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Influence of heavy metals on growth and ferrous sulphate oxidation by *Acidithiobacillus ferrooxidans* in pure and mixed cultures

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Abstract

The work described here concerns the study of the tolerance limits of the acidophilic bacterium, *Acidithiobacillus ferrooxidans*, in the presence of a range of heavy metal concentrations. With the aim of reducing the inhibitory effect that organic matter has on ferrous iron oxidation by *A. ferrooxidans*, a heterotrophic bacterium, *Acidiphilium*, sp., was added to media supplemented with organic matter. The maximum tolerated concentrations (MTC) were measured. *At. ferrooxidans* tolerates high levels of the metals tested: 0.4 g Cr(III)/l, 10 g Cu(II)/l, 10 g Cd(II)/l, 30 g Zn(II)/l and 30 g Ni(II)/l. The addition of heterotrophic bacteria, *Acidiphilium*, changes the tolerance limits in different ways depending on the metal: 0.4 g Cr(III)/l, 4 g Cu(II)/l, 10 g Cd(II)/l, 40 g Zn(II)/l and 15 g Ni(II)/l.

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

1. Introduction

The accumulation of heavy metals in water, air or soils is a serious environmental problem. In recent years, several technologies have been developed with the aim of reducing or removing heavy metals from 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* are acidophilic bacteria usually found in acid mine effluents. *At. ferrooxidans* is an autotroph, i.e., its carbon source is carbon dioxide and it oxidises ferrous iron or reduces sulphur compounds for growth [4]. *Acidiphilium* is a heterotroph and uses organic matter as a carbon source [5]. Both microorganisms show a particular tolerance to several heavy metals [6,7,8]. These tolerance characteristics, along with their ability to transform metal ions, means that *At. ferrooxidans* and *Acidiphilium* can be used to solve certain environmental problems caused by the presence of heavy metals.

With the aim of studying the potential application of these microorganisms in the removal or partial removal of heavy metal ions from contaminated media, it is necessary to ascertain the tolerance of these bacteria to the contaminants in question. Therefore, the purpose of this work was to determine the tolerance levels of *At. ferrooxidans* to the following heavy metal ions: Cr(III), Cu(II), Cd(II), Zn(II) and Ni(II). Natural media usually contain organic matter, which produces an inhibitory effect on ferrous iron oxidation by *At. ferrooxidans* [9]. In media that contain organic matter it is possible to add heterotrophic bacteria, e.g., *Acidiphilium*, which is commonly found in the environment of *At. ferrooxidans*, in order to decrease the negative effect on autotrophic bacteria.

2. Materials and methods

2.1. Microorganisms

The bacterial strains used in this work were *At. ferrooxidans* and *Acidiphilium* (a mixed culture of *Acid-*

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ocella 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 [10], 9K medium containing ferrous sulphate (10 g/l as the energy source), $(NH_4)_2SO_4$ (3.0 g/l), $MgSO_4$ (0.5 g/l), K_2HPO_4 (0.5 g/l), KCl (0.1 g/l) and $Ca(NO_3)_2$ (0.01 g/l). The mixed culture was cultivated in SMS salt medium [7], which contained $(NH_4)_2SO_4$ (0.2 g/l), $MgSO_4$ (0.4 g/l), K_2HPO_4 (0.1 g/l), KCl (0.1 g/l), yeast extract (0.25 g/l) and ferrous sulphate (5 g/l).

2.3. Experimental conditions

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

To study the influence of heavy metal ions on the bacterial growth and oxidative capacity, pure and mixed cultures were exposed to different concentrations of each of the following ions: 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 sulphate was monitored by determining its residual concentration in the medium using a 1,10-phenanthroline method [11]. This method is based on the complexation reaction between ferrous iron and 1,10-phenanthroline; the orange complex being measured by spectroscopy (HP8453) at 515 nm. The concentration of total iron in solution was measured by reducing the ferric iron 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 concentrations.

The bacterial concentration was determined by counting in a Neubauer chamber in conjunction with an optical microscope (Olympus BH-2) according to the method described by Gómez and Cantero [12].

Table 1
MTC, T85 and BP for a pure culture of *At. ferrooxidans* in the presence of the heavy metals studied

Ion	MTC (g/l)	T85 (h)	BP (Mcell/mg $Fe(II)_{oxid}$ h)
Cr(III)	0.4 ± 0.008	56.57 ± 1.13	0.015 ± 0.0003
Cu(II)	10 ± 0.20	79.71 ± 1.59	0.070 ± 0.0014
Cd(II)	10 ± 0.20	68.27 ± 1.37	0.130 ± 0.0260
Zn(II)	30 ± 0.60	45.01 ± 0.90	0.070 ± 0.0014
Ni(II)	30 ± 0.60	60.01 ± 1.20	0.060 ± 0.0012
Control	–	21.34 ± 0.43	0.39 ± 0.0080

3. Results and discussion

The tolerance of *At. ferrooxidans* in a pure or mixed culture to five heavy metal ions [Cr(III), Cu(II), Cd(II), Zn(II) and Ni(II)] was studied.

The study was carried out by performing several series of experiments with different concentrations of each metal in tandem with a control culture.

The results obtained from the experiments allowed three parameters to be calculated and these were used in the interpretation of the results: T85, the time period required to oxidise 85% of the substrate (hours); BP (bacterial production), the bacterial concentration in relation to the quantity of ferrous iron oxidised per hour [Mcell/mg $Fe(II)_{oxid}$ h] and maximum tolerated concentration (MTC), defined as the maximum concentration at which bacterial growth is observed. The values of the three parameters for each ion and each culture studied are shown in Tables 1 and 2.

It is well known that bacterial heavy metal resistance depends on the nature of the strain. A wide range of MTC can be found in the literature according to strains studied in each case. MTC determined by other authors for several strains of *At. ferrooxidans* and *Acidiphilium* are shown in Table 3. From these data, it is possible to make a comparison between MTCs found in the literature (Table 3) and MTCs obtained in this work (Tables 1 and 2). The data reveal a high degree of variability in the resistance to heavy metals for different strains of the same bacteria.

It is clear that the presence of heavy metals decreased the oxidative capacity of the bacterial species studied and,

Table 2
MTC, T85 and BP for a mixed culture of *At. ferrooxidans* and *Acidiphilium* in the presence of the heavy metals studied

Ion	MTC (g/l)	T85 (h)	BP (Mcell/mg $Fe(II)_{oxid}$ h)
Cr(III)	0.4 ± 0.008	55.29 ± 1.10	0.86 ± 0.017
Cu(II)	4 ± 0.08	42.56 ± 0.85	0.87 ± 0.017
Cd(II)	15 ± 0.30	49.42 ± 0.20	1.25 ± 0.025
Zn(II)	40 ± 0.80	63.70 ± 0.99	0.27 ± 0.005
Ni(II)	20 ± 0.40	43.30 ± 0.87	0.34 ± 0.007
Control	–	23.00 ± 0.46	2.01 ± 0.040

Table 3
Literature MTC values for pure cultures of *At. ferrooxidans* and *Acidiphilium* strains in the presence of heavy metals

Bacteria	Heavy metal	MTC (g/l)	Reference
At. ferrooxidans	Cr(III)	0.52	[13]
		0.78	[14]
		3.9	[15]
	Cu(II)	0.6–10	[16]
		19	[17]
		16	[18]
		10	[19]
		5	[20]
	Cd(II)	5.6	[21]
		1.12	[22]
	Zn(II)	40	[23]
		40	[19]
	Ni(II)	0.6–9.4	[16]
		6.3	[24]
		10	[25]
Acidiphilium	Cu(II)	0.6–1.9	[7]
	Cd(II)	<0.02–112.4	
	Zn(II)	0.6–13.1	
	Ni(II)	1.2–23.5	

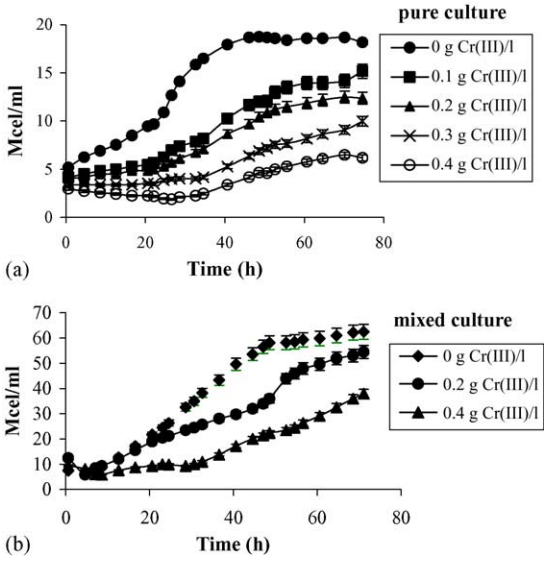

Fig. 2. Evolution of bacterial growth (a) for a pure culture of *At. ferrooxidans* (b) for a mixed culture of *At. ferrooxidans* and *Acidiphilium* in the presence of several concentrations of chromium(III).

furthermore, cellular growth was inhibited as the metal concentration in the medium increased. Two examples of the evolution of ferrous sulphate oxidation in the presence of different metal ion concentrations are shown in Fig. 1a and b. An increase in the metal concentration produces a progressive increase in the oxidation time. This rise is mirrored by a decrease in cellular growth, as can be seen in the examples represented in Fig. 2a and b, where high metal concentrations cause a significant lag phase.

The tolerance study of *At. ferrooxidans* with respect to Cr(III) was carried out in cultures supplemented with 0.1, 0.2, 0.3, 0.4 and 0.5 g Cr(III)/l. Growth was not observed at all at the higher concentration tested (data not shown). The maximum tolerated concentration was 0.4 g Cr(III)/l and for this culture the time necessary to oxidise 85% of the initial ferrous content increased from 21.34 h (control culture) to 56.57 h. This effect was accompanied by a significant decrease in the bacterial production (0.015 Mcell/mg $Fe(II)_{oxid}$ h). The presence of *Acidiphilium* in cases where organic matter is added does not seem to affect the tolerance or the capacity of *At. ferrooxidans* to oxidise ferrous iron in the presence of Cr(III). In this case the MTC was the same [0.4 g Cr(III)/l] and the oxidation time was very similar (56.27 h). The chromium ion proved to be the most toxic of the metal ions studied for both cultures (pure and mixed). The strains employed in this work seem to be very sensitive to Cr(III)—strains have been reported in the literature that are able to tolerate 3.9 g Cr(III)/l [15].

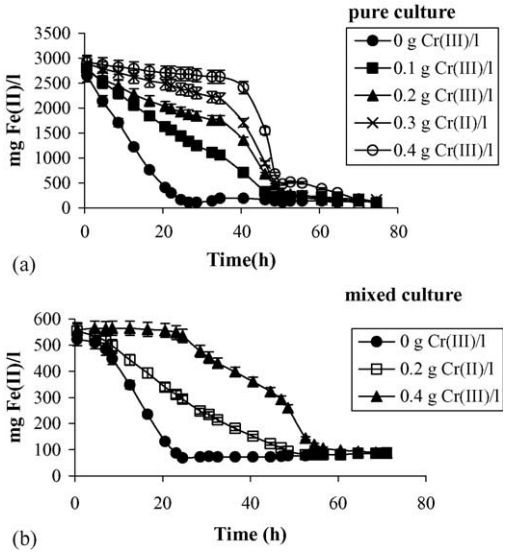

Fig. 1. Evolution of ferrous sulphate oxidation (a) for a pure culture of *At. ferrooxidans* (b) for a mixed culture of *At. ferrooxidans* and *Acidiphilium* in the presence of several concentrations of chromium(III).

The study in the presence of Cu(II) was performed in cultures with concentrations in the range 2–12 g Cu(II)/l. The pure culture, *At. ferrooxidans*, tolerates up to 10 g Cu(II)/l. This value is within the range reported by other authors [16–20] for certain strains of this bacteria in the presence of Cu(II) (Table 3). The presence of heterotrophic bacteria produces a significant decrease in the tolerance limit [4 g Cu(II)/l]. This fact could be due to the inhibition that this metal produces on *Acidiphilium* strains. Mahatrapa and Banerjee [7] reported a tolerance study involving several strains of *Acidiphilium* genus in the presence of various metals and the tolerance range for copper was 0.6–1.9 g Cu(II)/l.

The study with cadmium was carried out with concentrations in the range 3–15 g Cd(II)/l. *At. ferrooxidans* showed growth up to 10 g Cd(II)/l and for these cultures the oxidation time increased to 68.27 h. The MTC is higher

those found in the literature for other strains of *At. ferrooxidans* [21,22]. The addition of heterotrophs seems to facilitate the oxidative capacity of *At. ferrooxidans*. The mixed culture tolerated 15 g Cd(II)/l and, moreover, the oxidation time decreased (49.24 h). This positive effect could be due to the high resistance to cadmium ions of *Acidiphilium* strains. Indeed, Mahatrapa and Banerjee [7] reported a wide tolerance range for this metal: <0.02–112 g Cd(II)/l. A particular behavioural trend was observed in the presence of cadmium in that bacterial production did not decrease to the same extent as with other metals (Tables 1 and 2).

The highest concentration of zinc tested was 50 g Zn(II)/l. This ion gives rise to the lowest toxic effect on both pure and mixed cultures. *At. ferrooxidans* tolerated 30 g Zn(II)/l and the time required for 85% substrate oxidation was only 45.01 h. In this case, the presence of *Acidiphilium* also seemed to facilitate the tolerance to Zn(II), with an MTC value of 40 g Zn(II)/l. Kondratyeva et al. [23] reported the same value for a pure culture of *At. ferrooxidans*.

The nickel tolerance was studied using concentrations below 40 g Ni(II)/l. A high tolerance to nickel was found, with *At. ferrooxidans* showing growth up to 30 g Ni(II)/l and an oxidation time of around 60 h. This value is higher than those reported by other authors, with the maximum tolerated concentration in those cases being approximately 10 g Ni(II)/l [16,24,25]. The mixed culture was more sensitive to the presence of nickel and only 20 g Ni(II)/l were tolerated. This value is consistent with the tolerance limit of a pure culture of *Acidiphilium* strains: 1.2–23.5 g Ni(II)/l.

4. Conclusions

On the basis of the results obtained, it is possible to establish the same order of toxicity for both cultures: Cr(III) > Cu(II) > Cd(II) > Ni(II) > Zn(II). The tolerated metal concentrations found for the pure and mixed cultures are similar or higher than concentrations usually found in contaminated media for heavy metal ions.

The strain studied, which was isolated from a mine environment, has a particular behaviour pattern; similar or higher tolerance levels for Cu(II), Cd(II) and Ni(II) were found in comparison to other studies, but the Cr(III) and Zn(II) tolerances were lower. This trend could be due to the presence of certain metallic species in the mine environment, which makes the bacteria resistant to high levels of certain metals. The decrease in the oxidative capacity of *At. ferrooxidans* when organic matter was supplemented was counteracted by the addition of heterotrophic bacteria. The metal tolerance of the mixed culture had a different behaviour, which depended on the metal studied and the particular tolerance of *Acidiphilium* to each metal in a pure culture.

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