Engineering in Life Sciences

Eng. Life Sci. **2017**, *17*, 382–391 www.els-journal.com

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Research Article

Biomachining: Preservation of *Acidithiobacillus ferrooxidans* and treatment of the liquid residue

Biomachining has become a promising alternative to micromachining metal pieces, as it is considered more environmentally friendly than their physical and chemical machining counterparts. In this research work, two strategies that contribute to the development of this innovative technology and could promote its industrial implementation were investigated: preservation of biomachining microorganisms (*Acidithiobacillus ferrooxidans*) for their further use, and making valuable use of the liquid residue obtained following the biomachining process. Regarding the preservation method, freeze-drying, freezing, and drying were tested to preserve biomachining bacteria, and the effect of different cryoprotectants, storage times, and temperatures was studied. Freezing at –80°C in Eppendorf cryovials using betaine as a cryoprotective agent reported the highest bacteria survival rate (40% of cell recovery) among the studied processes. The treatment of the liquid residue in two successive stages led to the precipitation of most of the total dissolved iron and divalent copper (99.9%). The by-products obtained (iron and copper hydroxide) could be reused in several industrial applications, thereby enhancing the environmentally friendly nature of the biomachining process.

Keywords: *Acidithiobacillus ferrooxidans* / Biomachining / Liquid residue treatment / Preservation of microorganisms

Received: May 1, 2016; *revised:* August 1, 2016; *accepted:* September 19, 2016

DOI: 10.1002/elsc.201600124

1 Introduction

Recent advances in biotechnology have led to the development of biomachining as a promising alternative to the physical and chemical micromachining methods used in manufacturing microelectromechanical devices. The technology refers to the use of microorganisms to selectively form microstructures on a workpiece through the removal or dissolution of metal [1, 2], and regarding material removal, it is considered more environmentally friendly than other techniques, due to lower energy consumption, lower cost, and the nonuse of hazardous components [1, 3–5].

Acidithiobacillus ferrooxidans has been widely used in the bioleaching and indirect biomachining of copper [6–10]. This bacterium utilizes the energy generated by oxidation of ferrous ions to ferric ions to fix carbon dioxide in the air (Eq. 1). In a second step (Eqs. 2-3), the biogenic ferric ion brings about the dissolution of copper metal [4, 7, 11].

$$2\,Fe^{2+} + 2\,H^{+} + 0.5O_2 \rightarrow 2\,Fe^{3+} + H_2\,O \quad (1)$$

$$2\,Fe^{3+} + 2e^{-} \rightarrow 2\,Fe^{2+} \quad (2)$$

$$Cu^{0} + 2e^{-} \rightarrow Cu^{2+} \quad (3)$$

Full-scale biomachining has not yet been implemented, despite the numerous advantages of this method as complement or alternative to traditional machining processes. Therefore, having a deeper knowledge of the process and optimizing operational aspects such as the preservation of microorganisms and the valorization of the biomachining liquid residue, could promote the industrial implementation of this green technology.

A reliable preservation method that enables long-term storage of *A. ferrooxidans* while maintaining cell viability would ensure the ready availability of the bacteria and prevent mutations and

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Abbreviation: BLR, biomachining liquid residue

contamination of cultures [12–15]. Successful techniques for preserving *A. ferrooxidans* have already been studied [16–19]. *A. ferrooxidans* is highly susceptible to cryoinjury [17] and the addition of a suitable protective agent is therefore critical for cell survival or for improving cell viability [17,19–21]. In the particular case of *A. ferrooxidans*, protective agents such as GP (glycerate 3-phosphate molecule), glycine betaine, skimmed milk, sucrose, sucrose + mannitol, and glycerol have already been tested in the different preservation methods [16, 17, 19]. Preservation in the absence of cryoprotective agents is also possible with the aid of cryoballs, which are now widely used [22].

Regarding the treatment of the liquid residue generated in the biomachining process, no research has been carried out with the aim of making use of this by-product.

In this research work, strategies for promoting the industrial application of this innovative micromachining technology have been investigated. Three methods of preserving *A. ferrooxidans* cells were tested (freeze-drying, freezing, and drying), and the effect of different cryoprotectants and storage times, among other key parameters, were evaluated. In addition, a procedure for treating the liquid residue generated in the process was also described, with the aim of obtaining a less contaminating residue and of recovering the dissolved metals as high purity precipitates.

2 Materials and methods

2.1 Preservation of *A. ferrooxidans*

2.1.1 Culture medium

A. ferrooxidans (DSM-14882) bacterial cells were cultured in a specific broth (9K) the chemical composition and initial pH value (adjusted with H_2SO_4) of which are summarized in Table 1.

In addition, three different culture media (A, B, and C) were tested separately in order to assess their suitability for determining cell viability. The chemical composition and the initial pH (adjusted with H_2SO_4) of the three solid media tested are shown in Table 1.

Table 1. Culture media: Chemical composition and initial pH

Medium	Chemical composition (g/L)	Reference
Liquid broth		
9K	30 $FeSO_4{\cdot}7H_2O$ 3 $(NH_4)_2SO_4$ 0.5 $Mg(SO_4){\cdot}7H_2O$ 0.5 K_2HPO_4: 0.1 KCl 0.01$Ca(NO_3)_2{\cdot}4H_2O$ pH = 1.8	[39]
Solid medium		
A	3.0 $(NH_4)_2SO_4$ 0.1KCl 0.5 K_2HPO_4 0.5 $MgSO_4{\cdot}7H_2O$ 0.01$Ca(NO_3)_2$ 16.5 $FeSO_4{\cdot}7H_2O$ 0.7% (w/v) Agarose pH 2.5	[24]
B	Solution A: 20% (w/v) $FeSO_4$ (36.57 g/L $FeSO_4{\cdot}7H_2O$) pH 2.0 Solution B: 1.8 $(NH_4)_2SO_4$ 0.7 $MgSO_4{\cdot}7H_2O$ 0.35 TSB pH 2.5 Solution C: Agarose 2.8% (w/v) No pH adjustment A:B:C solutions mixed in 1:14:5 ratio	[25]
C	Solution A: 0.5 $(NH_4)_2SO_4$ 0.5 $Mg(SO_4){\cdot}7H_2O$ 0.5 $K_2HPO_4{\cdot}7H_2O$ 5.0 mL H_2SO_4 (15 N) pH 1.3 Solution B: 167 g $FeSO_4{\cdot}7H_2O$ 50 mL H_2SO_4 (15 N) pH 1.3 A:B solutions mixed in 4:1 ratio and agarose added to 0.7% (w/v)	[26]

2.1.2 Cell preservation

Freeze-drying, freezing, and drying were tested as methods of preserving *A. ferrooxidans*.

2.1.2.1 Freeze-drying. Two different experiments (FD1 and FD2) were carried out to determine the effects of the duration of primary drying (12 or 35 h, respectively) and of three cryoprotective agents (sucrose, glycerol, and trehalose) on the viability of freeze-dried *A. ferrooxidans* cells. The conditions for each experiment are defined in Table 2.

In a third experiment (FD3), the influence of using younger cells from the beginning of the process was studied and a fourth cryoprotectant was also tested (betaine) (Table 2). In this third experiment, duplicate samples were freeze-dried in order to study how storage at 4°C for one month (4 weeks) affected cell viability.

In the three experiments, the first step in sample preparation was the removal (by deposition) of the solid ferrous residues that may be present in solution after cell cultivation. Thus, the cell cultures (200–400 mL) were incubated at room temperature for 10 min without stirring and then the steps indicated in Table 3 were performed.

In all three experiments, an additional sample was resuspended in distilled water as a control.

The viability of *A. ferrooxidans* was tested before and immediately after freeze-drying the samples obtained in all experiments (FD1, FD2, FD3, and FD3 after 4 weeks) (see "Cell viability" section).

2.1.2.2 Freezing. The effect of two freezing procedures on the viability of *A. ferrooxidans* was tested. The first method consisted of cryofreezing the samples in Eppendorf cryovials using different cryoprotectants, while the second method involved the use of commercial Cryoinstant vials (Scharlab, Barcelona/Spain). The

Table 2. Freeze-drying experiments

	FD1	FD2	FD3
Culture time (days)	14	18	4
Initial cell concentration (CFU/mL)	$1.8 \cdot 10^9$	$5.2 \cdot 10^8$	$2.1 \cdot 10^9$
Primary drying temperature (°C)	5	5	5
Primary drying duration (h)	12	35	35
Secondary drying temperature (°C)	30	30	30
Secondary drying duration (h)	3	3	3
Cryprotectant (% w/v in distilled water)	Glycerol (5%) Sucrose (18%) Trehalose (15%)	Glycerol (5%) Sucrose (18%) Trehalose (15%)	Glycerol (5%) Sucrose (18%) Trehalose (15%) Betaine (6%)
Storage time	None	None	None 4 weeks at 4°C

experiments were carried out using several storage temperatures and times as these aspects are key to obtaining a high survival rate. In both cases, the process started with the procedure summarized in Table 3 (steps I–IV).

2.1.2.2.1 Freezing in Eppendorf cryovials. The freezing procedure was based on the protocols reported by Wu et al. [12], Wu et al. [17], and Cleland et al. [19] and is summarized in Table 3 (steps V–VIII). The composition of each cryoprotectant (step VI) was: autoclaved glycerol solution (30% in distilled water, w/v); filter sterilized betaine solution (6% in distilled water, w/v) and a sterile-mixed solution containing glycerol and betaine (30% glycerol and 6% betaine in distilled water, w/v). The aliquots resuspended in distilled water were considered as controls.

The initial cell concentration of the samples prior to freezing was determined by spread plating one of the control samples onto the surface of a Petri plate containing medium B. The initial cell concentration (approximately $1.4 \cdot 10^9$ CFU m/L) was assumed to be the same for all samples before freezing.

Cell viability was calculated on days 14 and 28, to evaluate the effect of preservation time on the viability of *A. ferrooxidans* as described in the "Cell viability" section. For this purpose, the Eppendorf vials were thawed in a water bath (at 37°C), and the cryoprotectant was removed by centrifugation (10 000 rpm, 10 min, 4°C). The cell pellet was washed twice with 1 mL of fresh 9 K broth (by centrifuging at 10 000 rpm, 10 min, 4°C) before being resuspended and diluted tenfold in distilled water.

2.1.2.2.2 Freezing in commercial cryovials. Two samples, each of 1 mL, subjected to steps I–IV as summarized in Table 3, were frozen in commercially available Cryoinstant vials (Scharlab, Barcelona/Spain) containing porous beads in culture medium and glycerol as a cryoprotectant. One of the cryovials was stored at –20°C for 28 days. The second vial was frozen at –20°C for 1 h before being stored at –80°C for 28 days. On days 14 and 28, the commercial cryovials were thawed at room temperature and two beads were extracted from each. Cell viability was then determined by the methodology indicated in the "Cell viability" section by spreading the beads on solid medium B until the development of bacterial colonies.

2.1.2.3 Drying. Prior to drying, a suspension of *A. ferrooxidans* in culture media 9 K (cell viability = $8.7 \cdot 10^7$ CFU m/L) was concentrated by filtering in a 0.22 μm nitrocellulose vacuum filter. The concentrated cells retained on the filter (cell viability = $3.5 \cdot 10^9$ CFU m/L) were dried at 30°C for 24 h in an empty sterile Petri plate. The filter containing the dry biomass was stored at 4°C and, after 14 days, the bacteria were resuspended in 10 mL of distilled water. Finally, dilution of the resuspended bacteria was spread on solid medium B and incubated at 30°C for 12 days.

2.1.2.4 Cell viability. Cell viability was determined, after resuspending the samples in 1 mL of distilled water, by calculating the number of colony forming units per milliliter (CFU m/L). Viability was determined before (CFU_0) and after (CFU_t) submitting the cells to the corresponding method of preservation. Both CFU_0 and CFU_t were calculated by making serial dilutions of the liquid samples and plating these onto the surface of Petri plates containing medium B (Table 1). The plates were incubated at 30°C for 12–14 days. The viability rate (V%) was expressed by the cell survival rate: $V\ (\%) = 100 \cdot CFT_t/CFU_0$ [23].

2.2 Treatment of the biomachining liquid residue (BLR)

2.2.1 Preparation of synthetic BLR

A synthetic BLR was prepared. The composition simulated the typical liquid residue generated in the biomachining process, i.e. an acidic liquid (pH = 2.3) containing approximately 3.0 g Fe^{2+} L^{-1}, 3.0 g Fe^{3+} L^{-1}, 6.7 g Cu^{2+} L^{-1}, and 1.0 g NH^{4+} L^{-1}. Thus, 3.8 g of $FeSO_4 \cdot 7H_2O$, 6.5 g of $(NH_4)Fe(SO_4)_2 \cdot 12H_2O$ and 6.6 g of $CuSO_4 \cdot 5H_2O$ were dissolved in 250 mL of deionized water. The initial pH was adjusted to 2.27 by adding H_2SO_4 (25% w/v). The initial value of the Redox potential was 436.9 mV.

2.2.2 BLR treatment procedure

A 100 mL sample of the BLR was subjected to two successive stages, in duplicate. The objective of this sequential treatment was to remove the ferrous iron and the copper (II) ion in Stages I and II, respectively, by recovering the metals as pure metal hydroxides.

In Stage I, iron (II) was oxidized to iron (III) by adding hydrogen peroxide (30% w/v) to a continuously stirred 100 mL BLR sample until a change in the redox potential trend was observed. The pH was then increased from 2.27 to 4.0 by adding NaOH

Table 3. Freeze-drying and freezing process steps

Freeze-drying					
	Process steps	**1**	**2**	**3**	**4**
	Description	Cell harvesting by centrifugation	Resuspension of the cell pellet	Mixing of the aliquots in a single sample and cell harvesting by centrifugation	Supernatant removal and resuspension of the cell pellet
	Conditions	10 000 rpm, 20 min, 4°C (Eppendorf 5810-R, Eppendorf AG, Hamburg/Germany)	15 mL of 9K medium (Table 1)	10 000 rpm, 15 min, 4°C (Eppendorf 5810-R, Eppendorf AG, Hamburg/Germany)	15 mL of distilled water
	Volume per aliquot (mL)	65	65	325	325
	Number of aliquots	5	5	1	1
	Process steps	5	6	7	8
	Description	Cell harvesting by centrifugation	Supernatant removal and resuspension of the cell pellet	Incubation	Freeze-drying
	Conditions	10 000 rpm, 20 min, 4°C (Eppendorf 5415-R, Eppendorf AG, Hamburg/Germany)	0.5 mL of cryoprotectant (Table 2)	30°C, 1 h	Freezing (–40°C) Primary drying (5°C, 0.250 mbar) Secondary drying (30°C, 0.250 mbar)
	Volume per aliquot (mL)	1.5	0.5	0.5	0.5
	Number of aliquots	18	18	18	18
Freezing					
Initial process	Process steps	**I**	**II**	**III**	**IV**
	Description	Cell harvesting by centrifugation	Resuspension of the cell pellet	Mixing of the aliquots in a single sample and cell harvesting by centrifugation	Supernatant removal and resuspension of the cell pellet
	Conditions	10 000 rpm, 15 min, 4°C (Eppendorf 5810-R, Eppendorf AG, Hamburg/Germany)	15 mL distilled water	10 000 rpm, 15 min, 4°C (Eppendorf 5810-R, Eppendorf AG, Hamburg/Germany)	15 mL distilled water
	Volume per aliquot (mL)	65	65	325	325
	Number of aliquots	5	5	1	1
Eppendorf cryovials	Process steps	**V**	**VI**	**VII**	**VIII**
	Description	Cell harvesting by centrifugation	Supernatant removal and resuspension of the cell pellet	Refrigeration and incubation	Freezing
	Conditions	10 000 rpm, 15 min, 4°C (Eppendorf 5415-R, Eppendorf AG, Hamburg/Germany)	1 mL of cryoprotectant	Refrigeration: 4°C, 30–40 min Incubation: –20°C, 1 h	–80°C, 28 days
	Volume per aliquot (mL)	1	1	1	1
	Number of aliquots	13	3 (glycerol) 3 (betaine) 3 (glycerol-betaine) 4 (distilled water)	3 (glycerol) 3 (betaine) 3 (glycerol-betaine) 4 (distilled water)	3 (glycerol) 3 (betaine) 3 (glycerol-betaine) 4 (distilled water)

(97 mg/mL). Finally, the precipitated $Fe(OH)_3$ was separated by simple filtration (paper filter, FilterLab-1240).

In Stage II, the pH of the solution obtained (by filtration) in the previous stage was increased to 8.0 by adding NaOH (97 mg/mL), and the resulting precipitate was filtered (paper filter, FilterLab-1240). Finally, a cylindrical piece of iron was submerged in the filtrate for 2 h to remove the remaining dissolved Cu^{2+} by reduction to Cu^0 in the presence of iron. The sample solution was stirred and, after 1 h and at the end of the experiment, the pH was measured and the concentrations of Cu^{2+}

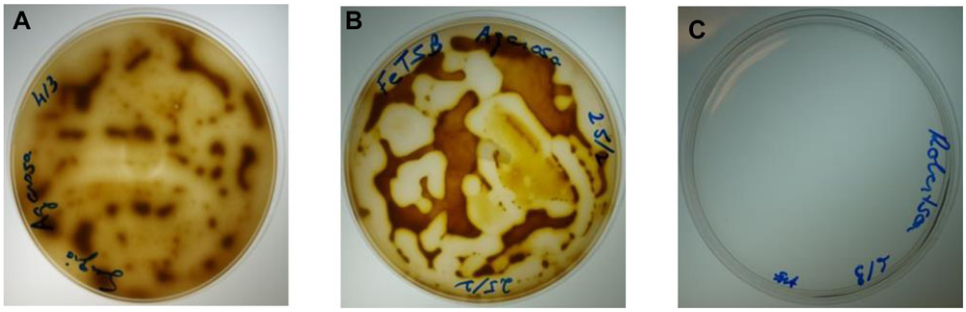

Figure 1. Growth of *A. ferrooxidans* on the three media tested: (A) Medium A, (B) Medium B, and (C) Medium C.

Table 4. Survival rate and concentration of *A. ferrooxidans* cells before and after freeze-drying in experiments FD1 (primary drying = 12 h), FD2 (primary drying = 35 h), and FD3 (primary drying = 35 h) using different cryoprotective agents

Cryoprotectant (% w/v in distilled water)		FD1	FD2	FD3	FD3–4 weeks
Control	Final cell concentration (CFU/mL)	$1.1{\cdot}10^5$	$9.0{\cdot}10^6$	$1.5{\cdot}10^5$	No bacteria
	Survival rate (%)	0.0057	1.7	0.0073	—
Glycerol (5%)	Final cell concentration (CFU/mL)	$2.8{\cdot}10^2$	$8.8{\cdot}10^6$	No bacteria	No bacteria
	Survival rate (%)	0.000015	1.7	—	—
Sucrose (18%)	Final cell concentration (CFU/mL)	$3.4{\cdot}10^3$	$4.5{\cdot}10^6$	$2.8{\cdot}10^5$	$7.0{\cdot}10^1$
	Survival rate (%)	0.00018	0.90	0.013	0.025
Trehalose (15%)	Final cell concentration (CFU/mL)	$8.1{\cdot}10^3$	$5.2{\cdot}10^6$	$2.6{\cdot}10^5$	$2.3{\cdot}10^3$
	Survival rate (%)	0.00044	1.0	0.013	0.87
Betaine (6%)	Final cell concentration (CFU/mL)	—	—	$4.6{\cdot}10^4$	$2.0{\cdot}10^1$
	Survival rate (%)	—	—	0.0022	0.043

and total Fe (Fe^T) in solution were determined. At the end of the process, the solution was characterized by determining the dissolved salt content as the weight of dry residue.

The same procedure was carried out by submerging a second piece of iron in an aqueous solution containing 20 g/L of Na_2SO_4 (main compound in the final filtrate). This was considered a blank solution for purposes of comparison.

2.3 Analytical methods

The redox potential was measured with a Thermo-Orion 920A+ potentiometer and the pH, with a Crison GLP 21 pH-meter.

The Cu^{2+} content was determined by atomic absorption (Perkin Elmer, Aanalyst 100, LabX, Midland/Canada). Total Fe (Fe^T) was measured by colorimetry. For this purpose, 5 mL of an ammonium acetate/acetic acid buffer solution (pH = 5.5) was added to a 50 mL flask containing 5 mL of sample. The sample was stirred and 2 mL of hydroxylamine hydrochloride solution (10% w/v) was added. The sample was stirred again and, after 5 min, 2 mL of a 2,2'-bipyridine solution (0.5% w/v) was added. After 5 min, the absorbance of the 50 mL diluted solution was measured at 520 nm by spectrophotometry (Vis/UV Thermo-Helios, Thermo Electron Corporation, Spain).

The dissolved salt content was determined by weighing the dry residue of the solution after evaporating the water content of an aliquot at 105°C to constant weight.

3 Results

3.1 Preservation of *A. ferrooxidans*

3.1.1 Culture medium

Colonies of *A. ferrooxidans* grew on medium A [24] and medium B [25] within 6–7 days of incubation at 28°C (Fig. 1A and B, respectively). However, no microbial growth was observed on medium C [26], even after incubation for 11 days (Fig. 1C).

3.1.2 Cell preservation

3.1.2.1 Freeze-drying. In FD1 (primary drying = 12 h), only the negative control was successfully freeze-dried, while the samples resuspended in glycerol, sucrose, and trehalose remained damp at the end of the process. However, when the length of the primary drying stage was increased to 35 h in experiments FD2 and FD3, freeze-drying of the control and the sample resuspended in the trehalose solution was successful.

Cell counts in the samples preserved in FD1 using cryoprotectants ranged between $2.8{\cdot}10^2$ CFU/mL and $8.1{\cdot}10^3$ CFU/mL (Table 4). The survival rate increased when the duration of the primary drying was prolonged to 35 h in experiment FD2. Nevertheless, by comparison to FD2, cell viability in experiment FD3 was reduced although in this case the primary drying was also established at 35 h. It should be noted that no *A. ferrooxidans* colonies were detected in the sample in which glycerol was used as a cryoprotectant.

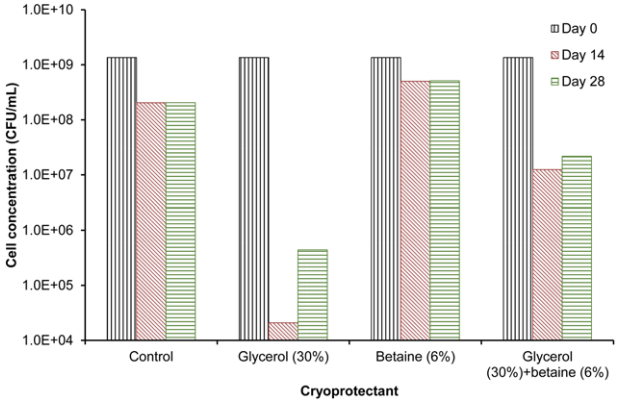

Figure 2. Concentration of *A. ferrooxidans* cells after freezing at –80°C in Eppendorff vials for 14 and 28 days and using different cryoprotectants.

Cell viability in the aliquots preserved for 4 weeks using sucrose, trehalose and betaine decreased respectively to $7.0 \cdot 10^1$ CFU/mL, $2.3 \cdot 10^3$ CFU/mL and $2.0 \cdot 10^1$ CFU/mL. No colonies were detected in the control sample or in the sample supplemented with glycerol after storage for 4 weeks.

3.1.2.2 Freezing. Regarding the samples stored in Eppendorf tubes, cell concentration was highest in the one with 6% betaine ($5.1 \cdot 10^8$ CFU/mL) after 28 days (Fig. 2). In the rest of the samples, the cell concentration varied from $4.4 \cdot 10^5$ CFU/mL to $2.1 \cdot 10^8$ CFU/mL.

In the samples stored in commercial cryovials, the cell viability decreased with time in both the sample frozen at –20°C and the one frozen at –80°C (Fig. 3). Cell growth was not observed after 28 days in the samples stored at –20°C (Fig. 3C), while the bacterium was recovered from those samples stored at –80°C for the same period of time (Fig. 3F).

3.1.2.3 Drying. In this study, no growth was observed after drying the filter at 30°C for 24 h. No microbial growth was observed after incubation of samples for 12 days at 30°C, even when samples obtained by washing the filter were seeded directly on medium B.

3.2 Treatment of synthetic BLR

The Fe^{2+} and Cu^{2+} content in the synthetic BLR was reduced in two consecutive stages: Stage I (Fe^{2+} removal) and Stage II (Cu^{2+} removal), as described below.

In Stage I, the initial redox potential (436.9 mV) of the synthetic BLR solution increased as a consequence of the continuous addition of H_2O_2. Once 0.45 mL of hydrogen peroxide had been added, this trend changed, and in the addition of another drop of H_2O_2, the Redox potential decreased from 616.8 to 590.3 mV, indicating complete oxidation of Fe^{2+} to Fe^{3+}.

In the next step (Stage II), 9.3 mL of NaOH was added to the continuously stirred BLR solution, until the pH of the solution increased to 4.0. During this step, the total Fe^{3+} precipitated as $Fe(OH)_3$ that was then recovered by filtering the solution.

Approximately 95 mL of the initial 100 mL of BLR solution was recovered after the removal of $Fe(OH)_3$ by filtration.

The color changes in the BLR solution during Stage I are shown in Fig. 4A–C.

In Stage II, the pH was increased to 8.27 by adding 5.3 mL of NaOH. Regarding the solution volume, 80 mL of liquid was recovered after removal of $Cu(OH)_2$ by filtration. Figs. 4D-4E show the color of the BLR solution before and after removal of $Cu(OH)_2$ by filtration. The submersion of an iron piece in the solution did not have the expected effect on Cu^{2+} removal (data not shown) and, therefore, this method was not considered effective for the desired purpose. The dry residue in the final solution, which was measured experimentally, was 25.7 g/L.

4 Discussion

4.1 Preservation of *A. ferrooxidans*

Several methods could be used for the estimation of cell viability. Several novel counting methods, including fluorescence analysis, staining technique, or protein measurement have been developed to date. These methods may require expensive consumables, complex equipment, and also incur high costs per test [27]. Compared to the aforementioned techniques, the conventional bacteria counting method based on plate colony counting is simple and is therefore usually used in laboratories. Nevertheless, it is characterized by being labor intensive, time consuming, and dependent on the operator's ability [21, 28], which could make the method both error-prone and less efficient.

In this case, for *A. ferrooxidans* plate colony counting, three solid media were tested in order to test bacterial growth. Of these, bacterial growth was only observed in medium A and medium B (Table 1). The latter of these two was chosen to determine the cell viability for the aforementioned bacteria counts due to it being less complex than medium A.

Focusing on the possibility of the industrial application of the biomachining process as a green alternative to conventional physical-chemical manufacturing procedures, one of the most challenging aspects would be to define a preservation process for the reliable maintenance of production strains. Even if serial subculturing techniques are well established and widely used, their low stability and high labor cost, render them less adequate for long-term storage [29].

Methods of storing microorganisms generally include passage culture, sterile sand tube preservation, freeze-drying, and freezing [30]. When one of the latter methods is mentioned in relation to the preservation of microorganisms, it is nearly always in regard to long-term storage [20, 28]. Wherever possible, long-term storage must be considered as the most appropriate option, as this involves halting the growth of the cells and maintaining them in a viable state [22].

Freeze-drying, also known as lyophilisation, was tested in three experiments (FD1, FD2, and FD3), as it is currently one of the most economical and preferred methods of preserving food products, biological materials, and is also used in drug delivery systems worldwide [31–34]. In this procedure, samples are frozen and the water content is then reduced in two consecutive steps: sublimation (primary drying) and desorption (secondary

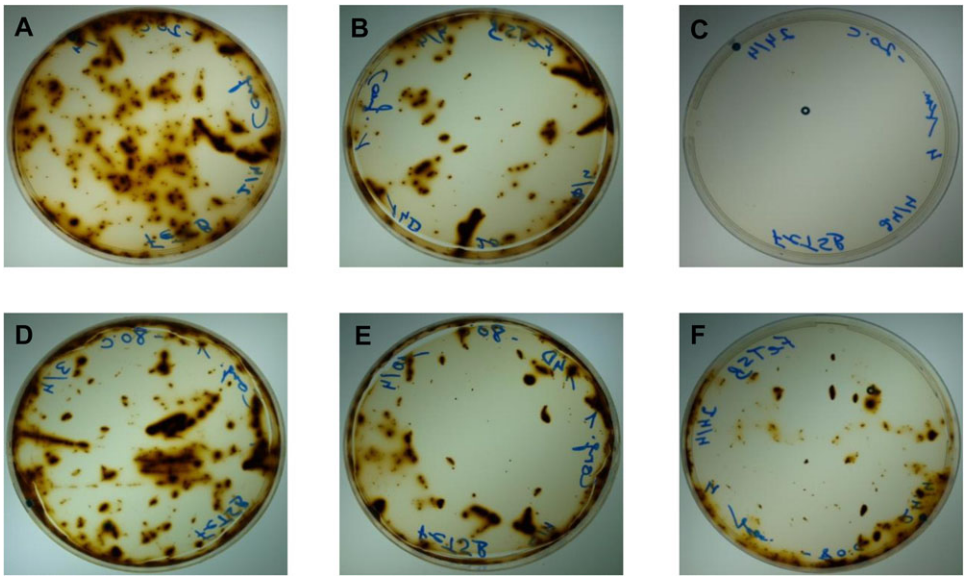

Figure 3. Cell viability in the samples stored at –20°C (A–C) and –80°C (D–F) in commercial cryovials: (A) T = –20°C, after 7 days; (B) T = –20°C, after 14 days; (C) T = –20°C, after 28 days; (D) T = –80°C, after 7 days; (E) T = –80°C, after 14 days and (F) T = –80°C, after 28 days.

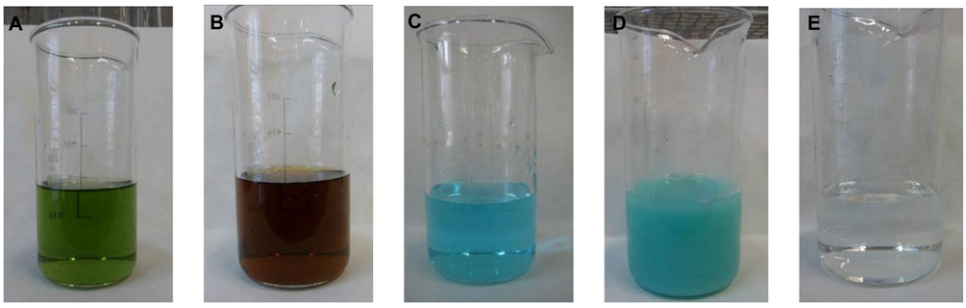

Figure 4. (A) Initial BLR, (B) BLR after Fe^{2+} oxidation (Stage I.i), (C) BLR after $Fe(OH)_3$ precipitation and filtration (Stage I.iii), (D) BLR solution after precipitation of Cu^{2+} and (E) BLR solution after removal of $Cu(OH)_2$ by filtration.

drying) [22]. It is essential to determine the duration of culture prior to harvesting cells and also to select an appropriate cryoprotective agent [33,34]. The cryoprotectants used in this study were: glycerol (5% w/v, 0.007 €/mL), sucrose (18% w/v, 0.012 €/mL), trehalose (15% w/v, 16.26 €/mL), and betaine (6% w/v, 0.012 €/mL).

The cell survival rate may vary greatly depending on the equipment and the conditions of the process [30, 33]. As expected, the length of the primary drying stage influenced the effectiveness of the freeze-drying process, obtaining best results when this period was set at 35 h of duration. Morais et al. [34] indicated that the primary drying step is usually long, having a significant impact on the overall cost of the process. Thus, defining an optimal duration for this step is essential in order to ensure both successful freeze-drying and an acceptable cost.

After FD1 (primary drying = 12 h), the cell viability decreased strongly, and the survival rate was below 0.1% in all the samples (Table 4). The best results were obtained in the control sample, although cell viability decreased by at least four orders of magnitude (from $1.8{\cdot}10^9$ CFU/mL to $1.1{\cdot}10^5$ CFU/mL). With an increase in the duration of the primary drying in FD2, 0.90–1.7% of the cells survived after freeze-drying; survival rates were highest in the control and the sample supplemented with glycerol. Finally, in FD3 the survival rate was higher in the samples supplemented with sucrose and trehalose (around 0.013% in both cases).

Even if the survival rate of the FD1 and FD2 experiments were low, it has been suggested that a survival rate of 0.10% of the original cell population after freeze-drying may be sufficient [35]. However, much higher poststorage survival rates (70–80%) have been reported as being necessary [22, 36].

Regarding FD3, the different survival obtained in comparison to FD2 may be related to the early stage at which the samples were harvested prior to freeze-drying (18 days for FD2 compared with 4 days for FD3). Indeed, the operational conditions were the same as in FD2 (primary drying stage, 35 h). This result could be in accordance with the ones obtained for microalgae freezing preservation by Morschett et al. [21] who reported that, contrary to what is commonly accepted, the preservation of stationary phase cells proved superior to freezing cells at their growing phase.

Furthermore, the absence of bacterial growth in the sample with glycerol as cryoprotectant may be due to the complicated retrieval procedure involved, which makes glycerol unsuitable as a cryoprotectant for many of the bacteria used in bioleaching [17].

The influence of storage time on cell survival was determined in the samples from experiment FD3, although cell survival immediately after freeze-drying was extremely low. Storing the

samples in FD3 for 4 weeks at 4°C after freeze-drying had a negative impact on all samples, as the survival rate decreased significantly (Table 4). The samples stored in the presence of sucrose, trehalose, and betaine accounted for the 0.025, 0.87, and 0.043%, respectively, of viable cells remaining immediately after freeze-drying. Dimitrellou et al. [37] also noted the negative effect that storing samples for 15 days (at 4°C) after being freeze-dried had on cell viability in *L. casei* cells.

With this all, it could be concluded that freeze-drying adversely affected the viability of the strain studied regardless of the primary drying length and cryoprotectant used. The viability rate did not exceed 2% in any of the cases presented herein.

Together with freeze-drying, freezing is considered to be the most feasible and effective method for long-term storage of pure strains and mixed cultures [30]. Cryopreservation of bioleaching bacteria and microalgae was first reported about 40 years ago [17, 21]. However, several protocols and cryoprotectants have since been developed. In this study, glycerol (30% w/v, 0.041 €/mL), betaine (6% w/v, 0.012 €/mL), and a glycerol (30% w/v)-betaine (6% w/v) mixture (0.026 €/mL) were used as cryoprotective agents.

This storage method is based on freezing cells that have been suspended in a liquid medium, which may or may not contain a cryoprotective agent (although inclusion is advisable). Storage of samples at temperatures below 0°C maintains the intracellular and extracellular water in a solid state [22]. The low temperature reduces cell metabolism and cell growth is prevented due to the absence of liquid water.

Storage time (14 or 28 days) did not have a significant impact on cell survival in the samples stored at –80°C in Eppendorf tubes and treated with the same cryoprotectant, except in those to which glycerol was added (Fig. 2). However, the cryoprotectant influenced the viability of cells after freezing. Cell viability was highest in samples stored in 6% betaine, with a 37% survival rate after 28 days. The latter parameter was somewhat lower in the sample preserved in the absence of the protective agents (control, 15.2%) and much lower in the samples stored with the glycerol and glycerol + betaine solution (0.032 and 1.6%, respectively).

In the cases in which samples were stored in commercial cryovials, cell viability was influenced by both storage time and freezing temperature. The decrease was more significant in the samples stored at –20°C (Fig. 3A–C) than in those stored at –80°C (Fig. 3D–F). In general, the intracellular recrystallization of water decreased with storage temperature, thus enabling the cells to remain viable for longer [22].

Hence, the freezing procedure in Eppendorf vials showed high dependency on the type of cryoprotectant used, with betaine obtaining the highest recovery percentage. From an industrial point of view, it is interesting to point out that betaine was the less expensive cryoprotectant among the agents used for this preservation technique in this study. By contrast, freezing time had no influence on cell viability (up to 28 days). Conversely, the viability of the samples frozen in commercial cryovials decreased significantly during the freezing time. The temperature also proved to be an important factor, with better results in the samples stored at –80°C.

Finally, desiccation negatively affected cell viability, being inadequate for the preservation of *A. ferrooxidans*. Thus, drying has proven to be unsuitable for the preservation of *A. ferrooxidans* as it annulled all possibilities of microbial growth after processing.

4.2 Treatment of synthetic BLR

The indirect mechanism of the biomachining process is a cyclic combination of chemical and microbiological processes (Eqs. 1–3). This indirect pathway uses an intermediate redox couple (such as Fe^{2+}/Fe^{3+} ions) to dissolve the metal from the workpiece surface, and no direct contact is established between the bacteria and the metal. Thus, the biomachining liquid residue will contain a certain concentration of both Fe^{2+} and Fe^{3+}. In addition, when the biomachining is carried out on a copper workpiece, Cu^{2+} is released to the liquid residue and dissolved Cu^{2+} concentration increases with time. Both iron and copper are considered to be toxic heavy metals at high concentrations, so the release of these metal ions in high quantities could be dangerous for the environment [38]. Therefore, their total or partial removal from the liquid residue is of great importance in order to avoid any pollution caused by the effluent generated in the biomachining process. Additionally, if iron and copper are removed from the liquid residue as pure precipitates, two useful by-products can be obtained: $Fe(OH)_3$ and $Cu(OH)_2$.

The sequential treatment for the synthetic BLR consisted in the oxidation of Fe^{2+} to Fe^{3+}, its precipitation as $Fe(OH)_3$, and the precipitation of Cu^{2+} as $Cu(OH)_2$ (Eqs. 4–6).

$$2\,Fe^{2+}\,(aq) + H_2O_2\,(aq) + 2\,H^+\,(aq) \rightarrow 2\,Fe^{3+}\,(aq) + 2\,H_2O \quad (4)$$

$$Fe^{3+}\,(aq) + 3\,NaOH\,(aq) \rightarrow Fe(OH)_3\,(s) + 3\,Na^+\,(aq) \quad (5)$$

$$Cu^{2+}\,(aq) + 2\,NaOH\,(aq) \rightarrow Cu(OH)_2\,(s) + 2\,Na^+\,(aq) \quad (6)$$

Regarding reactive consumption, in Stage I 0.5 mg of H_2O_2 and 1.5 mg of NaOH was consumed per mg of oxidized Fe^{2+} and per mg of precipitated Fe^{3+}, respectively. In Stage II, 0.77 mg of NaOH was consumed per mg of Cu^{2+} dissolved in the BLR. Therefore, based on the price of these chemicals in the market (Sigma-Aldrich), the treatment of a residue containing 3 gFe^{2+}/L, 3gFe^{3+}/L, and 6.7 gCu^{2+}/L would have a total cost of 2.5 € per liter of treated dissolution.

The pH played an important role in both precipitation reactions. An increase in the pH above 4.0 during $Fe(OH)_3$ precipitation Eq. (5) could lead to the coprecipitation of Cu^{2+} and the consequent reduction of the quality of the precipitate. Regarding $Cu(OH)_2$ precipitation reaction Eq. (6), ammonium can be partly transformed to NH3 when the pH increases above 8.0. The NH_3 and the Cu^{2+} can form a soluble complex that would solubilize the precipitated $Cu(OH)_2$, thus reducing the amount of product obtained. Therefore, the pH was carefully controlled in both steps.

The Fe^T and Cu^{2+} contents were both greatly reduced in the process. Precipitation of both $Fe(OH)_3$ and $Cu(OH)_2$ led to 99.9% of the initial Fe^T and Cu^{2+} contents being insolubilized, as the concentrations of Fe^T and Cu^{2+} were reduced from 6.0 g/L in the initial BLR to respectively 3.6 and 3.4 mg/L in the final solution. The concentration of Fe^T was presumably

related to the Fe^{2+} that had not been completely oxidized in the first step; optimization of the Fe^{2+} to Fe^{3+} oxidation step could therefore reduce the concentration of Fe^{T} in the final solution.

This result of the dry residue was consistent with the theoretical Na_2SO_4 concentration in the solution (25.1 g/L), calculated by taking into account the stoichiometric Na^+ concentration (from the NaOH consumed in both Fe^{3+} and Cu^{2+} precipitation steps) and assuming that all the Na^+ was in the form Na_2SO_4. Thus, it can be concluded that the dry residue essentially consists of Na_2SO_4 (17.0 g/L and 8.1 g/L SO_4^{2-} and Na^+ in solution, respectively).

The method used in this study generated very pure $Fe(OH)_3$ and $Cu(OH)_2$ that are valuable chemicals that could be sold in order to yield a return that would reduce the overall cost of the treatment. For example, $Fe(OH)_3$ can be used as a cement additive, and $Cu(OH)_2$ can be redissolved and recovered as highly pure copper by electrolysis.

As a general conclusion, regarding the preservation study of *A. ferrooxidans*, it could be concluded that freezing at –80°C using Eppendorf cryovials is the procedure that provides the best results regarding the feasibility for the microorganisms studied in these assays. The procedure developed for treating the liquid residue generated in the biomachining process enabled the metal content of the effluent to be reduced, which then only contained significant amounts of sulfates and sodium as well as the ammonia nitrogen (from the 9K broth). Moreover, metal recovery as high purity precipitates that could be used in industrial applications was proven to be possible. These two aspects are of great interest for the implementation of future biomachining industrial-scale plants.

Practical application

The use of microorganisms to remove material from metal workpieces, known as biomachining, provides an effective and sustainable alternative to conventional chemical and physical manufacturing processes. Despite this technology being researched thoroughly nowadays because of its potential benefits, its industrial application is still pending. The research here presented could promote the industrial implementation of this technology as it presents solutions to two important aspects of the process. First, the possibility of preserving the bacteria used in the process offers the opportunity to have the biomachining tool always ready for use. Second, treating the liquid residue obtained in the process is a way of boosting its "environmentally friendly" nature, reducing wastewater treatment costs and obtaining potentially useful by-products.

Authors are grateful to Basque Government (BFI 2011–119) and the University of the Basque Country UPV/EHU (UFI 11/29 and Convocatoria de ayudas para la Contratación de Doctores Recientes hasta su Integración en Programas de Formación Postdoctoral del Vicerrectorado de Investigación de la UPV/EHU) for providing financial support. GUSERBIOT, S.L. is acknowledged for collaboration and technical support.

The authors have declared no conflicts of interest.

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