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Immobilization of *Acidithiobacillus Ferrooxidans* on Two Hydrogels

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Bioleaching is considered a sustainable and effective alternative to conventional micromachining and extracting processes for recovering valuable metals from electronic waste. Nevertheless, the industrial scaling-up of these applications is limited by several factors.
Among these factors, the use of free cells and low microbial density have been reported to reduce process efficiency. Recent research has therefore focused on bacterial immobilization on different support materials as a solution for increasing microbial density inside the bioreactor and protecting microorganisms from toxic compounds. Furthermore, biomass immobilization facilitates biomass replacement whenever required. The increasing amount of metals in the solution is also a factor to be controlled, as it can inhibit bacterial activity.
This study set out to assess the suitability of two hydrogels, polyvinyl alcohol (PVA) and biocellulose (BC) as support materials for *Acidithiobacillus ferrooxidans* (A. *ferrooxidans*) immobilisation, and to analyse the effectiveness of the active material generated for use in metal bioleaching processes. In addition, an assessment was conducted of the influence of the amount of dissolved copper (0-20 g Cu^{2+}/L) on the time required for complete Fe^{2+} oxidation to Fe^{3+}.
The two hydrogels tested, PVA and BC, were viable as support materials for *A. ferrooxidans* immobilisation. Both active materials successfully transformed all the Fe^{2+} contained in the nutrient medium to Fe^{3+}. Nevertheless, BC was specifically selected for further studies because of its higher efficiency (shorter oxidation time needed for complete iron transformation), longer integrity maintenance, and higher resistance to dissolved copper up to 20 g Cu^{2+}/L.

1. Introduction

Bioleaching is a microorganism-promoted hydrometallurgical dissolution process that has traditionally been used at industrial scale for the recovery of metals from their ores. Nowadays, the scarcity of natural resources and the need to favour the transition towards a circular economy have extended the focus of bioleaching to applications such as biomachining, as an alternative to conventional micromachining (Díaz-Tena et al., 2017) and the recovery of metals from secondary solid wastes (Srichandan et al., 2019).
A variety of microorganisms such as mesophilic (Singh et al., 2018) and thermophilic autotrophic bacteria (Díaz-Tena et al., 2018), heterotrophic bacteria (Pradhan et al., 2012), fungi (Brandl et al., 2001) and mixed cultures (Vermeulen et al., 2017) have been used in the bioleaching process. In particular, the acidophilic mesophiles *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans* and the moderate thermophile *Acidithiobacillus caldus* (Martínez-Bussenius et al., 2016) have frequently been reported.
Regarding the operating conditions, the shacking rate, temperature, iron concentration and pH are widely known to affect bacterial activity, and therefore to influence the metal removal rate (Díaz-Tena et al., 2017). The use of free cells (weak physical strength) and low microbial density (low metabolic activity) have been reported as two of the factors limiting the industrial scaling-up of the most recent applications of biomachining (Giaveno et al., 2008). Therefore, research has focused on bacterial immobilization on different support materials as a solution for increasing microbial density inside the bioreactor and facilitating biomass replacement when required (Zhu et al., 2017). The selection of a suitable support material has a significant

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role to play on the performance of the process. Materials for cell immobilization need to meet different requirements, such as insolubility, non-toxicity, and high chemical and mechanical stability under operating conditions, as well as low cost (Zineb et al., 2018). Moreover, microbial adhesion capacity on the material and biofilm formation are also two key parameters for successful results when using the active material. Over the past ten years, hydrogels have been investigated as good candidates for bacterial immobilization due to their unique properties, such as high wettability and porosity (Tsou et al., 2016).
An additional factor to be controlled during the bioleaching process is the increasing amount of metals in the solution. One of the most commonly bioleached metals using *A. ferrooxidans* is copper. This metal is essential for several biological reactions, and serves as an electron donor during *A. ferrooxidans* growth (Lilova et al., 2007), but in high concentrations it becomes toxic and inhibits bacterial activity (Mykytczuk et al., 2011). Nevertheless, metal tolerance has been reported to vary significantly among *A. ferrooxidans* strains (Mykytczuk et al., 2011), and studies have shown that tolerance limits can be increased by bacterial adaptation (Pourhossein and Mousavi, 2018).
The objective here was to assess the feasibility of two hydrogels, polyvinyl alcohol (PVA) and biocellulose (BC), as support materials for *A. ferrooxidans* immobilization in metal bioleaching processes. Furthermore, the influence of the amount of dissolved copper (0-20 g Cu^{2+}/L) on the time required for complete Fe^{2+} oxidation by the active material was also assessed.

2. Materials and methods

2.1 Immobilization materials

Two hydrogels, polyvinyl alcohol (PVA) and biocellulose (BC), were selected as immobilization materials. PVA sheets were prepared using PVA (M_w 130000 g/mol) purchased from Sigma-Aldrich Corporation (Missouri, United States). PVA 10 wt% solution was prepared by dissolving PVA in deionized water at 95 ºC under vigorous stirring and reflux for 3 h. The homogenous PVA solution obtained was subsequently placed into a petri dish and cooled to ambient temperature. The freeze-thawing method was used to obtain the hydrogel (Hassan et al., 2000). Samples underwent 24 h freezing at -21 °C and 3 h of thawing at 25 ºC for three cycles.
BC hydrogel was biosynthesised by *Gluconacetobacter xylinus* bacteria in our lab. This kind of cellulose membrane produced by bacteria has unique structural and mechanical properties, constituting a naturally 3D nanoporous membrane. BC was biosynthesized by adding 1 vol% inoculum to the acidic culture medium and incubating under static conditions at 28 ˚C for several days. After incubation, the BC membrane was collected and treated with an alkaline solution to remove medium components and bacterial cells (Retegi et al., 2010).
In both cases, synthesized hydrogels were cut into small pieces (~ 5 cm^2). Additionally, a pre-treatment was designed to ensure the hydrogels were fully cleaned before their use. The protocol consisted of two successive washing stages, first in distilled water and then in a nutrient medium containing 9 g Fe^{2+} L^{-1}. This pre-treatment allowed the successful preparation of the synthetized materials for the immobilization step.

2.2 Immobilization protocol

Bacteria were immobilized on PVA and BC by immersing each hydrogel into an Erlenmeyer flask with a nutrient medium containing 9 g Fe^{2+} L^{-1}. The relationship between the nutrient medium (mL) and the exposed surface area of the support material (cm^2) was 1:0.6 in both cases. A 5 % inoculum of *A. ferrooxidans* in the exponential growth phase was added to each Erlenmeyer flask. The active material (AM) was obtained by cultivating the cells until the complete oxidation of Fe^{2+} to Fe^{3+}. The immobilization protocol is illustrated in Figure 1.

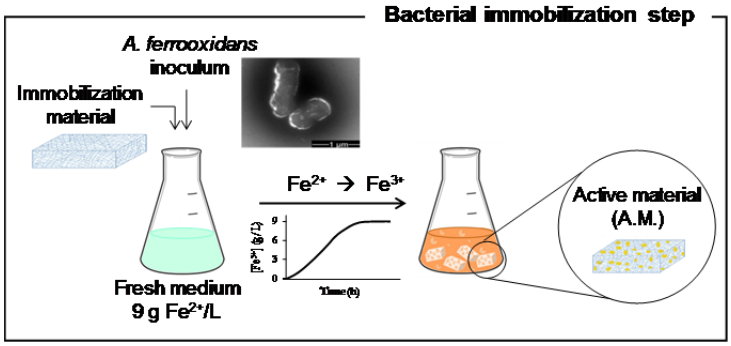

Figure 1. Schematics of the bacterial immobilization step

2.3 Performance of the active material

Each active material with the attached bacteria (active PVA and active BC) was cleaned with fresh medium and separately immersed in a fresh nutrient medium containing 9 g Fe^{2+} L^{-1} in the same mL:cm^2 ratio as in the immobilization step (1:0.6). The oxidation cycle was completed when all the Fe^{2+} was transformed into Fe^{3+}. This oxidation cycle was repeated twice. The use of the active material generated in successive oxidation steps and the operating conditions are illustrated in Figure 2.

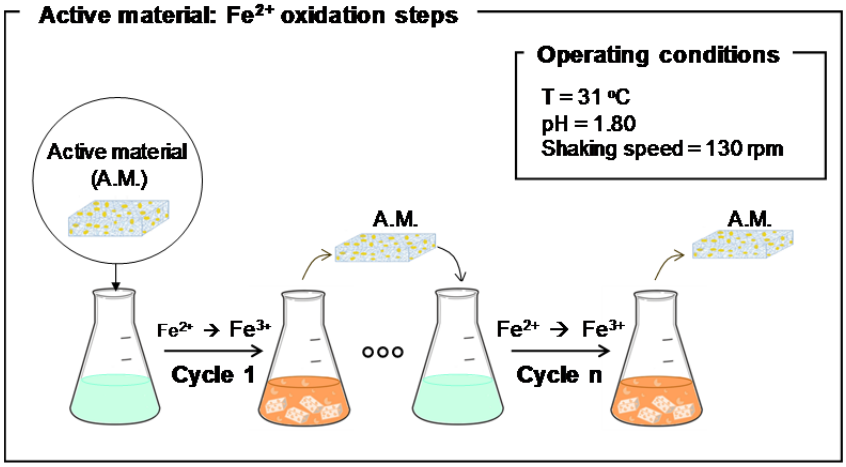

Figure 2. Schematics of the iron oxidation steps using the active material

2.4 Influence of dissolved Cu^{2+} in the performance of the active materials

The effect of copper concentration on the activity of the immobilized bacteria was studied by cultivating each active material in nutrient media containing 0, 5, 10, 15 and 20 g Cu^{2+} L^{-1}, respectively, under the same operating conditions as in the immobilization and successive oxidation steps, i.e., T = 31 ^{o}C, pH = 1.80, shaking speed = 170 rpm.

2.5 Analytical methods

The ferrous (Fe^{2+}) and total iron concentrations were determined using the 2,2'-dipyridyl molecular absorption spectrophotometry method (adapted from the '3500-Fe B' colorimetric procedure of the Standard Methods for the Examination of Water and Wastewater (Eaton et al., 1998)). The pH was measured with a Crison Basic 20 pH-meter equipped with a sensION+ 5010T pH electrode, and the oxidation-reduction potential (ORP) was measured with a Thermo-Orion 920+ device equipped with an Orion 9778BNWPO Sur-Flow® electrode at 25 ^{o}C.

3. Results

3.1 Immobilization materials

PVA and BC hydrogels were successfully prepared following the described method. Both hydrogels were homogenous and bright white (Figure 3). Furthermore, both synthesis methods produced hydrogels of the desired size, shape and thickness. This is an advantage for future applications and scaling-up, as hydrogels can be prepared according to the specific requirements of each design process.

3.2 Effectiveness of PVA and BC as active materials for Fe^{2+} oxidation

The bacteria *A. ferrooxidans* was successfully immobilized on both hydrogels. The microbial activity rendered an increasing concentration of Fe^{3+} in the solution, which was responsible for the materials changing colour from bright white to brown/yellowish, as observed in Figure 3. It is noteworthy that the operating pH (1.80) avoided jarosite precipitates forming on the surface of the materials.

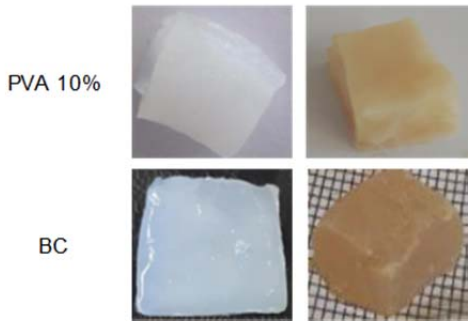

Figure 3. Polyvinyl alcohol (PVA) and biocellulose (BC) hydrogels: Freshly synthetized materials (left) and microorganisms containing materials (right).

Fe^{2+} was successfully oxidized to Fe^{3+} during the immobilization step, with the process being 21 % faster when using BC than when using PVA as support material (Figure 4). It should be noted that the presence of trace amounts of the reagents used in the preliminary synthesis of both support materials hindered microbial growth, thereby making the cleaning of the support material mandatory before bacterial immobilization.
Regarding the active materials, both active BC and active PVA were suitable for the proposed application. Nevertheless, the time required for the complete oxidation of Fe^{2+} when using the active BC in successive oxidation cycles was ~20 % lower than when using active PVA (Figure 4).

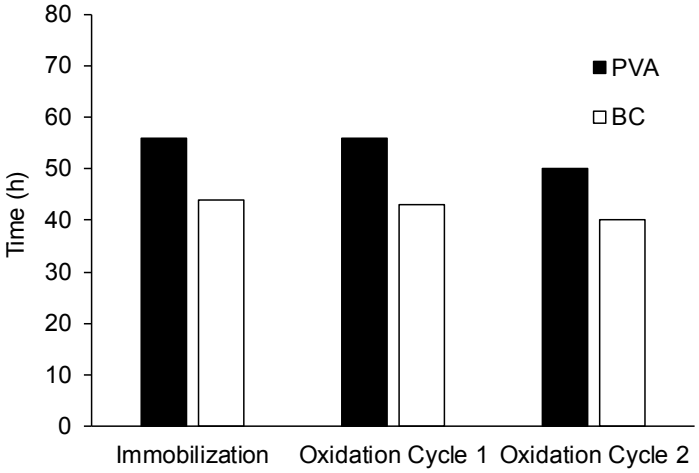

Figure 4. Oxidation time for the immobilization and oxidation steps

3.3 Influence of dissolved Cu^{2+} on the performance of the active materials

Several authors have stated that microbial resistance to copper varies among *A. ferrooxidans* strains. The inhibitory concentration reported in the literature for different *A. ferrooxidans* strains ranges between 0.32 (Mykytczuk et al., 2011) and 25 g Cu^{2+}/L (Novo et al., 2000). In this study, the presence of dissolved copper modified the performance of the process, although none of the copper concentrations used fully inhibited the biological activity of the active materials (Figure 5). In the 5-15 g Cu^{2+}/L range, the oxidation time remained almost constant at ~70 h and ~45 h for active PVA and active BC, respectively, regardless of the Cu^{2+} concentration. Conversely, in the presence of 20 g Cu^{2+}/L, the process slowed, particularly in the samples using active BC. When the highest concentration of dissolved copper (20 g Cu^{2+}/L) was tested, the oxidation time increased by around 19 % and 37 % in the reactors containing active PVA and active BC, respectively, compared to the oxidation time required with 15 g Cu^{2+}/L.
The variation of Fe^{3+} concentration with time when the active material was exposed to increasing dissolved copper concentrations was similar to that shown for the reference sample ([Cu^{2+}] = 0 g/L) in Figure 5.

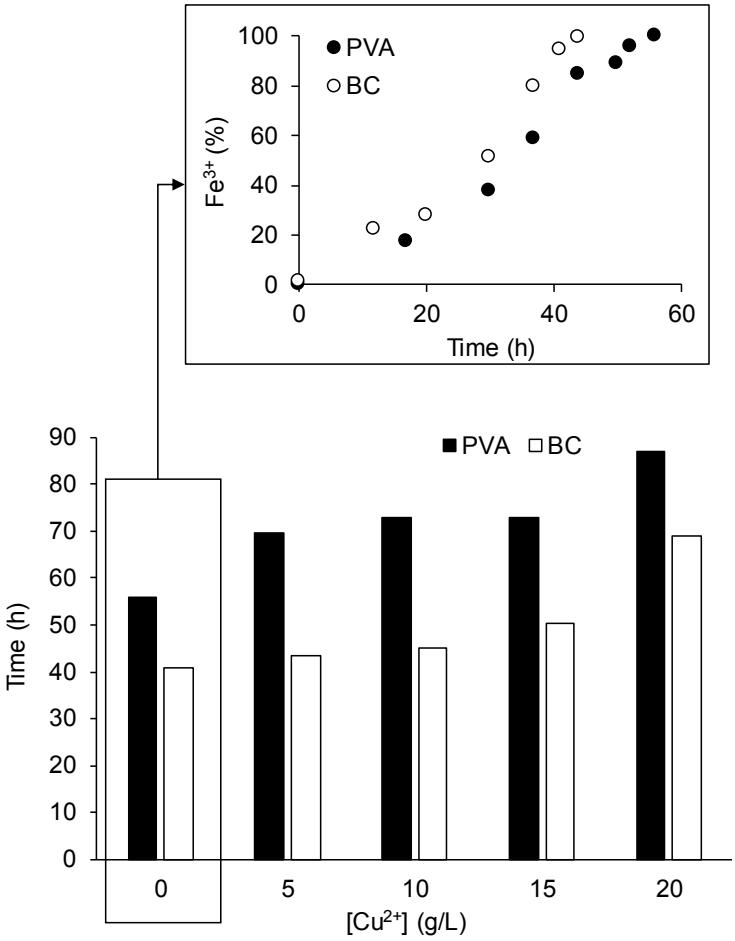

Figure 5. Oxidation time at different Cu^{2+} concentrations and evolution of Fe^{3+} concentration with time in the reference sample ([Cu^{2+}] = 0 g/L)

4. Conclusions

In this study, PVA and BC hydrogels were synthesized and investigated as support materials for *A. ferrooxidans* immobilization. The results revealed that the pre-treatment procedure designed was a crucial step for obtaining the active materials. Both hydrogels recorded good microbial adhesion after immobilization, with this step being faster for the BC hydrogel than for the PVA material.

Furthermore, the study also considered the behaviour of the two active materials during the consecutive Fe^{2+} oxidation cycles under the experimental operating conditions. Both active materials showed high structural integrity and bioactivity for Fe oxidation over several cycles, which was attributed to effective microbial immobilization. It may thus be concluded that both active materials can be used for the regeneration of the oxidant (Fe^{3+}) in bioleaching processes. Although the presence of copper in the solution has been reported to halt the process, the microbial activity in the two materials was not fully inhibited even at 20 g Cu2+/L.

The BC hydrogel was selected as the most suitable support for further studies because of its higher efficiency (20 % faster oxidation process than with the PVA hydrogel) and the maintenance of its integrity throughout the process during consecutive cycles. The oxidation time with this material varied slightly in the presence of copper up to 15 g Cu^{2+}/L.

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