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Simultaneous culture and biomachining of copper in MAC medium: A comparison between *Acidithiobacillus ferrooxidans* and *Sulfobacillus thermosulfidooxidans*

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ABSTRACT

Biomachining will not be considered as a full-scale manufacturing technology until a stable, controlled and continuous metal removal rate (MRR) is achieved. In this research work, a novel strategy that could promote its industrial implementation, namely simultaneous bacterial growth and machining of copper contained in oxygen-free copper (OFC) workpieces, was investigated. This proposal has the major advantage of being a single-stage process, thereby reducing total operating times and becoming more economical in comparison with conventional biomachining (downtime due to bacterial growth would disappear). The study was carried out using mesophilic (*Acidithiobacillus ferrooxidans*) and thermophilic (*Sulfobacillus thermosulfidooxidans*) extremophile bacteria in order to prevent the progressive decrease in the amount of metal removed per unit time. A constant MRR of 43 mg h^{-1} was achieved with *A. ferrooxidans* in the simultaneous process. Despite the accomplishment of a constant MRR, this value is lower than the maximum MRR obtained in conventional biomachining (109 mg h^{-1}), probably due to the inability of ferric ions to come into contact with the metallic surface. With regard to the culture period in MAC medium, *S. thermosulfidooxidans* showed a slower growth rate (0.11 h^{-1}) and lower ferrous ion oxidation level (0.12 g Fe^{2+} L^{-1} h^{-1}) than *A. ferrooxidans* (0.17 h^{-1} and 0.22 g Fe^{2+} L^{-1} h^{-1}, respectively) under optimal pH (1.5) and Fe^{2+} concentration (6 g L^{-1}) conditions.

KEYWORDS*: biomachining, bioleaching, copper, MAC medium, A. ferrooxidans, S. thermosulfidooxidans*
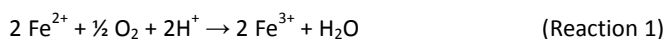
INTRODUCTION

Copper has been identified as a key metal for industrial and social progress. Indeed, both copper and its many alloys (e.g., oxygen-free copper (OFC) pieces) have become strategic materials due to their economic and environmental benefits[1]. It is estimated that two-thirds of the 550 million tonnes of copper produced since 1900 are still in productive use[2]. Its excellent properties, such as mechanical strength, conductivity, corrosion resistance, machinability, ductility and recyclability, make copper ideal for use in a large number of products and a wide range of applications, including electronics, communication, electricity and transport. For instance, a typical medium-sized car may contain up to 22.5 kg of this metal, and new generation aircraft and trains between 2 and 4 tons[1].

With regard to OFC microfabrication, physicochemical methods, such as milling, turning, drilling, laser, electrical discharge machining and chemical machining, have traditionally been applied. However, the high fabrication rates achieved have masked the drawbacks associated with these processes, namely high production costs, thermal and/or structural damage, and the application of harmful compounds for both employees' health and the environment. As a result, and given the environmental impact caused by the traditional manufacturing sector, concepts such as ecological and sustainable production are gaining more and more importance, and industries are increasingly being forced to retrain their processes (either by integrating new processes or transforming existing ones), thereby moving towards a greener manufacturing technology. According to Pusavec et al.[3], any environmentally sustainable process is based on four basic principles: (1) process cost depletion (including energy consumption and manufacturing process costs), (2) generated waste reduction and waste-treatment upgrading, (3) occupational health progress, and (4) a decrease in environmental impact.

Numerous approaches based on traditional manufacturing processes that improve progress towards so-called "green manufacturing" have arisen recently. These new processes are more efficient and eco-friendly than existing ones. Although their application to date has been limited to a laboratory scale, biomachining, which is defined as a process based on microbiological activity to form microstructures on a metal workpiece by metal removal (or dissolution) using microorganisms, is one of the possible alternatives for OFC microfabrication purposes[4]. Biomachining is characterised by machining components with minimum heat or residual stress and without damaging the metallurgical properties of the workpieces. Microorganisms responsible for the metal processing are not only commercially available, but also can be cultured and be readily available at any moment. In addition, energy consumption is low, thus resulting in a subsequent cost saving in the manufacturing of high quality pieces[5, 6].

Two main mechanisms (direct and indirect pathways) have been postulated to explain metal bioetching[7]. Regarding the direct mechanism, bacterial adhesion on metal surface allows the enzyme activity to be responsible for metal oxidation and dissolution[5]. Nevertheless, according to the literature, this process is not the priority pathway for zero-valence copper (Cu^0) biooxidation[6, 8]. Xenofontos et al.[8] observed that even if the effect of microorganisms on copper foil surface roughness was significant, the direct mechanism contributed only 5 % to the total biomachining process.

Copper biomachining via indirect mechanism typically involves two consecutive stages[9]. Initially, chemolithotrophic and acidophilic bacteria are grown using the energy generated by the oxidation of ferrous ions into ferric ions (Reaction 1), and in a second step, the biogenic ferric ion causes the dissolution of copper metal present in the bacterial medium (Reaction 2).

$$2\ Fe^{2+} + \tfrac{1}{2}\ O_2 + 2H^+ \rightarrow 2\ Fe^{3+} + H_2O \qquad \text{(Reaction 1)}$$

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$$Cu^0 + 2\ Fe^{3+} \rightarrow Cu^{2+} + 2\ Fe^{2+} \hspace{3cm} \text{(Reaction 2)}$$

Ideally, a closed cycle should be formed by joint interaction between the biological (Reaction 1) and chemical (Reaction 2) reactions. However, the relatively low biological metabolism in comparison with the fast chemical copper dissolution rate results in a depletion of the Fe^{3+} concentration in the liquid medium (Figure 1a), thereby decreasing the metal removal rate (MRR) over time[10]. In previous studies[4], the MRR was optimized by means of regeneration periods between successive stages of biomachining (Figure 1b). Thus, during the chemical solution bioregeneration, the piece of metal was submerged in a second bioreactor in order to continue the leaching process at the highest possible MRR[11].
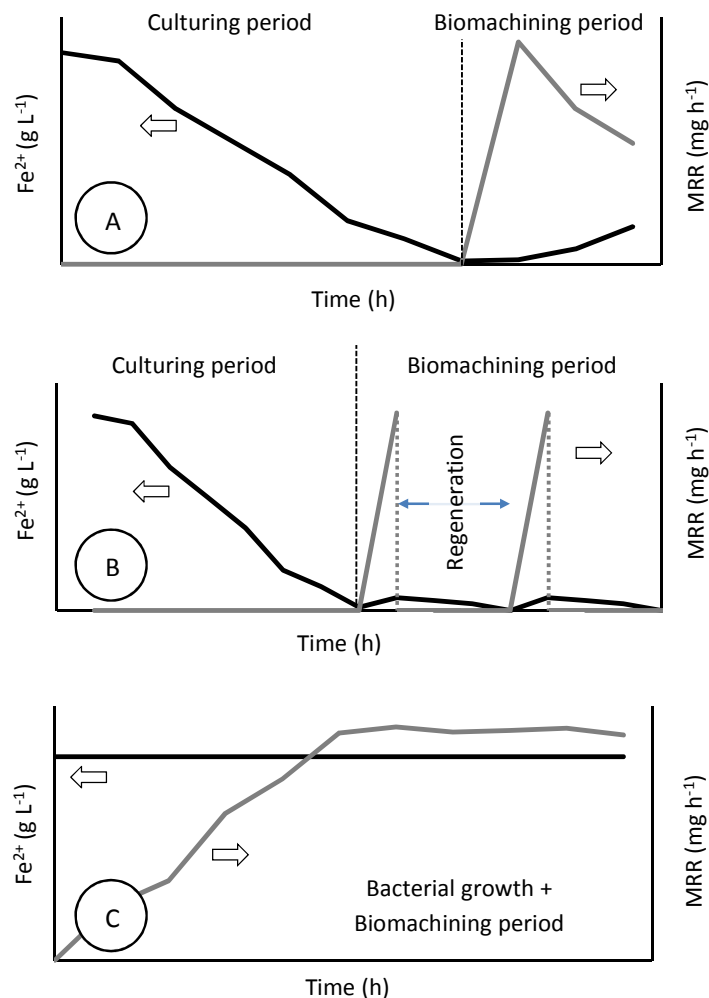

Figure 1. Evolution of Fe^{2+} concentration (black line) and metal removal rate (grey line) for a conventional biomachining process (A), an alternative biomachining process including regeneration periods (B), and a simultaneous bacterial growth and biomachining process (C).

The work described herein explores the possibility of carrying out the bacterial growth and machining stages simultaneously in order to provide a continuous, stable and sustainable biomachining process. The total operating time would be reduced in comparison with conventional biomachining as the growth period (Reaction 1) would act as a producing stage that occurs alongside copper dissolution (Reaction 2). In addition, the energy source for bacterial growth (i.e., Fe^{2+}) would be readily available since the ferric ions generated would be rapidly reduced upon interaction with elemental copper (Cu^0) (Reaction 2). Such an alternative approach could provide an endless energy source for the microorganisms, thus

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meaning that they would be immersed in a growth stage at a suitable activity level for machining purposes (Figure 1c).

The most commonly used bacterium in biomachining is the autotrophic, acidophilic and mesophilic bacterium *Acidithiobacillus ferrooxidans* (*A. ferrooxidans*). Thermophilic bacteria typically employed in the bioleaching of sulfide ores and environmental detoxification of heavy metals have not yet been investigated as possible agents for increasing the biomachining rate. As such, experimental data regarding the growth and machining capacity of *Sulfobacillus thermosulfidooxidans* (*S. thermosulfidooxidans*) were also compared with those for *A. ferrooxidans* in this study.

EXPERIMENTAL SECTION

Preparation of copper samples

OFC workpieces with a minimum purity of 99.99 % and a size of 10 × 15 × 2 mm were manufactured in the Department of Mechanical Engineering of the University of the Basque Country (UPV/EHU) using an 800-grit abrasive disk wheel (Petrographic cleaner LS1, REMET, Italy). Each workpiece had a hole (2 mm in diameter and 2 mm thick) to allow complete immersion in the active medium when performing the tests (SI Figure S1). Prior to immersion in MAC medium, the OFC blocks were rinsed with deionized water and ethanol (96 % v v^{-1}) and then heated to remove surface moisture.

Microorganisms and culture media

A. ferrooxidans DSM-14882 was kindly provided by the Department of Chemical Engineering and Food Technology of the University of Cádiz (UCA) and *S. thermosulfidooxidans* was obtained from Copahue geothermal ponds (Neuquen, Argentina).

The mineral medium (MAC[12]) composition (g L^{-1}) was: 1) 1L basalt solution, comprising: 0.132 $(NH_4)_2SO_4$, 0.031 $MgSO_4 \cdot 7H_2O$, 0.147 $CaCl_2 \cdot 2H_2O$, 0.022 K_2HPO_4; 2) 1 mL trace elements, comprising: 0.099 $MgCl_2 \cdot 4H_2O$, 0.068 $ZnCl_2$, 0.101 $CoCl_2 \cdot 6H_2O$, 0.031 H_3BO_3, 0.012 $Na_2MoO_4 \cdot 2H_2O$, 0.085 $CuCl_2 \cdot 2H_2O$. $FeSO_4 \cdot 7H_2O$ was added in different quantities depending on the final Fe^{2+} concentration required.

Study of the effect of initial pH and Fe^{2+} concentration on *A. ferrooxidans* and *S. thermosulfidooxidans* kinetics

Both bacteria were cultured in 250 mL conical flasks containing 150 mL of MAC medium supplemented with $FeSO_4 \cdot 7H_2O$, which served as bacterial energy source. Bacteria were incubated under agitation conditions (180 rpm) (ShakerPro 6004, Vicking, Argentina) at 30 °C and 45 °C for *A. ferrooxidans* and *S. thermosulfidooxidans*, respectively.

As the pH was not adjusted during the culture period, this stage was concluded when complete oxidation of Fe^{2+} to Fe^{3+} was achieved (less than 1 % of the initial Fe^{2+} concentration), which indicated that bacterial growth was satisfactory, or jarosite precipitation due to progressive basification was observed[4].

To analyse the effect of pH and Fe^{2+} concentration, two pH values (1.5 and 2.0) and three Fe^{2+} concentrations (3, 6 and 9 g Fe^{2+} L^{-1}) were tested. pH tests below 1.5 were not performed since a marked reduction in *A. ferrooxidans* viability has already been observed by Gomez and Cantero[13] when working at a pH of 1.25. With regard to *S. thermosulfidooxidans*, prior research indicated that ferrous ion oxidation rates were similar between pH 1.4–2.0, with an optimum pH of 1.7[14]. Deionized water was added during the experiments to compensate water losses due to evaporation. Blank tests following the

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same procedure were performed without bacteria to verify the oxidation capacity ($Fe^{2+} \rightarrow Fe^{3+}$) of dissolved oxygen. An additional test was performed for *S. thermosulfidooxidans* at pH 1.5 and 6 g Fe^{2+} L^- ^{1} in the presence of 300 μL of yeast extract (10 % w v^{-1}). Each experiment was performed in duplicate and the mean results were used.

Biomachining process

Conventional biomachining process

Biomachining tests were carried out by suspending pre-weighed OFC copper blocks (Sartorius Practum 124-1S analytical Balance, Spain) in 250 mL conical flasks containing the aforementioned culture media (*A. ferrooxidans* or *S. thermosulfidooxidans*, both cultured up to stationary phase[15]) under the optimal conditions based upon the results obtained in "*Parameters affecting the culture period*" section (pH of 1.5 and 6 g Fe^{2+} L^{-1}) (Table 1). The influence of workpiece dimensions[16] was avoided by selecting analogous pieces in terms of both weight and total surface area. A pH threshold value of 1.8 was maintained in all experiments by addition of sulfuric acid (25 % v v^{-1})[4].

Biomachining during the culture period

Tests to analyse the effect of conducting bacterial culture (*A. ferrooxidans* or *S. thermosulfidooxidans)* and biomachining simultaneously were performed in four different solutions, as shown in Table 1. In this case, copper bioleaching was considered to be complete either when dissolutions presented jarosite formation (in the samples with no pH control) or the MRR ceased to increase over time. Irrespective of the fact that copper OFC samples were placed within the flasks together with the uncultured inoculum (SI Figure S1), the same conditions as those mentioned in the previous section were applied in these experiments.

Table 1. Experimental conditions regarding conventional and alternative biomachining procedures.

Experiment	Bacteria	Initial pH	Temperature (°C)	$[Fe^{2+}]$ (g L^{-1})	pH control
Conventional biomachining	*A. ferrooxidans*	1.5	30	6	Yes
	S. thermosulfidooxidans	1.5	45	6	Yes
Simultaneous culture and biomachining	*A. ferrooxidans*	1.5	30	6	No
	A. ferrooxidans	1.5	30	6	Yes
	S. thermosulfidooxidans	1.5	45	6	No
	S. thermosulfidooxidans	1.5	45	6	Yes

The metal samples tested in the latter two sections were removed from the solution at user-defined intervals, rinsed with deionized water and ethanol (96 % v v^{-1}), dried, weighed, and then reintroduced into the liquid samples. Each experiment was performed in duplicate and the mean results were used. The copper removal rate (MRR) achieved was calculated as follows[9]:

$$MRR\ \left(mg\ h^{-1} \right) = \frac{Weight\ loss\ (mg)}{Time\ (h)} \qquad (1)$$

Cu^{2+}, Fe^{2+}, Fe^{3+} and total Fe determination

Fe^{2+} concentration was quantified using the *o*-phenanthroline method with a NanoColor® UV/VIS spectrophotometer (Macherey-Nagel GmbH & Co., KG, Germany)[17]. Cu^{2+} and total Fe concentrations

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were determined for filtered samples by atomic absorption spectrophotometry (AA-6650, Shimadzu, France). The Fe^{3+} concentration was calculated as the difference between total and ferrous ion. Samples were rapidly filtered (0.45 µm FilterLab membrane filter) prior to Fe^{2+} and Fe^{3+} determination to accurately disrupt the activity of remaining bacteria present in the media[4].

Other analyses

The total cell number was calculated using a counting chamber (Improved Neubauer counting chamber, Zuzi, Spain) in conjunction with a phase-contrast microscope (Labophot, Nikon, USA). Additionally, cell densities were compared using the 4',6'-diamidino-2-phenylindole (DAPI) stain technique[18]. Supporting data, namely pH (GLP 21+ pH-meter equipped with a sensION+ 5014T glass combination pH electrode, Crison, Spain) 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, 4M KCl)) connected to a Thermo-Orion 920A+ meter, ThermoFisher Scientific, Germany), were measured to provide additional information regarding the stage of the process. All potentials in this paper are given with respect to the reference electrode (+220 mV vs. Ag/AgCl).

RESULTS AND DISCUSSION

Effect of initial pH and Fe^{2+} concentration on *A. ferrooxidans* and *S. thermosulfidooxidans* kinetics

A. ferrooxidans

An optimal culture medium formulation and suitable growth conditions are essential in any biological process in which bacteria are involved in order to attain their maximal growth rate and replication. For *A. ferrooxidans* culture in MAC medium, samples with an initial pH of 2.0 presented a slightly lower growth rate in comparison with samples with an initial pH of 1.5. A difference of approximately 10 % in the bacterial concentration increase was observed when samples with a Fe^{2+} concentration of 3 g L^{-1} but different pH values (1.5 or 2.0) were compared (Figure 2). For the sake of clarity, samples with an initial pH of 2.0 and the highest Fe^{2+} concentrations (6 g L^{-1} and 9 g L^{-1}) were not included in Figure 2. In these latter two cases, jarosite precipitation (SI Figure S2) was evident when the pH rose above 2.3, which was in accordance with Nazari et al[19]. As expected, a higher bacterial density was achieved in those samples with a higher iron concentration, which was visually corroborated by means of DAPI analysis (SI Figure S3).

Biological oxidation of Fe^{2+} to Fe^{3+} in samples grown at a pH of 1.5 resulted in an average increase in the pH value of 0.5 as a result of the consumption of hydrogen ions. Proton depletion was similar irrespective of the Fe^{2+} content (Figure 2). The time required by the inoculum to accomplish Fe^{2+} oxidation and achieve the maximum redox potential (590–630 mV vs. Ag/AgCl) essentially depended on the amount of ferrous ion in the media (SI Figure S4). In all cases, the culture period was completed within 38 h. Blank tests (abiotic tests developed to observe ferrous ion oxidation associated with dissolved oxygen) showed a negligible Fe^{2+} depletion in samples at pH 1.5 or pH 2.0. For the same culture period (24 h), minor increases (6–17 mV (↑ 2–5 %)) in the redox potential associated with the conversion of Fe^{2+} into Fe^{3+} were measured, which confirmed the applicability of the redox potential as a rapid measurement for gaining information about the status of the biomachining process[4].
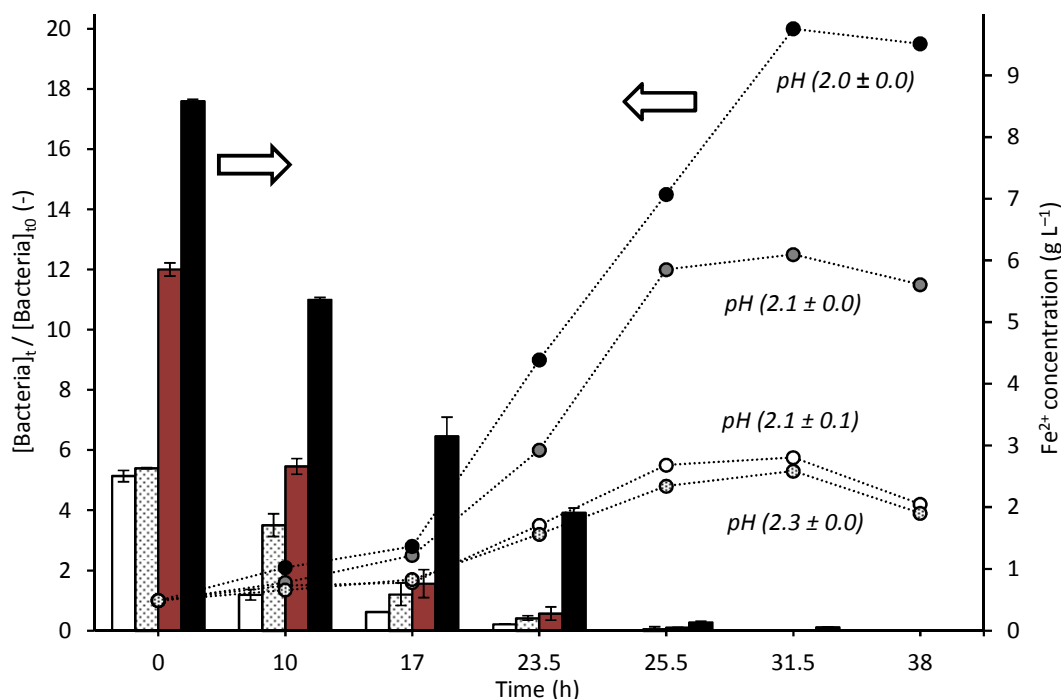
Figure 2. Evolution of Fe^{2+} (columns) and bacteria (circles) concentration during the culture of *A. ferrooxidans* in MAC medium for the three Fe^{2+} concentrations: 3 g Fe^{2+} L^{-1} (pH 1.5 (white) and pH 2.0 (dotted)), 6 g Fe^{2+} L^{-1} and pH 1.5 (grey), and 9 g Fe^{2+} L^{-1} and pH 1.5 (black). Error bars represent one standard deviation of the duplicates.

Taking into account that the variables with the highest influence on *A. ferrooxidans* metabolism are ferrous and ferric ion concentration, pH, temperature, dissolved oxygen concentration, carbon dioxide concentration and inert solids[13], the effect of pH and Fe^{2+} concentration on the Fe^{2+} oxidation rate ($v_{Fe^{2+}}$), specific bacteria growth rate (μ) and specific substrate consumption (q_s) were addressed in this section in order to establish the optimal conditions for *A. ferrooxidans* culture in MAC medium (Table 2). The μ and $v_{Fe^{2+}}$ rates were calculated during the bacterial exponential growth phase, as defined by Blanch and Clark[20]. q_s was obtained as the ratio between bacterial growth rate and cell yield, which is defined as the amount of cells produced per unit amount of substrate consumed[20].

Previous studies have demonstrated that the rate of Fe^{2+} bio-oxidation by *A. ferrooxidans* is highest when the dissolved oxygen level is maintained above 20 % of the saturation value[21, 22]. Given the absence of suspended and finely ground inert solids in the Erlenmeyer flasks and that the dissolved oxygen concentration far outweighed the minimum value established in the literature ($\sim$1.5–2 mg L^{-1}) throughout the experiments carried out, the influence of dissolved oxygen concentration and inert solids presence was discarded in this study.

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Table 2. Fe^{2+} oxidation rate ($v_{Fe^{2+}}$), specific bacteria growth rate (μ) and specific substrate consumption (q_s) for *A. ferrooxidans* and S. *thermosulfidooxidans* cultured in MAC medium.

Bacteria	Strain	Medium	$[Fe^{2+}]_{initial}$ (g L^{-1})	pH	$v_{Fe^{2+}}$(g Fe^{2+} L^{-1} h^{-1})	μ (h^{-1})	q_s(mg Fe^{2+} Mcell^{-1} h^{-1})	Reference
	DSM 14882	MAC	3	1.5	0.096	0.14	0.008	This study
	DSM 14882	MAC	3[a]	2.0	0.089	0.12	0.007	This study
	DSM 14882	MAC	6	1.5	0.217	0.17	0.009	This study
	DSM 14882	MAC	9	1.5	0.248	0.14	0.007	This study
A. ferrooxidans	ATCC 19859	9K	2–3	1.9	–	0.12–0.13	–	(23)
	–[b]	9K[c]	2	1.8	0.06[d]	0.14	–	(24)
	ATTC 13728	9K[c]	3	–	–	0.145	0.0037	(25)
	TfH6[e]	9K	9	1.5	–	0.080	0.0052	(26)
	SIMA 8621	9K	9	2.2		0.183	0.0036	(27)
	–[f]	MAC	6	1.5	0.120	0.11	0.039	This study
	DSM 9293^T	Watling[g]	10	1.6–1.8	0.12[d]			(14)
S. thermosulfidooxidans	DSM 9293	Norris	2.5	1.5	0.179	–	–	(28)
	DSM 9293	Norris	5	1.5	0.480	0.22	0.028	(29)
	DSM 9293	Norris	20	1.5	0.564	0.25	0.064	(29)

[a] Samples with an initial pH of 2.0 and the highest Fe^{2+} concentrations (6 g L^{-1} and 9 g L^{-1}) were not included due to jarosite formation, a physical barrier which prevents effective diffusion of the reagents and products.
[b] Isolated from the Rio Tinto mine in Huelva (Spain)
[c] The inorganic salt medium employed by Gomez & Cantero[24] and MacDonald & Clark[25] was based on that proposed by Silverman & Lundgren[30] except for the $FeSO_4$ content, which was modified in order to achieve an initial Fe^{2+} concentration of 2 g L^{-1} and 3 g L^{-1}, respectively.
[d] Graphically inferred.
[e] Isolated from Hutti Gold mines (India)
[f] Isolated from Copahue geothermal ponds (Argentina)
[g] Basal salt medium was prepared by adding 1.5 g L^{-1} $(NH_4)_2SO_4$, 0.25 g L^{-1} KH_2PO_4, 0.25 g L^{-1} $MgSO_4$ and supplemented with 0.1 g L^{-1} yeast extract.

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The ferrous ion oxidation rate was higher in those samples with higher ferrous ion concentration, probably due to the fact that increasing amounts of Fe^{2+} resulted in greater densities of cells that are able to transform more Fe^{2+} ions into Fe^{3+} [9]. The linearity between initial ferrous ion concentration and its oxidation rate found in this study is not fully supported in the literature. Thus, Danis et al.[31] observed that increasing Fe^{2+} concentrations up to 2.6 g L^{-1} gave increased Fe^{3+} levels, but higher ones led to lower Fe^{3+} concentrations. Karamanev and Nikolov[32] showed that the *A. ferrooxidans* oxidation rate was almost unaffected by ferrous ion concentration from 4 g L^{-1} upwards.

The stagnation or even decrease of the ferrous ion oxidation rate is related to the inhibition suffered by *A. ferrooxidans*[31, 32]. This fact was evident in our study, since both the specific bacterial growth rate and specific substrate consumption decreased at the highest ferrous ion concentration (9 g Fe^{2+} L^{-1}). Similarly, Barron and Lueking[23] observed that medium Fe^{2+} concentrations (2–3 g L^{-1}) favoured rapid cell growth rates at low cell densities, whereas higher Fe^{2+} concentrations (>4 g L^{-1}) should be selected to provide high cell yields but not optimum bacterial growth rates. In our study, the maximum growth rate (0.17 h^{-1}) and substrate consumption (0.009 mg Fe^{2+} $Mcell^{-1}h^{-1}$) were slightly higher than those found by other authors, although it should be noted that different strains and nutrient media were employed in those reports[23–27].

Consequently, the optimal conditions were found to be pH 1.5 and a ferrous ion concentration of 6 g L^{-1}, which ensured the highest bacterial growth rate, with moderate acid consumption and limited possibility of jarosite formation.

S. thermosulfidooxidans

Bioleaching of copper using mesophilic iron-oxidising acidophilic bacteria, such as *A. ferrooxidans*, which develop relatively high redox potentials during leaching processes by maintaining high ferric to ferrous ion ratios, has been extensively studied. However, the use of moderately thermophilic bacteria could be beneficial to boost bioleaching processes since higher temperatures improve both bacterial leaching kinetics and metal dissolution[33]. Cruz et al.[33] observed that greater and faster nickel extraction was achieved from nickel sulfide ores when using *S. thermosulfidooxidans* in comparison with *A. ferrooxidans*, mainly due to the temperature effect and the rapid increase in solution redox potential. In addition, *S. thermosulfidooxidans* has been reported to be tolerant to copper ions during ferrous ion biooxidation, and its cell-growth inhibition has been established at 22 g L^{-1} [14].
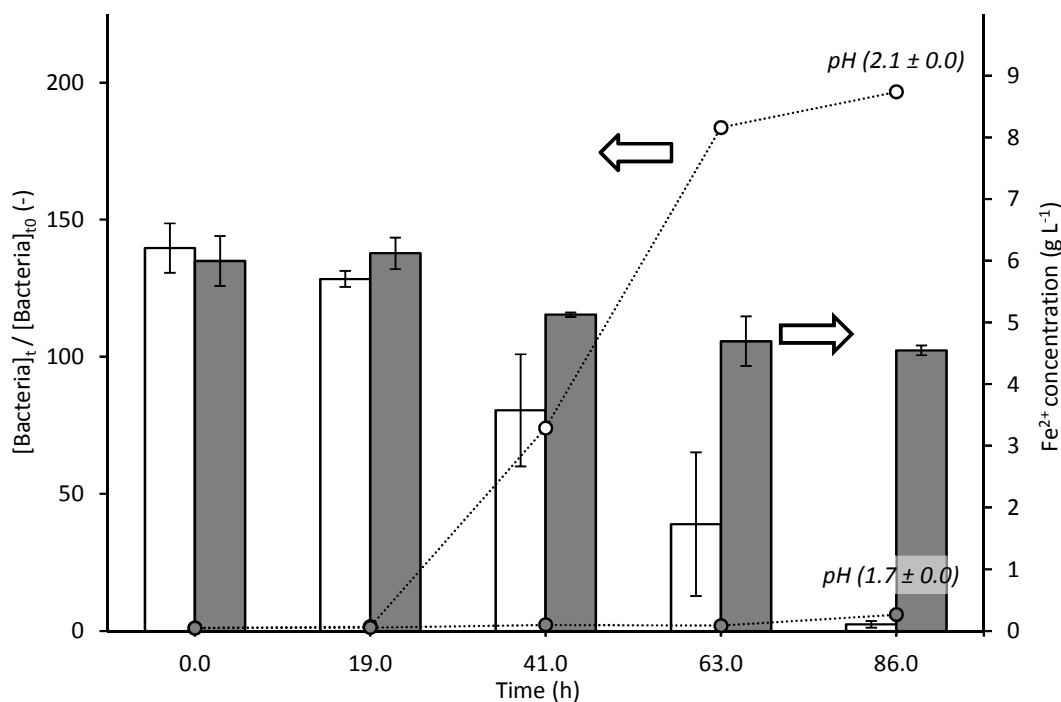
Figure 3. Evolution of Fe^{2+} (columns) and bacterial (circles) concentration during the culture of *S. thermosulfidooxidans* in MAC medium with an initial pH of 1.5 and Fe^{2+} concentration of 6 g L^{-1} with yeast extract (white) and without supplements (grey). Error bars represent one standard deviation of the duplicates.

In this study, only the sample supplemented with yeast extract was able to complete Fe^{2+} oxidation. Thus, *S. thermosulfidooxidans* acted as a relatively poor ferrous ion oxidiser when grown in pure culture (Figure 3). This is in accordance with Pina et al.[29], who highlighted the benefits of mixotrophic conditions at the expense of autotrophic ones (Table 2). *S. thermosulfidooxidans* showed slower growth kinetics (μ = 0.11 h^{-1}) and lower ferrous ion oxidation rates ($v_{Fe^{2+}}$ = 0.12 g Fe^{2+} L^{-1} h^{-1}) than *A. ferrooxidans* under the same conditions. In light of the results obtained, and setting aside the benefits owing to the higher temperatures applied (which also involves the handicap of an additional energy consumption), no potential benefit for the process can be expected in this case when using *S. thermosulfidooxidans* instead of *A. ferrooxidans*.

Biomachining experiments

Proteobacteria of the genus *Acidithiobacillus*, and more specifically *A. ferrooxidans* or *A. thiooxidans*, are the most widely studied bacteria in copper biomachining[8, 16], although reports involving bacteria of the genus *Staphylococcus sp.* and a strain of *Aspergillus niger* fungi have been published recently[34, 35]. However, to the best of our knowledge, little information regarding the ability of moderately thermophilic bacteria in this field is available. In this section, the performance of mesophilic (*A. ferrooxidans*) and thermophilic bacteria (*S. thermosulfidooxidans)* in MAC medium during copper biomachining process is compared (Figure 4). A progressive decrease in the amount of copper removed per unit time was observed for both bacteria after the maximum value had been reached during the first hour, with a maximum MRR of 109 and 117 mg h^{-1} being reached for *A. ferrooxidans* and *S. thermosulfidooxidans*, respectively. These values are in accordance with those obtained by Jadhav et al.[36], who used *A. ferrooxidans* 13823 culture supernatant in 9 K medium and also observed a sustained decrease after the first hour (25–30 % and 40–45 % decrease after 2 and 3 h, respectively).
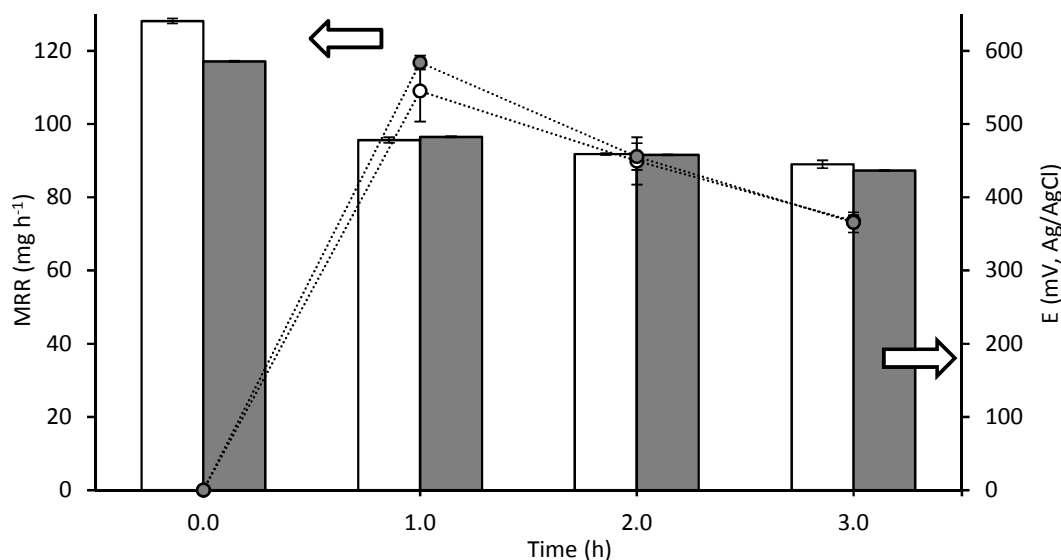
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Figure 4. Evolution of redox potential (columns) and metal removal rate (circles) for *A. ferrooxidans* (white) and *S. thermosulfidooxidans* (grey) in MAC medium with an initial pH of 1.5 and Fe^{2+} concentration of 6 g L^{-1} (with yeast extract supplementation for the thermophilic bacteria). Error bars represent one standard deviation of the duplicates.

As stated above, the decrease in metal leaching rate with Fe^{3+} concentration has prevented biomachining from being implemented industrially. To address this problem, previous studies published by us[4] proposed the use of a multi-module carousel in which each module contains a ferric ion solution and a microbial consortium that is able to oxidize Fe^{2+} ions. The workpiece remains submerged in each solution as long as the MRR is a maximum. During that period, acidophilic bacteria (i.e., *A. ferroxidans)* regenerate the ferric ion concentration in the rest of the modules. The novelty of this study lies in the achievement of a stable, controlled and continuous biomaching process by means of a unique dissolution tank in which bacterial culture and biomachining take place at the same time ("*Biomachining during culture period*" section). In this case, continuous bacterial growth at its maximum degrading activity would be accomplished since the higher kinetics of the chemical reaction would ensure the availability of the substrate (Fe^{2+} ions) (Figure 1c). The key is therefore to overcome the difference between chemical and microbiological reaction rates to achieve a good balance and the desired MRR stability.

Biomachining during culture

A copper bioleaching process in which bacterial growth and workpiece biomachining take place at the same time has been envisaged in this section. Jarosite accumulation was visually observed in those samples with no pH control after 38 h (pH_{final} 2.3) and 86 h (pH_{final} 2.5) for *A. ferrooxidans* and *S. thermosulfidooxidans*, respectively, thereby reducing iron availability in the solution (precipitation above 60 % of total iron) and concluding the process prematurely.

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In those experiments with pH control, *S. thermosulfidooxidans* showed a seven-times lower MRR in comparison with *A. ferrooxidans* after operation for 1 day (1.72 vs 12.6 mg h^{-1}). In addition, growth of *S. thermosulfidooxidans* was limited in comparison with its performance during standard culture (SI Figure S5), which might be related to its low toleration to copper cations, thereby significantly reducing previous inhibition limits reported in the literature for different strains of the same species[14]. Similarly, Watking et al.[37] previously observed that metal tolerance (Fe, Cu, Zn, Ni and Co) varied significantly between strains of the same specie. Regarding *A. ferrooxidans* cell density, bacteria seemed to grow faster when biomachining was performed together with the growth step (SI Figure S6). In addition, the bacteria developed in the latter experiments reached a smaller size, which could be related to the increase in ferrous-oxidizing activity[38].

The biomachining test using *A. ferrooxidans* showed an increase in MRR (~43 mg h^{-1}) for the first 50 hours (Figure 5), subsequently remaining constant over the following 30 h. Although a stable copper removal rate over time was achieved, the maximum MRR reached was less than half the value attained during conventional biomachining (109 mg h^{-1}) (Figure 4). Moreover, the copper removal rate remained constant even though the amount of Fe^{3+} available also increased throughout the experiment. Jarosite formation was discarded as a cause of this stagnation since the total iron content in the medium remained approximately constant throughout the test (~6 g L^{-1}). One possible explanation might be related to biofouling as a result of the adhesion of microorganisms and subsequent biofilm formation on the workpiece surface[39]. The resulting passivating layer would prevent ferric ion migration, thus inhibiting the biomachining process[40].
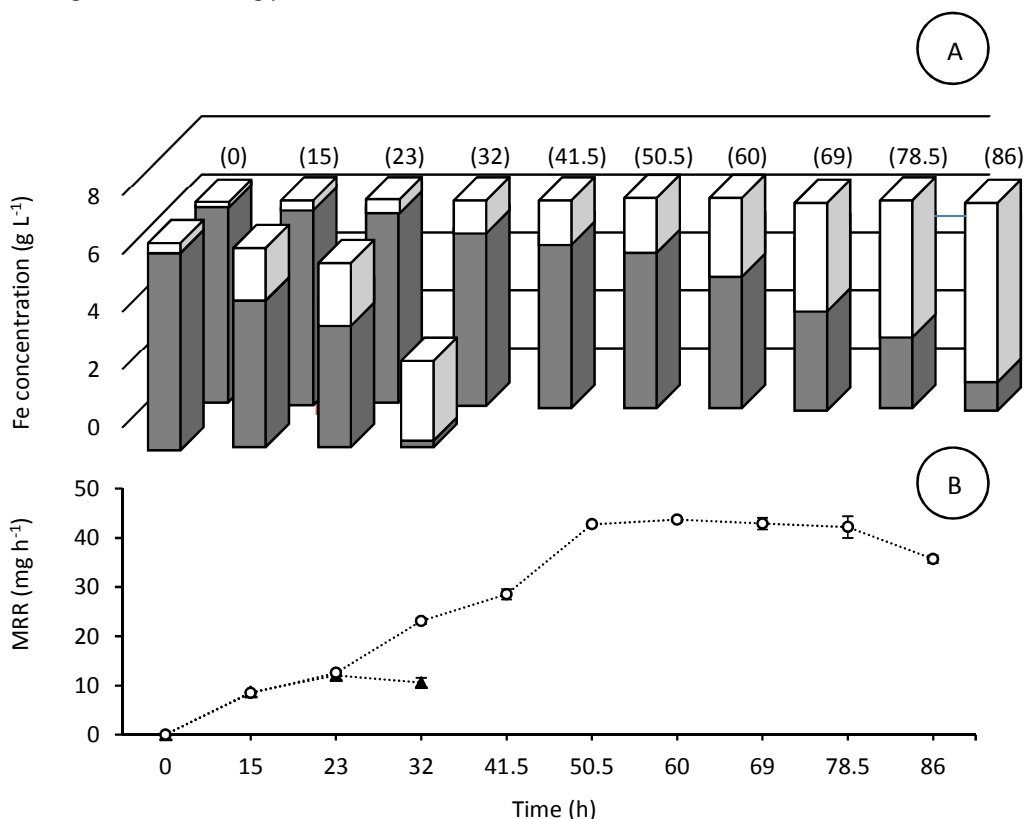

Figure 5. Evolution of Fe^{2+} (grey columns) and Fe^{3+} (white columns) concentration during simultaneous bacterial culture and biomachining for *A. ferrooxidans* in MAC medium (initial pH 1.5 and Fe^{2+} concentration of 6 g L^{-1}) with no pH control (front row) and with a constant pH threshold value of 1.8 (back row) (A); metal removal rate observed in these experiments (with no pH control (black triangles) and for a maximum pH of 1.8 (white circles)) (B).

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SUMMARY

Despite the potential benefits of biomachining (e.g., environmentally friendly nature) as an alternative to conventional chemical and physical manufacturing processes, its industrial application has not yet been completed. However, more in-depth studies of the chemical and biological aspects of this process, as well as the influence of the most significant process parameters, could promote its industrial implementation. In this case, the use of extremophile mesophilic (*A. ferrooxidans*) and thermophilic (S. *thermosulfidooxidans*) bacteria to remove copper from OFC workpieces has been investigated. Unlike the obligate chemolithoautotroph *A. ferrooxidans*, *S. thermosulfidooxidans* only exhibited measurable growth rates in mixotrophic conditions, therefore its nutrient medium had to be supplemented with yeast extract. Despite this, *A. ferrooxidans* showed a higher specific bacterial growth rate and specific substrate consumption under the optimal conditions (initial pH and Fe^{2+} concentration of 1.5 and 6 g L^{-1} and 30 °C (for *A. ferrooxidans*) or 45 °C (for *S. thermosulfidooxidans*)) in MAC medium. Jarosite formation and precipitation was observed when the pH exceeded the value of 2.2 for samples with a total iron content $\geq$6 g L^{-1}.

As expected, when the *A. ferrooxidans* growth (cultivation period) and machining stages were performed simultaneously, a stable copper removal rate in the range 40–45 mg h^{-1} was obtained. This method avoided Fe^{3+} depletion in the medium, which is the key factor governing the machining rate. In contrast, the maximum MRR achieved was less than half of the value attained during conventional biomachining with pH adjustment (109 mg h^{-1}). A linear Fe^{3+} concentration increase in the medium (51 mg h^{-1}, R^2 = 0.951)) from the hour 20 onwards suggested a decrease in cation diffusion, probably caused by the attachment of microorganisms to the metallic surface, thus acting as a weak diffusion barrier.

SUPPORTING INFORMATION

Image of an OFC workpiece placed within a conical flask together with the uncultured inoculum in MAC medium during simultaneous bacterial growth and copper machining test, jarosite precipitation in samples with an initial pH of 2.0 and Fe^{2+} concentration of 6 g/L and 9 g/L, DAPI-stained microscopy images of *A. ferrooxidans* and *S. thermosulfidooxidans* grown with and without the presence of copper OFC samples within the flasks and the correlation between ferrous iron concentration depletion and redox potential increase during *A. ferroxidans* culture period.

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SYNOPSIS

Simultaneous bacterial growth and copper machining in oxygen-free copper workpieces enables a constant metal removal rate by extremophile bacteria.

ABSTRACT GRAPHIC (for table of contents use only)