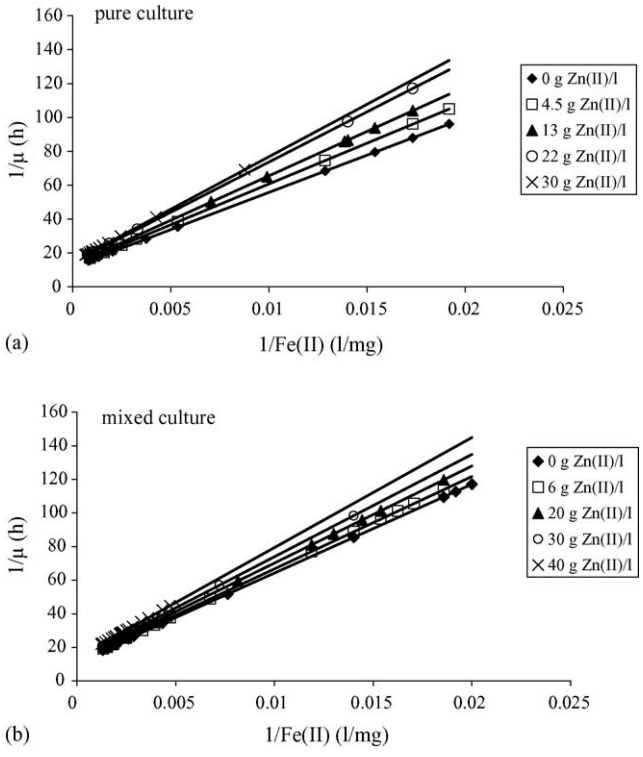

Fig. 2. Representation of reciprocal specific growth rate vs. reciprocal substrate concentration for *At. ferrooxidans* in the presence of zinc(II) (a) pure culture and (b) mixed culture with *Acidiphilium* sp.

Table 4
Constants of inhibition (K_I) for each heavy metal ion for *Acidithiobacillus ferrooxidans*, pure and mixed culture

	K_I (g/l)				
	Cr(III)	Cu(II)	Cd(II)	Zn(II)	Ni(II)
Pure culture	0.42	20.92	47.01	131.52	89.23
Mixed culture	0.48	54.43	42.31	125.27	98.57

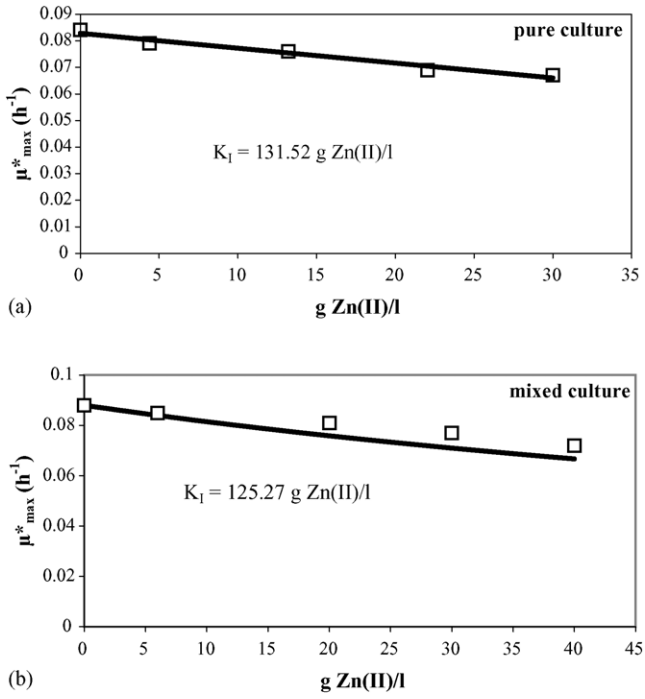

Fig. 3. Representation of apparent maximum specific growth rate of *At. ferrooxidans* vs. zinc(II) concentration (a) pure culture and (b) mixed culture with *Acidiphilium* sp.

If these values of K_I are compared with values obtained by Kupka and Kupsáková [18] for ferrous sulphate oxidation by *At. ferrooxidans* in presence of copper and nickel ion, it can be seen K_I for copper obtained in this work (20.92 g/l) is very similar than the obtained for these authors (21.80 g/l). In the nickel case, this value differs enough, K_I was 13.67 g/l for Kupka and Kupsáková [18] and it was 89 g/l in this work. This high value is in accordance with maximum tolerated concentration for this ion, 30 g Ni(II)/l, while the maximum tested concentration in Kupka and Kupsáková's work [18] was 8 g Ni(II)/l, these difference indicates that *At. ferrooxidans* strain used in this work shows a high tolerance to nickel ion and it is superior than strain used in the other work.

In regard to mixed cultures values, it is difficult to do a comparison because of the absence of works on kinetic modelling of these cultures in ferrous sulphate solution.

Finally, it can be affirmed that the growth of *At. ferrooxidans* in pure or mixed culture with *Acidiphilium* sp. in aqueous ferrous sulphate solution in the presence of heavy metal is affected by a non-competitive inhibition. This effect is showed with suitable precision in the growth equation proposed. The equation predictions of specific growth rate were closely to experimental data and provide high coefficient.

Acknowledgement

The authors wish to express sincere gratitude to Consejería de Medio Ambiente (Junta de Andalucía) for funding this research through Project 18.IN.PR.0010.

References

[1] Ballester A, González F, Blázquez ML. The use catalytic in bioleaching. Hydrometallurgy 1992;29:145–60.
[2] Zouboulis AI, Matis KA, Handcock IC. Biosorption of metals from diluted solutions. Sep Purif Method 1997;26(2):255–95.
[3] White C, Sharman AK, Gadd G. An integrated microbial process for the bioremediation of soil contaminated with toxic metals. Nat Biotechnol 1997;16:572–5.
[4] Nemati, Webb C. A kinetic model for biological oxidation of ferrous iron by *Thiobacillus ferrooxidans*. Biotechnol Bioeng 1997;53(5):478–86.
[5] Harrison Jr AP. *Acidiphilium crytum* gen. nov. sp. nov., heterotrophic bacterium from acidic mineral environments. Int J Syst Bacteriol 1981;108:328.
[6] Leduc LG, Ferroni GD, Trevors JT. Resistance to heavy metals in different strains of *Thiobacillus ferrooxidans*. World J Microb Biotechnol 1997;13:453–5.
[7] Tuovinen OH, Niemela SI, Gyllemberg HG. Tolerance of *Thiobacillus ferrooxidans* to some metals. Anton van Lee JMS 1997;37:489–96.
[8] Baillet F, Magnin P, Cheruy A. Cadmium tolerance and uptake by a *Thiobacillus ferrooxidans* biomass. Environ Technol 1997;18:631–8.
[9] Ghosh S, Mahapatra NR, Banerjee. Metal resistance in *Acidocella* strains and plasmid-mediated transfer of this characteristic to *Acidiphilium multivorum* and *Escherichia coli*. Appl Environ Microbiol 1997;63(11):4523–7.
[10] Mahatrapa NR, Banerjee PC. Extreme tolerance to cadmium and high resistance to copper, nickel and zinc in different *Acidiphilium* strains. Lett Appl Microbiol 1996;23:393–7.
[11] MacDonald DG, Clark RH. The oxidation of aqueous ferrous sulphate by *Thiobacillus ferrooxidans*. Can J Chem Eng 1970;48:669–76.
[12] Lacey DT, Lawson F. Kinetics of the liquid-phase oxidation of acid ferrous sulphate by the bacterium *Thiobacillus ferrooxidans*. Biotechnol Bioeng 1970;12:29–50.
[13] Liu MS, Branion RMR, Duncan DW. The effects of ferrous iron, dissolved oxygen, and inert solids concentrations on the growth of *Thiobacillus ferrooxidans*. Can J Chem Eng 1989;66:445–51.
[14] Suzuki I, Lizama HM, Tackaberry PD. Competitive inhibition of ferrous iron oxidation by *Thiobacillus ferrooxidans* by increasing concentrations of cells. Appl Environ Microbiol 1989;55(5):1117–21.
[15] Kumar R, Gandhi KS. Modelling of Fe^{2+} oxidation by *Thiobacillus ferrooxidans*. Appl Microbiol Biotechnol 1990;33:524–8.
[16] Pagella C, Silvestri P, de Faveri DM. Hydrogen sulphide removal with a biochemical process: the biological step. Chem Biochem Eng Q 1996;10:165–74.
[17] Gómez JM, Caro I, Cantero D. Kinetics equation for growth of *Thiobacillus ferrooxidans* in aqueous ferrous sulphate solutions. J Biotechnol 1996;48:147–52.
[18] Kupka D, Kupsáková I. Iron(II) oxidation kinetics in *Thiobacillus ferrooxidans* in the presence of heavy metals. In: Bio-

hydrometallurgy and the environment toward the mining of the 21st century. Process metallurgy, vol. 9A, 1998. p. 387–98.
[19] Usami S, Sugitani T. Effect of organic substances on the growth and iron oxidation activity bacteria. Hakko Kogaku Zassi 1971;49(7):587–91.
[20] Silverman MP, Lundgren DG. Studies on the chemoautotrophic iron bacterium *Ferrobacillus ferrooxidans*. I. An improved medium and a harvesting procedure for securing high cell yields. J Bacteriol 1959;77:642–7.
[21] Vogel AI. Vogel's text book of quantitative chemical analysis. 5th ed. London: Longman Group Ltd.; 1989.
[22] Gómez JM, Cantero D. Modelling of ferrous iron oxidation by *Thiobacillus ferrooxidans* in discontinuous culture: influence of temperature, pH and agitation rate. J Ferment Bioeng 1998;86(1):79–83.
[23] Cabrera FG, Oxidación bacteriana de sulfato ferroso por microorganismos acidófilos en presencia de metales pesados. MSc Thesis. Spain: University of Cadiz; 2001.
[24] Marquardt DW. An algorithm for least-squares estimation of nonlinear parameters. J Soc Ind Appl Math 1963;11:431–41.