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New advances in copper biomachining by iron-oxidizing bacteria

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ABSTRACT

The machining of copper contained in oxygen-free copper workpieces by extremophile bacteria has been studied. The effect of the main parameters affecting the continuity of the process and which decrease the removal rate were analysed during the incubation, biomachining and regeneration phases. The presence of copper affected the bacterial culture, while the enhancement of process performance due to the simultaneous presence of *A. ferrooxidans* and *L. ferrooxidans* was relatively limited. pH was maintained below 1.80 to avoid Fe^{3+} losses in the form of jarosite precipitates. Measurement of the redox potential allowed a rapid monitoring of the process status.

1. Introduction

The favourable properties of oxygen-free copper (OFC) (high electrical and thermal conductivity, high ductility and corrosion resistance), combined with its homogeneous structure, make OFC the optimum substrate material for numerous components with highly specific requirements, such as spent nuclear fuel canisters, lens moulds, high voltage electrodes in vacuum interrupters and other sophisticated applications in the electronic and electrotechnical industries [1,2].

The manufacturing process for OFC workpieces requires polishing or machining in order to add, remove or shape the material within small tolerances. As the conventional chemical and physical processes that are currently employed increase heat or residual stress during these steps, biomachining has arisen as an alternative sustainable and precise machining tool. Biomachining has been defined as a controlled microbiological technique for the selective formation of microstructures on a workpiece by metal removal or dissolution, and it is characterised by machining components with minimum heat or residual stress and without exerting a cutting force during the process [3]. The microorganisms used in biomachining processes replace the toxic chemical compounds (such as $FeCl_3$) typically employed in chemical processing [4]. In addition, energy consumption is low, thus resulting in a subsequent cost saving in the manufacturing of high quality pieces [5]. Furthermore, the environmental benefit of biomachining is a valuable issue in current industrial policy, as many industries focus their efforts on both improving productivity and complying with environmental regulations [6,7].

Biomachining is a process based on the removal of metallic material by solubilisation through pure chemical reactions in solution. When OFC is the metallic material, chemolithotrophic and acidophilic microorganisms (archaea and bacteria) act as a catalyst by oxidizing ferrous to ferric iron. Fe^{3+} is subsequently reduced back to Fe^{2+} due to copper leaching ($Cu^0 \rightarrow Cu^{2+}$). In a third stage, the Fe^{2+} resulting from this reaction is regenerated to Fe^{3+} by the cells, thus allowing them to obtain energy for growth and bringing the cycle between Fe^{3+} and Fe^{2+} to a close [8].

The main drawback of controlled microbiological corrosion is the progressive decrease in the amount of metal removed per unit

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of time [5,9,10]. Thus, even if a maximum rate is initially achieved, the specific material removal rate (SMRR) has been reported to be very low after several hours. Several factors, such as a decrease in ferrous sulfate concentration, an increase in Cu^{2+} concentration, hydrolysis phenomena or oxygen depletion, have been proposed to have a critical impact on the SMRR. Despite this, Kumada et al. [11] correctly predicted that the machining rate could be controlled by adjusting the Fe^{3+} concentration in the liquid medium. Moreover, although the microbial consortium has proven to be very efficient at regenerating Fe^{3+}, the chemical mechanism of copper dissolution is too fast when compared with the bio-oxidation of Fe^{2+} [12,13]. Given this assumption, a rest period to allow the complete bio-regeneration of Fe^{3+} after each biomachining testing time was included in our previous work (4 or 8 h of rest after 1 or 4 h of biomachining, respectively) using the autotrophic, acidophilic and mesophilic bacterium *Acidithiobacillus ferrooxidans* [14]. Thus, the Cu^0 removal rate increased in a cyclical fashion after each rest period. The discrepancies found during the determination of the iron ion concentration in this study [14], prevented us from developing a continuous, stable and sustainable biomachining process.

The work described herein explores the effect of several process parameters (pure culture *vs* mixed culture, inoculum concentration, jarosite formation, Cu^{2+} and Fe^{3+} concentrations) that might affect the three stages described in our previous study, namely incubation, biomachining, and regeneration period, in an effort to ensure a constant removal rate over time. The mechanism of copper removal during the biomachining process was also evaluated. Finally, a methodology for the correct storage and determination of Fe^{2+} and Fe^{3+} in the bioleaching media is presented and the applicability of the redox potential as a rapid measurement for gaining information about the status of the biomachining process is evaluated.

2. Material and methods

2.1. Microorganisms and culture media

A. ferrooxidans (DSM-14882) was obtained from the Department of Chemical Engineering and Food Technologies of the University of Cádiz (UCA). *Leptospirillum ferrooxidans* (ATCC 29047) (CINDEFI, National University of La Plata, Argentina), a bacterium previously employed in bioleaching activities, was selected to constitute a consortium together with *A. ferrooxidans* [15]. In contrast to *A. ferrooxidans*, *L. ferrooxidans* is less inhibited by ferric iron and sustains higher bio-oxidation activity at higher redox potentials, thus allowing the maintenance of an elevated rate of Fe^{2+} consumption even at very low Fe^{2+} concentrations [16].

Both bacteria were cultured in a modified Lundgren-Silverman 9 K liquor. This medium was composed of mineral salts ($(NH_4)_2SO_4$ 3.0 g/L, K_2HPO_4 0.5 g/L, $MgSO_4{\cdot}7H_2O$ 0.5 g/L, KCl 0.1 g/L, $Ca(NO_3)_2$ 0.01 g/L) and 30 g/L of $FeSO_4{\cdot}7H_2O$ (final concentration of 6 g Fe^{2+}/L), which served as the energy source for *A. ferrooxidans* and *L. ferrooxidans*. Bacteria were incubated under agitation conditions (130 rpm) at a temperature of 30 °C until complete oxidation of Fe^{2+} to Fe^{3+} was achieved, which indicated that bacterial growth was satisfactory. The specific bacterial (*A. ferrooxidans* or *L. ferrooxidans)* broth volume mixed with modified 9 K medium for each experiment is detailed in the following sections.

2.2. Preparation of copper samples

OFC workpieces with a minimum purity of 99.99% and a size of $10 \times 15 \times 2$ mm were abraded using an 800-grit abrasive disk wheel (REMET petrographic cleanser LS1) [4,17]. Each workpiece had a hole (2 mm in diameter and 2 mm thick) for suspension in the active medium when performing the tests. Prior to their immersion in 9 K medium, the OFC blocks were rinsed with deionized water and ethanol (96%) and then heated to remove surface moisture.

2.3. Copper biomachining procedure

Biomachining tests were carried out by suspending preweighed OFC blocks into the aforementioned culture medium in conical flasks at 30 °C and 130 rpm (shaking water bath ST 30, Nüve). The metal samples were removed from the solution at user-defined intervals, rinsed with deionized water and ethanol (96%), dried, weighed, and finally reintroduced into the liquid sample. The SMRR achieved was calculated as follows:

$$\text{SMRR (mg/(h cm}^2\text{))} = \frac{\text{Weight loss (mg)}}{\text{Time (h) Area (cm}^2\text{)}} \quad (1)$$

2.4. Effect of inoculum concentration

The effect of the inoculum concentration at 2, 4 and 6% v/v (IN-1:IN-3) on the cultivation period, which corresponded to a bacterial concentration of 3.33×10^6, 6.66×10^6 and 10^7 cell/mL, respectively, was studied. The initial Fe^{2+} concentration in the three 9 K medium samples (IN-1:IN-3) was established at 6 g Fe^{2+}/L. The redox potential and ferrous iron concentration were determined until oxidation of ferrous iron was almost complete (less than 1% of the initial concentration). In all cases, the results showed that the final number of bacteria was 1.3×10^8 cell/mL. Each experiment was performed in duplicate and the mean results were taken.

2.5. Effect of Cu^{2+} concentration

The effect of Cu^{2+} concentration was quantified for the cultivation (C1:C4), biomachining (B1:B4) and regeneration stages (R1:R4). The modified 9 K medium used in all experiments contained 6 g Fe^{2+}/L and the inoculum concentration was established at 6% v/v. Each experiment was performed in duplicate and the mean results were taken.

With regarding to the cultivation period, four culture solutions (C-1:C-4), each of which contained increasing concentrations of Cu^{2+} (Table 1), were prepared. The experiment lasted until the oxidation of ferrous iron was almost complete (less than 1% of the initial concentration).

In the biomachining step, *A. ferrooxidans* broth was initially pre-inoculated at 6% v/v (in the absence of copper); when the culture reached the stationary phase (full oxidation of the ferrous iron), it was divided into four equal aliquots (B-1:B-4) and increasing concentrations of Cu^{2+} were added to each sample (Table 1). A single OFC workpiece was sequentially bio-oxidized for one hour in the four media (B-1 → B-2 → B-3 → B-4) in order to avoid the surface effect of the process [9]. The SMRR was then determined.

In the regeneration step, *A. ferrooxidans* was initially pre-inoculated at 6% v/v (in the absence of copper); when the culture reached the stationary phase, an OFC workpiece was biomachined in the solution for two hours. The resulting leachate, which contained a Cu^{2+} concentration of 1.5 g/L due to the metal released during the biomachining step, was divided into four equal aliquots (R-1:R-4) and additional increasing concentrations of Cu^{2+} were added to each sample (Table 1). The evolution of Fe^{2+} concentration over time was analysed.

2.6. Effect of Fe^{3+} concentration

The influence of Fe^{3+} concentration during the biomachining stage was examined. *A. ferrooxidans* was cultured in three flasks of 9 K medium (inoculated at 6% v/v) containing 0.6, 3 and 6 g Fe^{2+}/L,

Table 1
Cu^{2+} concentration in the samples related to bacteria culturing, biomachining and regeneration phases.

Experiment[a]	Culturing (C)				Biomachining (B)				Regeneration (R)			
Sample	C-1	C-2	C-3	C-4	B-1	B-2	B-3	B-4	R-1	R-2	R-3	R-4
OFC workpiece	×	×	×	×	√	√	√	√	×	×	×	×
$[Cu^{2+}]$ (g/L)	0	1.2	3.6	6.1	0	1.2	3.6	6.1	1.5 (+0)	2.8 (+1.3)	5.2 (+3.7)	7.5 (+6.0)

[a] In all cases, *A. ferrooxidans* was cultured at 6% v/v in the modified 9 K medium (6 g Fe^{2+}/L).

respectively. A single OFC workpiece was sequentially bio-oxidized in the three media (0.6 g Fe^{2+}/L → 3 g Fe^{2+}/L → 6 g Fe^{2+}/L) and the SMRR determined. The experiment lasted for three hours, which was the time necessary to observe the phenomenon of decreasing SMRR during biomachining. Each experiment was performed in duplicate and the mean results were taken.

2.7. Effect of a mixed culture

The use of mixed cultures (two or more microbial species) appears to be advantageous with respect to a pure culture because of the potential for consortia to be more robust in the event of environmental fluctuations. As far as the biomachining process is concerned, a mixed culture system was proposed to increase Fe^{2+} bio-oxidation kinetics since the regeneration step has been identified as the system bottleneck [14].

A diagram of the experimental set-up for the procedure is provided in Fig. 1. Two samples of 150 mL each (MC-1 and MC-2) were prepared in flasks according to the methodology indicated in section 2.1 (*A. ferrooxidans* culture inoculated at 6% v/v) and these were biomachined twice for 1 h. Prior to the regeneration step (which was established at 4 h in our previous work [14]), sample MC-2 was also inoculated with *L. ferrooxidans*, which was cultured separately (6% v/v) and concentrated in a laboratory centrifuge (Spectrafuge 24D, Labnet) in order to separate the supernatant. The biomass was subsequently resuspended in 5 mL of modified 9 K medium (50% v/v) and introduced into the lab jar. The experiment was performed in duplicate and the mean results were taken.

2.8. Fe^{2+}, Fe^{3+} and total Fe determination

The ferrous iron concentration was determined using the 2,2′-dipyridyl molecular absorption spectrophotometry method (adapted from the '3500-Fe B' colorimetric procedure of [18]). The procedure for the colorimetric detection of Fe^{2+} was as follows: a liquid sample (1 mL) was diluted (1:1000) in a volumetric flask and the pH of the solution was maintained at 5.5 by adding 5 mL of an acetic acid buffer. Subsequently, 2 mL of 2,2′-dipyridyl (0.5%, w/v in 96% ethanol) was added in order to form a red complex. The coloured solution obeyed the Beer–Lambert law, i.e., the absorbance was dependent on the Fe^{2+} concentration. The absorbance value was measured with a visible spectrophotometer (Thermo Scientific™ Helios Alpha) at a wavelength of 520 nm.

A similar procedure was designed to determine total iron concentration. In this case, Fe^{3+} reduction was undertaken by treatment with 2 mL of hydroxylamine hydrochloride (10%, w/v in deionized water) prior to the addition of 2,2′-dipyridyl. The ferric iron concentration was calculated as the difference between total and ferrous iron.

2.9. Sample processing and conservation

A. ferrooxidans activity during Fe^{2+} and Fe^{3+} concentration determinations must be rapidly and accurately disrupted since the remaining bacteria present in the media continue to obtain energy from the oxidation of ferrous iron and, thus, the experimental results may be distorted. Reductions in the initial Fe^{2+} contents (~3 g/L) of 90% and 52% were observed in 3 h in samples stored at ambient temperature (20 °C) or cooled in the fridge (4 °C) by means of biological action. Only freezing (−20 °C) and filtration (0.45 μm FilterLab membrane filter) techniques provided sufficient certainty to ensure the quality of the results.

2.10. Other analyses

The total cell number was calculated using a counting chamber (Improved Neubauer counting chamber, Zuzi) in conjunction with a phase-contrast microscope (Labophot, Nikon). The precipitates formed within the biomachining leachate were identified by X-ray diffraction (XRD) patterns obtained using a Philips Xpert PRO automatic diffractometer operating at 40 kV and 40 mA, in theta–theta configuration, equipped with a secondary monochromator with Cu-Kα radiation (λ = 1.5418 Å) and a PIXcel solid-state detector (active length in 2θ 3.347°). The spectra were recorded in the 5°–80° 2θ range with a 0.026° step size. Supporting data, namely pH (Crison GLP 21+ pH-meter equipped with a sensION+ 5014T glass combination pH electrode) and redox potential (Orion 9778BNWP Sure-Flow® electrode with epoxy body (combination of a platinum redox and a silver/silver chloride reference electrode in one body (Ag/AgCl, 4 M KCl)) connected to a Thermo-Orion 920A+ meter), were measured to provide additional information regarding the stage of the process. All potentials in this paper are given respect to the reference electrode (+220 mV vs. Ag/AgCl).

3. Results and discussion

3.1. Parameters affecting the incubation period

3.1.1. Effect of jarosite formation

The formation of hydroxysulfate precipitates (jarosite) during the oxidation of ferrous iron by free suspended cells of *A. ferrooxidans* was studied. Jarosite precipitation is a very important unwanted phenomenon that is observed in numerous industrial processes involving bacterial cultures in acidic and sulfate-rich environments. An excessively long incubation period leads the Fe^{3+} ions to form a sulfate complex within the periplasm of the *A. ferrooxidans* cell and this is subsequently precipitated as a polymeric ferric sulfate outside the cell wall [19]. As it has been reported that pH is the main parameter affecting jarosite formation [20], the main purpose of the work described in this section was to characterize the composition of the precipitate formed and to establish the optimal pH value for an efficient Fe^{2+} oxidation by *A. ferrooxidans* at which there is minimal risk of jarosite precipitation (reduction of Fe^{3+} concentration in the media).

The oxidation of Fe^{2+} by *A. ferrooxidans* in 9 K medium involves the consumption of hydrogen ions, thus meaning that the pH of the liquid medium increases as the reaction progresses. In this case, the pH increased from 1.75 to 2.00 after 48 h of the incubation process. The first signs of solid accumulation were observed visually after 72 h of incubation. In a similar manner, Liu et al. [21] showed that the formation of jarosite began when the *A. ferrooxidans* culture reached the logarithmic growth phase (above 46 h of incubation period) with pH values above 2.2.

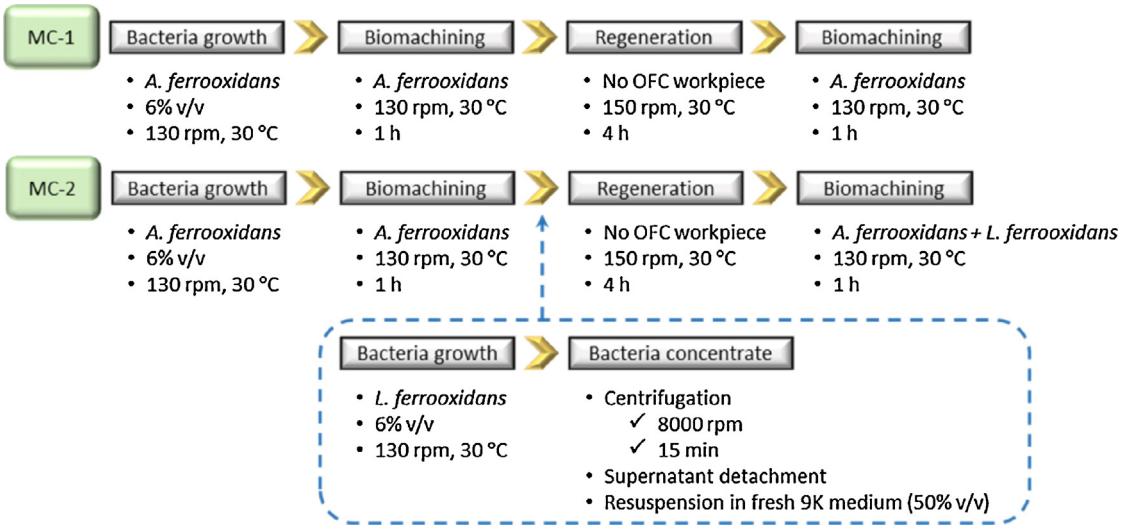

Fig. 1. Experimental set-up employed to study the effect of the addition of a second Fe^{2+} oxidizing strain during the biomachining process.

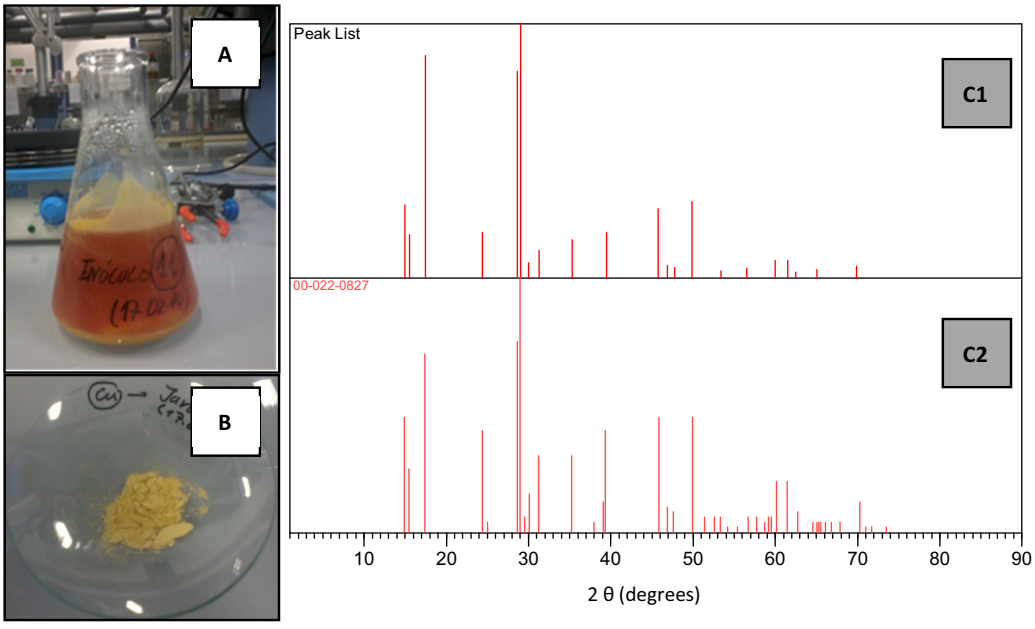

Fig. 2. *A. ferrooxidans* inoculum culture containing precipitated jarosite after 5 days of the incubation period at 30 °C and 130 rpm (A), filtered and dried mustard-coloured sulfate-mineral (B) and diffractograms obtained for a jarosite standard sample (Space Group: R-3*m*; ICDD PDF: 00-022-0827) (C1) and the solid precipitated during the experiment (C2).

The precipitate formed within the Erlenmeyer flask and collected after 5 days of incubation at 30 °C and 130 rpm was identified by X-ray powder diffraction as a compound related to jarosite-group minerals with the chemical formula $KFe_3(SO_4)_2(OH)_6$ (Fig. 2). The appearance of this solid halved the Fe^{3+} content in the 9 K medium from ~6 g Fe^{3+}/L to 3 g Fe^{3+}/L. It was therefore crucial to minimize jarosite formation in order to avoid loss of the Fe^{3+} ions needed to carry out solid copper biomachining. As such, a pH threshold value of 1.80 was maintained in the subsequent experiments by the continuous addition of sulfuric acid (25% v/v).

3.1.2. Effect of inoculum concentration

The evolution of the ferrous iron concentration and redox potential with time up to the end of the incubation period is represented in Fig. 3 for the three samples with different inoculum concentrations (IN-1:IN-3). A typical culture concentration of 2% v/v, as reported by other authors, was used as a reference value [8,22]. In

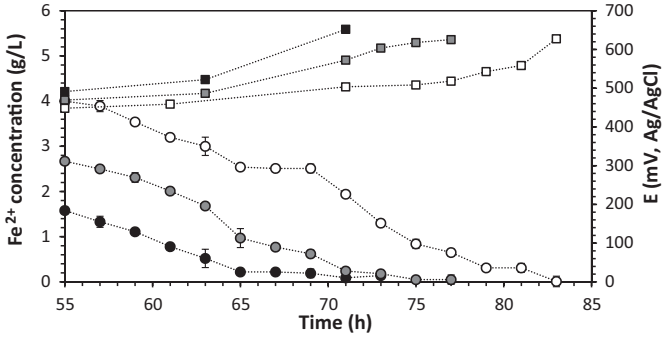

Fig. 3. Fe^{2+} concentration (circles) and redox potential values (squares) for IN-1 (white), IN-2 (grey) and IN-3 (black) samples. Error bars represent one standard deviation of the duplicates. For the sake of clarity, time values from the 55th hour onwards are shown as data collected before that time are less relevant.

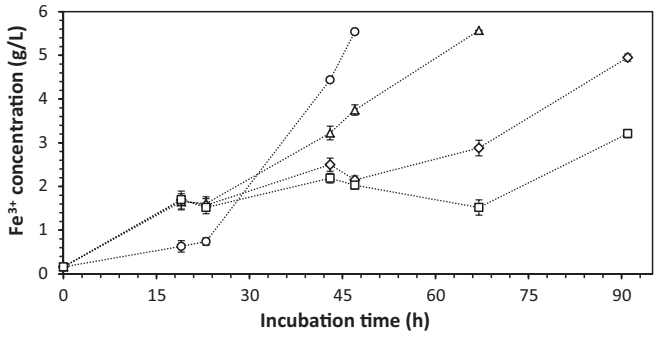

Fig. 4. Influence of Cu^{2+} concentration on the oxidative capacity of bacteria during the incubation period. Fe^{2+} concentration for C-1 (circle), C-2 (triangle), C-3 (diamond) and C-4 (square). Error bars represent one standard deviation of the duplicates. A maximum concentration of 6 g Fe^{2+}/L could be achieved in each case.

this study it was assumed that the culture reached the stationary phase once Fe^{2+} had been almost depleted from the solution [22].

It is clear from the figure that a higher inoculum concentration required less time to exhaust the ferrous iron and achieve the maximum redox potential (~630 mV vs. Ag/AgCl). These results are consistent with the data previously reported by Kumada et al. [11], who reported that a higher bacterial population would lead to a high Fe^{3+} level in the 9 K medium within a shorter cultivation time. In contrast to other studies, an optimal cell concentration was not found in our case. Hocheng et al. [23] suggested a bacterial concentration of 5×10^8 cells/mL for the bio-oxidation of 4 g Fe^{2+}/L. A faster transformation of Fe^{2+} was not achieved above this value. Similarly, Chang et al. [9] observed the positive effect of increasing inoculum concentrations on the metal removal rate up to 10^8 cells/mL.

3.1.3. *Effect of Cu^{2+} concentration*

The time elapsed for Fe^{3+} generation was used as a measure of bacterial activity in response to Cu^{2+} stress in this experiment (Fig. 4). Copper is essential for *A. ferrooxidans* development as it serves as an electron donor during bacterial growth and is found in the structure of rusticyanin, a protein that functions as the major electron carrier in the iron respiratory electron transport chain [24]. Nevertheless, *A. ferrooxidans* bacterium might not tolerate the excessive amounts of copper accumulated during the bioleaching process. Maximum tolerated concentrations up to 19 g Cu^{2+}/L have been reported in the literature [25,26], although other authors have established cell-growth inhibition below 0.3 g Cu^{2+}/L [27].

The presence of divalent copper decreased the oxidative capacity of the bacterial culture under study (Fig. 4). In the absence of Cu^{2+} (C-1), 99% of ferrous iron oxidation was accomplished after 47 h, whilst a similar oxidation degree (98%) was reached after 67 h for the test with 1.2 g Cu^{2+}/L (C-2). The oxidation process was incomplete for C-3 (88%) and C-4 (47%) even after 91 h. The reduction in the bio-oxidation capacity also affected cell growth, which also diminished as the metal concentration in the medium increased. Although the bacterial concentration increased from 10^7 cell/mL to 1.31×10^8 cell/mL in C-1, this increase was lower for samples C-2 and C-3 (9.12×10^7 and 1.20×10^7 cell/mL, respectively), and a reduction in bacterial concentration was even observed in C-4 (4.40×10^6 cell/mL).

3.2. *Parameters affecting the OFC sample biomachining period*

3.2.1. *Effect of the dominant copper removal mechanism (direct/indirect)*

Direct and indirect mechanisms have been proposed to explain the biomachining process [28]. In the direct mechanism, the attachment between bacteria and the metal is physical and constant

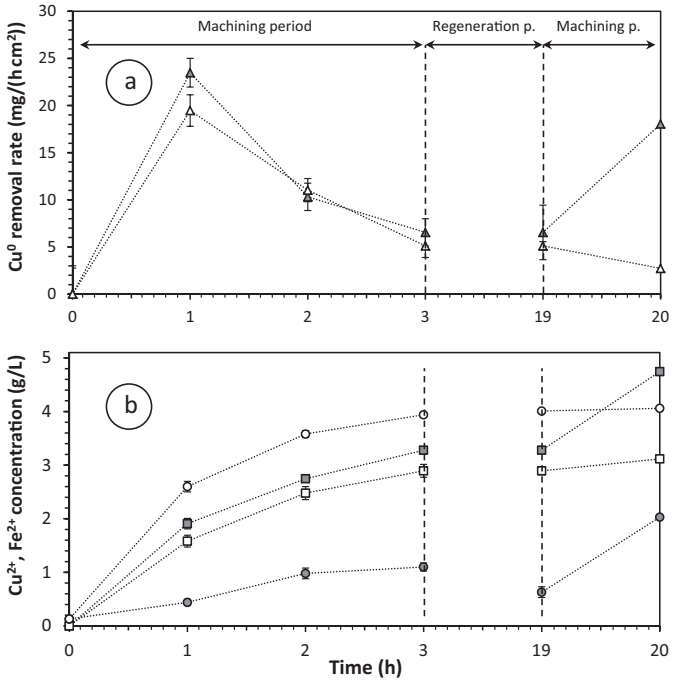

Fig. 5. Solid copper lixiviating rate (triangles) (a); Cu^0 (squares) and Fe^{2+} concentration (circles) for the abiotic sample (with filtration pretreament) (white) and for the biotic sample (standard sample) (grey) (b). Error bars represent one standard deviation of the duplicates.

as microorganisms exchange electrons with the metallic surface directly. In this case, the bacteria oxidize and dissolve Cu^0 by means of iron-oxidizing enzymes contained in the periplasmic space and inner membrane (Equation (2)).

$$2Cu^0 + 4H^+ + O_2 \rightarrow 2Cu^{2+} + 2H_2O \quad (2)$$

The indirect mechanism requires an intermediate redox couple, such as ferrous/ferric ions. The overall reaction (Equations (3) and (4)) is as follows:

$$4Fe^{2+} + O_2 + 4H^+ \rightarrow 4Fe^{3+} + 2H_2O \quad (3)$$

$$2Fe^{3+} + Cu^0 \rightarrow 2Fe^{2+} + Cu^{2+} \quad (4)$$

The Fe^{2+} contained in the bacterial growth medium, defined as the main energy source for *A. ferrooxidans*, is first bio-oxidized to Fe^{3+}. The resulting cation is a strong oxidant (standard reduction potential of +0.77 V(SHE)) that is able to readily solubilize pure copper by oxidizing Cu^0 to Cu^{2+}. The simultaneously formed divalent iron is trapped again by the bacteria and the process is repeated cyclically. As such, the indirect mechanism includes a redox cycle that combines microbiological (Equation (3)) and pure chemical (Equation (4)) processes [29].

The evolution of the rate of copper removal by means of a standard incubated sample (simultaneous presence of *A. ferrooxidans* bacteria and ~6 g Fe^{3+}/L) and a filtered sample (~6 g Fe^{3+}/L) is represented in Fig. 5a for a machining cycle involving a machining step (0–3 h), a regeneration step (3–19 h) and a second machining step (19–20 h). In both cases, the SMRR reached its peak during the first hour (23.5 mg/(h cm^2) (biotic sample) and 19.5 mg/(h cm^2) (abiotic sample)), and a similar total metal release of 3.28 g Cu^{2+}/L and 2.89 g Cu^{2+}/L for the non-filtered (biotic) and the filtered (abiotic) tests (Fig. 5b), respectively, was observed during the biomachining period (0–3 h). The difference between the two processes (11%) is in agreement with the value obtained by Xenofontos et al. [8], who concluded that direct biomachining contributed only 5% to the total biomachining process.

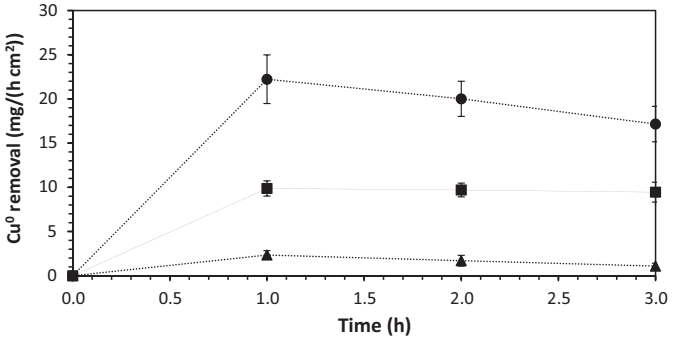

Fig. 6. Copper removal rate during the test for an initial Fe^{3+} concentration of 0.6 g/L (triangles), 3 g/L (squares) and 6 g/L (circles). Error bars represent one standard deviation of the duplicates.

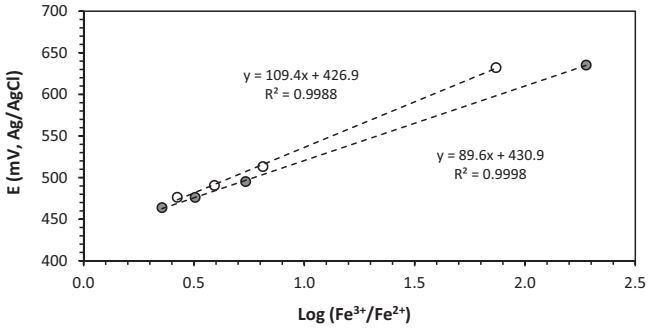

Fig. 7. Correlation between redox potential and $[Fe^{3+}]/[Fe^{2+}]$ concentration during the biomachining process for two samples with initial Fe^{3+} contents of 3 g/L (white circles) and 6 g/L (grey circles).

The biomachining process was restarted after a regeneration period of 16 h, during which accumulated Fe^{2+} ions entered *A. ferrooxidans* cells in the non-filtered sample and were reoxidized to Fe^{3+}. The results indicate that copper leaching was only marked in the sample with no Fe^{3+} depletion (non-filtered sample). Slight Cu^0 dissolution was observed at hour 19 for the filtered solution (2.7 mg/(h cm^2)) as the available ferric iron concentration dropped by 64% with respect to the start of the process. Consequently, the main bacterial contribution to this "corrosion" system is to keep iron ions in the higher oxidized state, with copper leaching being accomplished indirectly.

3.2.2. *Effect of Cu^{2+} concentration*

The removal of released cupric ions has been proposed by several authors as a prerequisite for obtaining better machining efficiency and maintaining a constant Cu^0 biomachining rate [17,30]. In our previous study, the Cu^{2+} produced during the biomachining of an OFC piece was removed by precipitation with H_2S [14]. However, no real enhancement in the Cu^0 removal process was observed on applying this strategy. In this case, the influence of Cu^{2+} concentration was evaluated by adding different Cu^{2+} contents to four already incubated solutions of 50 mL (Fe^{3+} ~6 g/L). The weight loss of the OFC workpiece submerged in each system is presented in Table 2.

A similar amount of metallic copper was removed in all four cases, with an average bioleaching rate of 18.1 ± 0.6 mg/(h cm^2). This confirmed that the biomachining process involving *A. ferrooxidans* is unaffected by the initial range of Cu^{2+} ions tested (0–7.4 g/L). Nevertheless, it must be taken into account that even though the biomachining period progressed without inhibition symptoms, the presence of Cu^{2+} could affect subsequent process steps, such as the regeneration period, as it has been shown that *A. ferrooxidans* metabolism is altered by Cu^{2+} concentrations over 3.6 g/L (section 3.1.3).

3.2.3. *Effect of Fe^{3+} concentration*

Metallic copper lixiviation occurs due to the presence of Fe^{3+} ions within the culture. Given the typical range of ferric iron concentrations employed in previous bioleaching studies using *A. ferrooxidans* (0–9 g Fe^{3+}/L) [31], the effect of the initial Fe^{3+} concentration on copper biomachining was investigated by comparing the response of three samples containing 0.6, 3 and 6 g Fe^{3+}/L. The Cu^0 removal over time for each Fe^{3+} concentration is plotted in Fig. 6. A linear correlation was observed between Fe^{3+} concentration and Cu^{2+} release, which means that twice the amount of Fe^{3+} would lead to a twofold increase in Cu^0 lixiviation (e.g., 29.0 mg Cu^0/cm^2 and 59.4 mg Cu^0/cm^2 for 3 and 6 g/L in 3 h, respectively; Fig. 6).

Despite the fact that a higher concentration of trivalent iron favours the loss of material, the addition of large amounts of Fe^{3+} would not be feasible industrially since a number of related problems might arise, such as an increase in the roughness of the final copper surface, higher sulfuric acid consumption or uncontrolled jarosite precipitation [12,14,32]. In addition, Kawabe et al. [33] reported that ferrous ion oxidation activity was reduced by more than half at concentrations above 14 g/L for three different *A. ferrooxidans* strains.

3.2.3.1. Relationship between redox potential and concentration of iron ions (Fe^{2+}, Fe^{3+}). Since the copper leaching process is determined by the ferric/ferrous redox couple, the experimental redox potential could be correlated with the $[Fe^{3+}]/[Fe^{2+}]$ concentration ratio, as described by the Nernst equation. The redox potential values, based on links established between Fe^{3+} depletion and Fe^{2+} generation, are plotted in Fig. 7 for bioleaching processes with 3 g Fe^{3+}/L and 6 g Fe^{3+}/L.

In both cases an essentially total correlation was obtained (correlation coefficient, R, around 1) between the redox potential (vs. Ag/AgCl) and the logarithm (Fe^{3+}/Fe^{2+}). This result demonstrates that the experimental results satisfy the Nernst equation. As such, measurement of the redox potential would be a faster and easier way to gain information about the status of the biomachining process (e.g., the end of the incubation phase) than other techniques employed in previous biomachining studies, such as bacterial counting or the observation of a vivid colour change in the media [4,9].

3.3. *Parameters affecting the* ferrooxidans *regeneration period*

3.3.1. *Effect of Cu^{2+} concentration*

Given the importance of a compulsory regeneration period for *A. ferrooxidans* cultures in order to achieve a stable and controlled bioleaching process, the effect of Cu^{2+} concentration on bacterial metabolism was studied as in the previous tests for incubation and biomachining periods. The influence of Cu^{2+} concentration on the bio-oxidation rate of Fe^{2+} is shown in Fig. 8. Recovery of the original ferric ion levels in the presence of 1.52 g Cu^{2+}/L (R-1) was completed in 4 h. A slight worsening was observed in the tests carried out with higher Cu^{2+} concentrations, and recovery yields of 90.6%, 89.3% and 88.5% were obtained for tests R-2, R-3 and R-4, respectively. The tendency shown by Cu^{2+} ions to slow down the regeneration phase was previously observed by Choi et al. [34], who noted a slight drop in the Fe^{2+} oxidation rate when a copper concentration of about 0.5 g/L was added to the inoculum medium.

3.3.2. *Effect of a mixed culture*

Bacterially assisted machining involves a cyclic combination of chemical and microbiological processes. However, the kinetics of copper oxidation by the ferric iron are faster than the bio-

Table 2
Effect of Cu^{2+} concentration during the biomachining period.

Sample	Workpiece mass (g)		Removal rate (mg/(h cm^2))	[Cu^{2+}]released (g/L)	[Cu^{2+}] (g/L)	
	Initial	Final			Initial	Final
B-1	2.071	2.001	17.3	1.28	0	1.28
B-2	2.001	1.925	18.5	1.37	1.20	2.57
B-3	1.925	1.849	18.7	1.38	3.60	4.98
B-4	1.849	1.777	17.7	1.31	6.10	7.41

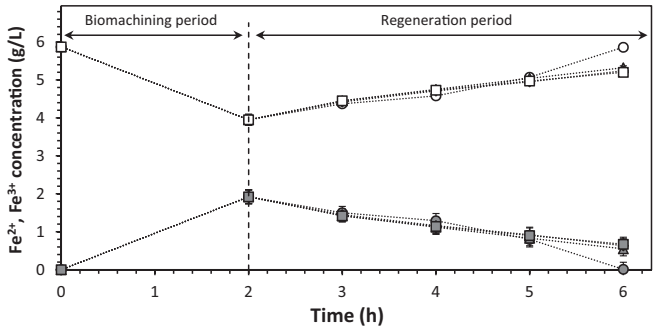

Fig. 8. Influence of Cu^{2+} concentration on the oxidative capacity of bacteria during the regeneration period. Fe^{2+} (grey) and Fe^{3+} (white) concentration for R-1 (circle), R-2 (triangle), *R*-3 (diamond) and R-4 (square). Error bars represent one standard deviation of the duplicates.

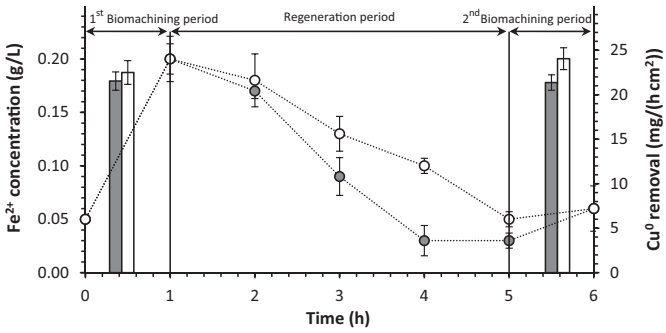

Fig. 9. Fe^{2+} concentration (circles) and copper removal rate (bar-chart) during the first stage of biomachining (0–1 h), regeneration period (1–5 h) and the last biomachining stage (5–6 h) for *A. ferrooxidans* culture MC-1 (grey) and mixed consortium (*A. ferrooxidans* + *L. ferrooxidans*) MC-2 (white). Error bars represent one standard deviation of the duplicates. The total dissolved iron (Fe^{2+} and Fe^{3+}) concentration remained constant (6 g/L).

conversion rate of the ferrous to ferric iron, a situation that causes an ion imbalance in the system over time. Lambert et al. [12] observed that the maximum copper leaching rate was ~6 times faster than the Fe^{2+} oxidation rate.

In order to accelerate the microbiological process, *L. ferrooxidans* was added to sample MC-2 during the regeneration period to form a microbial consortium of *A. ferrooxidans* and *L. ferrooxidans*. Both the pure culture and the mixed culture oxidized almost all of the ferrous iron after 4 h of regeneration (less than 1% of the initial ion concentration remained in each sample) (Fig. 9). It was expected that *L. ferrooxidans* could sustain a higher Fe^{2+} bio-oxidation rate in comparison with the *A. ferrooxidans* culture (MC-1) at the beginning of the second biomachining period due to its better adaptability to the high redox potential (563 mV vs. Ag/AgCl) and the low level of Fe^{2+} available (<0.05 g/L). A similar copper removal rate was observed in the second biomachining period for MC-1 (<1% variation), whereas sample MC-2 exhibited a slightly better performance – from 22.5 mg/(h cm^2) in the first biomachining period to 24.05 mg/(h cm^2) in the second one.

4. Conclusions

The use of extremophile bacteria to remove copper from OFC workpieces has been investigated. The performance of the biomachining process has been assessed in terms of the metal removal rate, and several parameters were examined during the manufacturing stages defined in our previous work (incubation, biomachining and regeneration phases) in order to avoid the progressive decrease in copper removal rate observed with time. It was found that the status of the process could easily be controlled by measuring the redox potential as a linear correlation was observed between ferric iron concentration and copper release.

As expected, OFC biomachining by means of *A. ferrooxidans* was found to be indirect in nature. The formation and precipitation of jarosite was observed in 9 K medium when the pH exceeded the value of 2.00. As such, the presence of divalent copper is detrimental to the bacterial culture performance, especially during incubation and regeneration periods, as the bacterial growth period increased by at least 50% when the concentration was above 6 g Cu^{2+}/L. The introduction of a second strain (*L. ferrooxidans* ATCC 29047) resulted in a limited enhancement in the copper removal rate (7% increase). The efficiency of the biomachining process was boosted by an increase in both the concentration of the inoculum and the ferric iron. The results obtained in this study provide evidence for the need to optimize the overall biomachining cycle in order to promote its industrial-scale implementation.

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References

[1] M. Ochoa, M.A. Rodríguez, S.B. Farina, Corrosion of high purity copper in solutions containing NaCl, Na_2SO_4 and $NaHCO_3$ at different temperatures, Procedia Mater. Sci. 9 (2015) 460–468.
[2] H. Rajainmaki, A. Helenius, M. Kolehmainen, The production and application of oxygen-free copper, JOM 45 (1993) 68–70.
[3] E. Diaz-Tena, A. Barona, G. Gallastegui, A. Rodriguez, L.N. Lopez de Lacalle, A. Elías, Biomachining: metal etching via microorganisms, Crit. Rev. Biotechnol. (2016), http://dx.doi.org/10.3109/07388551.2016.1144046.
[4] D. Johnson, R. Warner, A.J. Shih, Surface roughness and material removal rate in machining using microorganisms, J. Manuf. Sci. Eng. 129 (2007) 223–227.
[5] J. Istiyanto, A.S. Saragih, T.J. Ko, Metal based micro-feature fabrication using biomachining process, Microelectron. Eng. 98 (2012) 561–565.
[6] V.K. Mital, K.S. Sangwan, Prioritizing barriers to green manufacturing: environmental: social and economic perspectives, Procedia CIRP 17 (2014) 559–564.
[7] S.M. Masoumik, S.H. Abdul-Rashid, E.U. Olugu, Importance-performance analysis of green strategy adoption within the malaysian manufacturing industry, Procedia CIRP 26 (2015) 646–652.

[8] E. Xenofontos, A. Feidiou, M. Constantinou, G. Constantinides, I. Vyrides, Copper biomachining mechanisms using the newly isolated *Acidithiobacillus ferrooxidans* B1, Corros. Sci. 100 (2015) 642–650.
[9] J.H. Chang, H. Hocheng, H.Y. Chang, A. Shih, Metal removal rate of *Thiobacillus thiooxidans* without pre-secreted metabolite, J. Mater. Process. Technol. 201 (2008) 560–564.
[10] Y.P. Ting, A.S. Kumar, M. Rahman, B.K. Chia, Innovative use of *Thiobacillus ferrooxidans* for the biological machining of metals, Acta Biotechnol. 20 (2000) 87–96.
[11] M. Kumada, T. Kawakado, S. Kobuchi, Y. Uno, S. Maeda, H. Miyuki, Investigation of fine biomachining of metals by means of microbially influenced corrosion: differences between steel and copper in metal biomachining by using *Thiobacillus Ferrooxidans*, Corros. Eng. 50 (2001) 585–596.
[12] F. Lambert, S. Gaydardzhiev, G. Léonard, G. Lewis, P.F. Bareel, D. Bastin, Copper leaching from waste electric cables by biohydrometallurgy, Miner. Eng. 76 (2015) 38–46.
[13] G. Lewis, S. Gaydardzhiev, D. Bastin, P.F. Bareel, Bio hydrometallurgical recovery of metals from fine shredder residues, Miner. Eng. 24 (2011) 1166–1171.
[14] E. Díaz-Tena, A. Rodríguez-Ezquerro, L.N. López de Lacalle, L. Gurtubay, A. Elías, A sustainable process for material removal on pure copper by use of extremophile bacteria, J. Clean. Prod. 84 (2014) 752–760.
[15] J. Zhu, Q. Li, W. Jiao, H. Jiang, W. Sand, J. Xia, X. Liu, W. Qin, Q. Guanzhou, Y. Hu, L. Chai, Adhesion forces between cells of *Acidithiobacillus ferrooxidans, Acidithiobacillus thiooxidans or Leptospirillum ferrooxidans* and chalcopyrite, Colloids Surf. B 94 (2012) 95–100.
[16] D.E. Rawlings, H. Tributsch, G.S. Hansford, Reason why '*Leptospirillum*'-like species rather than *Thiobacillus ferrooxidans* are the dominant iron-oxidizing bacteria in many commercial processes for the biooxidation of pyrite and related ores, Microbiol 145 (1999) 5–13.
[17] J. Istiyanto, T.J. Ko, I.C. Yoon, A study on copper micromachining using microorganisms, Int. J. Precis. Eng. Manuf. 11 (2010) 659–664.
[18] A.D. Eaton, L.S. Clesceri, A.E. Greenberg, M.A.H. Franson, Standard Methods for the Examination of Water and Wastewater, American Public Health Association, American Water Works Association, Water Environment Federation, Washington DC, 1998.
[19] N. Lazaroff, Mineral leaching, iron precipitation and sulfate requirement for chemolithotrophic iron oxidation, Stud. Environ. Sci. 66 (1997) 61–75.
[20] J. Daoud, D. Karamanev, Formation of jarosite during Fe^{2+} oxidation by *Acidithiobacillus ferrooxidans*, Miner. Eng. 19 (2006) 960–967.
[21] J.S. Liu, B.M. Li, D.Y. Zhong, L.X. Xia, G.Z. Qiu, Preparation of jarosite by *Acidithiobacillus ferrooxidans* oxidation, J. Cent. South Univ. Technol. 14 (2007) 623–628.
[22] Y. Yang, X.B. Wang, Y.D. Liu, S. Wang, W. Wen, Techniques for micromachining using *Thiobacillus ferrooxidans* based on different culture medium, Appl. Mech. Mater. 16–19 (2009) 1053–1057.
[23] H. Hocheng, J.H. Chang, H.S. Hsu, H.J. Han, Y.L. Chang, U.U. Jadhav, Metal removal by *Acidithiobacillus ferrooxidans* through cells and extra-cellular culture supernatant in biomachining, CIRP J. Manuf. Sci. Technol. 5 (2012) 137–141.
[24] N.C.S. Mykytczuk, J.T. Trevors, G.D. Ferroni, L.G. Leduc, Cytoplasmic membrane response to copper and nickel in *Acidithiobacillus ferrooxidans*, Microbiol. Res. 166 (2011) 186–206.
[25] G. Cabrera, J.M. Gómez, D. Cantero, Influence of heavy metal on growth and ferrous sulphate oxidation by *Acidithiobacillus ferrooxidans* in pure and mixed cultures, Process Biochem. 40 (2005) 2683–2687.
[26] A. Das, J.M. Modak, K.A. Natarajan, Surface chemical studies of *Thiobacillus ferrooxidans* with reference to copper tolerance, Antonie Van Leeuwenhoek 73 (1998) 215–222.
[27] H.L. Liu, B.Y. Chen, Y.W. Lan, Y.C. Cheng, SEM and AFM images of pyrite surfaces after bioleaching by the indigenous *Thiobacillus thiooxidans*, Appl. Microbiol. Biotechnol. 62 (2003) 414–420.
[28] H. Hocheng, U. Jadhav, Process of biological machining, in: A.Y.C. Nee (Ed.), Handbook of Manufacturing Engineering and Technology, Springer-Verlag, London, 2015, pp. 1687–1710.
[29] K. Lilova, D. Karamanev, R.L. Flemming, T. Karamaneva, Biological oxidation of metallic copper by *Acidithiobacillus ferrooxidans*, Biotechnol. Bioeng. 97 (2007) 308–316.
[30] Y. Li, D. Zhang, Y. Wu, Biomachining of metal copper by *Thiobacillus ferrooxidans*, Acta Microbiol. Sin. 40 (2000) 327–330.
[31] S.O. Rastegar, S.M. Mousavi, S.A. Shojaosadati, R.S. Mamoory, Bioleaching of V, Ni, and Cu from residual produced in oil fired furnaces using *Acidithiobacillus ferrooxidans*, Hydrometallurgy 157 (2015) 50–59.
[32] Y. Xiang, P. Wu, N. Zhu, T. Zhang, W. Liu, J. Wu, P. Li, Bioleaching of copper from waste printed circuit boards by bacterial consortium enriched from acid mine drainage, J. Hazard. Mater. 184 (2010) 812–818.
[33] Y. Kawabe, C. Inoue, K. Suto, T. Chida, Inhibitory effect of high concentrations of ferric ions on the activity of *Acidithiobacillus ferrooxidans*, J. Biosci. Bioeng. 96 (2003) 375–379.
[34] M.S. Choi, K.S. Cho, D.S. Kim, D.J. Kim, Microbial recovery of copper from printed circuit boards of waste computer by *Acidithiobacillus ferrooxidans*, J. Environ. Sci. Health Part A Toxic/Hazard. Subst. Environ. Eng. 39 (2004) 2973–2982.