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Enhanced Gold Biosorption of *Lysinibacillus sphaericus* CBAM5 by Encapsulation of Bacteria in an Alginate Matrix

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Abstract: Given its variety of properties, including conductivity and slow corrosion, the industrial uses for gold are increasing dramatically. This means that greater amounts of gold are being released into the environment and that a biological approach to recycling gold is of great interest. *Lysinibacillus sphaericus*, a bacterium capable of metal accumulation inside the cell and adsorption in the external surface, was encapsulated in an alginate matrix to improve the capture of gold from aqueous media. In this study, *L. sphaericus* CBAM5 proved to have the greatest potential compared to other strains and, following its encapsulation, the efficiency for the removal of the precious metal, at a concentration of 60 ppm, was 100% after three hours of exposure. It was identified that the alginate spheres with bacteria could also be reused. In fact, an efficiency of 60% was retained after three cycles of utilization. Thus, alginate acts as an adequate immobilization matrix for bacteria as a highly effective gold capture mechanism, which also shows great potential as an alternative for biotechnological applications.

Keywords: gold; biosorption; encapsulation; alginate; *Lysinibacillus sphaericus*

1. Introduction

The use of gold for the development of new technologies based on gold nanoparticles in the pharmaceutical, electronic, and electrical industries is on the increase [1]. This means that significant quantities of this precious metal are being released into the environment through industrial waste water and mining activities [1,2]. Some conventional technologies are used to capture metals from wastewaters—for example, precipitation, chemical oxidation or reduction, reverse osmosis, coagulation, and electrochemical treatment, among others [3]. Nevertheless, these methods are expensive and are incapable of achieving high efficiencies [3]. Thus, the ability to recycle gold from different residual samples is of great interest and, in this respect, sorption by microorganisms is an expanding area of research [4,5].

Previous studies demonstrate that certain types of bacteria possess heavy metal resistance and detoxification mechanisms, which may be extracellular or intracellular and specific to a certain metal or to a variety of them [6]. This type of bacteria can therefore proliferate in metal-contaminated environments and may be able to capture these metals [7,8]. As an example, *Lysinibacillus sphaericus* is a gram-positive bacterium isolated from a uranium mining waste pile [9], which has a surface layer (S-layer) protein, a porous lattice that protects it and acts as an ion trap. Moreover, several studies have revealed *L. sphaericus*' ability to accumulate heavy metals such as Pb(II), Cr(VI), Cd(II), among others [10–13] due to its S-layer protein and heavy metal efflux pumps [11,14]. It has also recently been

found that *L. sphaericus* is able to accumulate and adsorb Au(III) and that there is evidence for the biosynthesis of gold nanoparticles or colloids [1,15].

Given the protection it provides against environmental stress, the immobilization of bacterial cells could improve their tolerance and capacity to remove contaminants [16]. Several matrices like chitosan, chitin, cellulose, alginate or polyvinyl alcohol are known to protect against UV radiation, toxins, desiccation, contaminants from the medium and possible competing microorganisms [17]. For example, alginate is an algal polysaccharide commonly used in the food industry [18] and it has been demonstrated that it may provide an efficient means to encapsulate bacteria to capture heavy metals such as Cr(VI) [12]. This type of matrix gives not only protection to the cell [12,13] but it also provides a controlled environment for bacterial cells [18] due to its porosity that allows the dissolved contaminants to pass through it, increasing the retention time in the matrix. Other advantages related to the immobilization of bacteria are the increase of biosorption rates as a result of a higher cell density, and the easy separation of the biomass in the solid phase from the liquid media [19].

Based on the above, an alginate matrix was used to encapsulate *L. sphaericus* cells in order to improve their gold biosorption performance while protecting them to reuse the encapsulated biomass for several cycles. A comparative study between free and encapsulated *L. sphaericus* cells was performed and the gold biosorption efficiency was evaluated.

2. Materials and Methods

2.1. Strains of *L. sphaericus* and Growth Conditions

The strains used in these experiments were selected based on information from previous studies that showed their potential to capture heavy metals from aqueous media [11,12,15,20]. Each of the selected *L. sphaericus* strains shown in Table 1 were incubated in nutrient broth (OXOID) for 15 h at 30 °C and 150 rpm. They were then harvested by centrifugation (11,000 rpm, 15 min and 4 °C) and resuspended in minimum salt medium (MSM). This medium contains: KH₂PO₄ 0.5 g/L, NH₄Cl 1 g/L, Na₂SO₄ 2 g/L, KNO₃ 2 g/L, CaCl₂ 0.001 g/L, FeSO₄ 0.0004 g/L, MgSO₄·7H₂O 1 g/L, sodium acetate 5 g/L. The resuspended bacteria had an optical density (OD_{605 nm}) of 0.9–1.2 and were then used for the gold biosorption assays.

Table 1. Strains of *L. sphaericus* evaluated in the biosorption assays.

<i>L. sphaericus</i> Strains	Site of Isolation	Reference
CBAM5	Hydrocarbon-contaminated soil	[14]
III(3)7	Oak forest soil	[14]
OT4b.31	Coleopteran larvae	[14]
MCB1	Nechi River	[15]
MCB2	Nechi River	[15]

2.2. Biosorption of Gold by Free-Living *L. sphaericus* Cells

Each of the five *L. sphaericus* strains (Table 1) were grown and the biosorption assay was conducted as previously described [15]. Briefly, the assay was undertaken in 50 mL flasks with MSM to a final volume of 15 mL, HAuCl₄·3H₂O (60 mg/L) and 3 mL of each strain suspension. For the assay with the mixture of strains, 1 mL of each bacteria suspension (*L. sphaericus* CBAM5, III(3)7 and MCB2) was used. The assays were incubated at 30 °C, 130 rpm. A control assay was made using the same conditions as above but without adding cells for the inoculation. The experiment was conducted in triplicate. Following this, aliquots of 1.5 mL were taken at times 0, 0.25, 0.5, 1, 1.5 and 2 h and centrifuged at 13,000 rpm and 4 °C. The supernatants were analyzed by flame atomic absorption spectroscopy (FAAS), described later.

2.3. *L. sphaericus* CBAM5 Immobilization in an Alginate Matrix

Due to its high efficiency for gold removal, *L. sphaericus* CBAM5 was the strain selected for the immobilization assays. A total of 0.6 g of sodium alginate was poured and homogenized in 60 mL of a *L. sphaericus* CBAM5 suspension in MSM and in 60 mL of MSM (negative control). These solutions were added separately dropwise to a CaCl_2 sterilized solution (2%) in distilled water. The resulting spheres were separated and washed with sterile distilled water. Using 0.5 g of spheres, the bacteria concentration was determined by crushing and mixing them with 1 mL of sterile saline solution (0.85%). Standard serial 10-fold dilutions were inoculated (10 μL) in nutrient agar and incubated for 20 h at 30 °C for the drop plate count method [21].

2.4. Biosorption of Gold by Encapsulated Cells of *L. sphaericus* CBAM5 in Alginate

For this assay, 10 g of alginate spheres with *L. sphaericus* CBAM5 and no cells (negative control) were added to MSM and $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (60 mg/L) to obtain a final volume of 15 mL in 50 mL flasks. The mixture was incubated at 30 °C and 150 rpm. Aliquots of 1.5 mL were taken from the flasks at times 0, 15, 30 min and 1, 1.5 and 2, 3 and 4 h and centrifuged at 13,000 rpm and 4 °C. FAAS was used to analyze the gold concentration in the supernatants as described below. The bioassay was conducted in triplicate. To identify the stability of *L. sphaericus* CBAM5 in the alginate matrix, the bacteria concentration after 24 h was determined as described above. This was also performed for the negative control spheres to confirm their sterility.

2.5. Reusability Test

To determine the potential reusability of the alginate spheres with encapsulated bacteria, gold biosorption assays were performed three times. Prior to each cycle, the spheres were washed with distilled water and exposed to gold as described above. Aliquots of 1.5 mL were taken from the flasks at times 0 and 4 h. Gold concentration in the supernatant was measured by FAAS and gold capture efficiency was calculated. The washing steps were performed after each cycle and all assays were conducted in triplicate.

2.6. Release of Bacteria from Alginate Spheres

The protocol for quantification of the bacteria released was described before [18]. Minor modifications were made as follows: 1 g of alginate spheres with encapsulated bacteria were suspended in 10 mL of sterile saline solution (0.85% w/v) in 15 mL flasks and incubated at 30 °C and 150 rpm. At times 0 and 72 h, 1 mL aliquots were collected and standard serial 10-fold dilutions were inoculated (10 μL) in nutrient agar for the drop plate count method after 20 h of incubation at 30 °C.

2.7. Flame Atomic Absorption Spectrometry (FAAS) Measurements

All the experiments for the quantification of gold were carried out using a high-resolution continuum source atomic absorption spectrometer (HR-CSAAS, ContrAA 800, commercially available from Analytik Jena, Jena, Germany) in flame mode (FAAS). Flame conditions were properly optimized before the measurements, using the absorption line at 242.7590 nm. All gold standards were made on the measurement day by diluting 1000 mg/L standard (HAuCl_4 , CertiPUR®, Merck, HCl 2 mole/L) to concentrations between 2 and 10 ppm. Distilled water and hydrochloric acid 1 mole/L (ACS reagent, 37%, Merck) were used to acidify and dilute all standards and samples at 5% (M/V).

2.8. Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray Spectroscopy (EDS) Analysis

The remaining spheres were observed using scanning electron microscopy (SEM). Spheres from the assays were collected and sequentially washed with ethanol at 70%, 90% and 100%. They were then frozen using liquid nitrogen and cut in half. An aluminum support was used for metallization and SEM observation of the samples. In addition, a semi-quantification of the metal with energy dispersive

X-ray spectroscopy (EDS) was performed using the equipment JEOL JSM-6490LV (JEOL, Tokyo, Japan) scanning electron microscope equipped with an Oxford INCA PentaFetX3 EDS detector.

2.9. Statistical Analysis

Data obtained from the bioassays were analyzed using one-way ANOVA followed by the Tukey test at $P < 0.05$. All the analyses were performed using Software RStudio (R 3.5.1) (Boston, MA, USA).

3. Results

3.1. Biosorption of Gold by Free-Living Cells of *L. sphaericus*

In order to identify the efficiency of gold capture by free cells of five strains of *L. sphaericus*, the metal concentration in the supernatants was followed by using FAAS during a period of 2 h because previous studies showed that gold adsorption occurs in a brief period of time (Figure 1). In terms of single strains, *L. sphaericus* CBAM5 showed the best efficiency with a decrease of 52% of Au(III) in the supernatant, followed by MCB2 and III(3)7, although non-significant differences were determined for these two strains. As those three strains (CBAM5, MCB2 and III(3)7) presented the highest efficiency, they were chosen to assess gold capture by a mixture of cells. After 2 h, a decrease of 33% of Au(III) was observed, showing that single *L. sphaericus* CBAM5 is more efficient for gold sorption. The control assay showed that the concentration of gold remained stable during the two hours of the experiment, which validates the results for the free cells.

Figure 1. Gold (60 ppm) biosorption assay with free cells of *L. sphaericus* strains and a mixture (MIX) of some of them (CBAM5, MCB2 and III(3)7) with an $OD_{605} = 0.9$ – 1.2 , $pH = 5$ – 6 , for 2 h. Points at 2 h denoted by a different letter differ significantly at $P < 0.05$ according to a one-way ANOVA and a Tukey analysis.

3.2. Biosorption of Gold by Encapsulated Cells of *L. sphaericus* CBAM5 in Alginate

As *L. sphaericus* CBAM5 was the most efficient strain in the assay with free cells, it was chosen for encapsulation in an alginate matrix. Its capacity to capture gold was examined by using FAAS within the first two hours of the experiment (Figure 2). A rapid decrease of the gold percentage in the supernatant was observed, and after two hours, 97% of Au(III) was captured by the cells in the alginate spheres, indicating an improvement when encapsulating the bacteria. The control assay showed that the spheres without cells can capture 60% of gold after 1.5 h and a desorption process occurs after two hours of the experiment and only 10% of gold is retained in the alginate matrix.

Figure 2. Gold (60 ppm) biosorption assays with free (CBAM5_free) and encapsulated (CBAM5_Alginate) cells of *L. sphaericus* CBAM5 with pH = 5–6. Control_Alginate: assay with alginate spheres without bacteria. Points at 2 h denoted by a different letter differ significantly at $P < 0.05$ according to a one-way ANOVA and a Tukey analysis.

In comparison with the assay using non-encapsulated cells, a more rapid uptake of gold was observed with cells within the alginate matrix because after the first hour of exposure to the metal, those cells captured 94% of the gold in the medium while the free cells captured only 41%.

The analysis with SEM showed the presence of *L. sphaericus* CBAM5 within the alginate matrix (Figure 3a) and bacilli morphology was identified. The detection of backscattered electrons (Figure 3b) allowed the identification of areas with different compositions. Using an EDS analysis of those areas (Figure 3c), the presence of gold aggregates and possible nanoparticles inside the matrix and on the surface of the cells was determined.

Figure 3. Cont.

(c)

Figure 3. SEM micrographs of *L. sphaericus* CBAM5 cells (a) inside the alginate sphere seen with retro-dispersed electrons. (b) Close-up of image (a) seen with retro-dispersed electrons. (c) EDS analysis of image (b) showing the presence of Au in the matrix.

3.3. Reusability Test and Release of Bacteria from Alginate Spheres

In order to characterize the alginate matrix for the encapsulation of bacteria and its possible use for biotechnological applications, this study determined the reusability of the spheres and the bacteria released from them.

Firstly, the alginate spheres were used to test the release of encapsulated bacteria with an initial concentration of 2.13×10^6 CFU/mL by their immersion in a saline solution and incubation for 72 h. Figure 4 shows that *L. sphaericus* CBAM5 was released from the alginate matrix immediately after exposure to saline solution and a bacterial concentration of 3.11×10^2 CFU/mL was determined in the medium.

Figure 4. Release of bacteria from alginate beads. The bacterial concentration (log(CFU/mL)) in the saline solution after 72 h of incubation is shown.

This release of bacteria was continuously observed after 24 and 48 h of incubation of the alginate spheres (data not shown). Finally, after 72 h, a bacterial concentration of 2×10^6 CFU/mL was determined inside the spheres and one of 4.83×10^7 CFU/mL in the saline solution. It could be said that bacteria are being released into the medium and are able to continue their division phase, which explains the high titer in the saline solution. They can also divide inside the matrix, which explains the decrease of only 6% of the bacterial concentrations inside the alginate spheres after 72 h of incubation.

In order to identify the potential reusability of the encapsulated bacteria in alginate, the efficiency of gold capture was tested for three cycles. The results in Figure 5a show a decrease in the percentage of gold biosorption after the third cycle of reuse. Nevertheless, the efficiency continues to be higher (60%) than that observed for the free *L. sphaericus* CBAM5 cells (51%) in their first cycle. This reveals a clear advantage in the use of encapsulated cells for metal removal.

Figure 5. (a) Reusability of the encapsulated *L. sphaericus* CBAM5 cells for 3 cycles of 4 h. (b) Bioassays with 1. Encapsulated CBAM5 and 2. Alginate spheres without bacteria, showing the difference of color in the matrix obtained after 3 cycles of reuse.

Moreover, a visual difference was identified between the control bioassay and the treatment with bacteria in alginate spheres, as shown in Figure 5b. The alginate matrix took on a red-purple color indicating a possible biosynthesis of gold nanoparticles, characterized by this color [22]. This result correlates with the SEM micrograph of the alginate spheres with bacteria taken after the first cycle. Figure 3b shows a close-up of a bacterial cell with gold particles of nanometric size, ranging from 200 to 600 nm.

4. Discussion

Sustainability is a current global challenge but the massive extraction of metals from the environment interferes with the possibility of reaching this goal [23]. Great efforts are being made to develop circular economies in an attempt to minimize waste by giving it value and by replacing mass production [24]. In this respect, the recovery and reuse of metals is an interesting alternative with which to approach sustainability. Biosorption has been described as a low-cost method for metal recovery and the use of microorganisms with bio-derived materials could serve as sustainable technology for industries [23] that operate with precious metals with high economic value, such as gold [25].

Alginate is a polysaccharide produced by algae and, given the presence of carboxylic groups in its structure, it has been studied as a metal-binding polymer [26,27]. It has also been used as an immobilization matrix given its simple preparation method [27]. In this study, *L. sphaericus*, a bacterium with a high metal-binding capacity [12,14,15], was chosen for encapsulation in an alginate matrix, improving its efficiency to capture gold to 100% in a period of just 3 h.

Several strains of *L. sphaericus* were chosen to determine their potential to capture gold from aqueous media. As shown in Figure 1, *L. sphaericus* CBAM5 showed the highest efficiency and it might present different mechanisms to achieve this. For instance, *L. sphaericus* CBAM5 is known for having an S-layer protein present in the external cell envelope, capable of self-assembly, and with a P4 symmetry [28,29]. This protein is known to have a high affinity to metals and its functional groups are able to bind Au(III) in a stable and efficient way [5]. The bacteria could present other resistance mechanisms like gold absorption and accumulation inside the cell by sequestration. There was no evidence of efflux pumps being activated during the bioassays because no oscillations of the gold concentration in the supernatant were observed (Figure 1).

Moreover, as shown in Figure 2, the encapsulated *L. sphaericus* CBAM5 was able to remove nearly 100% of the gold from the aqueous media in three hours, making alginate a suitable polymer for the optimization of metals sorption processes in biotechnological applications.

The relevance of alginate as an immobilization matrix lies in its biodegradable potential and its non-toxic nature [18]. It provides a controlled environment for the bacteria by protecting them from stress conditions such as UV radiation, contaminants, and desiccation, among others. Due to its tortuosity, the alginate matrix is also able to control the diffusion of the aqueous media towards the encapsulated cells, increasing the contaminant retention time [17,19] and improving *L. sphaericus* CBAM5's efficiency in capturing metals such as gold.

The tortuosity of the matrix also determines the release of bacteria into the medium. In this study, it was determined that *L. sphaericus* CBAM5 can be released from the alginate spheres after 72 h of incubation because a high titer of bacteria was found in the saline solution (Figure 4). Nevertheless, the bacterial concentration remained high inside the alginate matrix, with a decrease of only 6%. It is proposed that cell division can be achieved in a saline solution and inside the alginate matrix due to energy reserves available for the microorganisms acting as autotrophs in nutrient-limiting conditions. These promising results showed that the alginate beads could be reused for enhanced gold biosorption for at least 72 h because high titers of bacteria remain in the spheres, despite the fact that they could be released into the environment. Future studies with a longer period of time should be conceived.

To study the reusability of the encapsulated cells (Figure 5), the alginate spheres with *L. sphaericus* CBAM5 were reused for three cycles of exposure to a gold solution. An efficiency of 60% was retained after the cycles. This decrease in the gold biosorption might indicate a level of saturation of the proteins capable of metal adsorption on the external layer of the bacteria, specifically the S-layer protein, which is abundant in *L. sphaericus* [5].

Finally, a model explaining the possible interactions of gold with the bacteria and the matrix is shown in Figure 6. Gold in solution is absorbed by the alginate beads due to their porous structure that controls the diffusion of the aqueous media. The metal can also be adsorbed by the matrix which is made up of mannuronic and glucuronic acid, which are able to bind with Au(III) complexes [27]. *L. sphaericus* CBAM5 inside the matrix can also adsorb gold through its S-layer protein assembled in the cell wall. An accumulation of gold inside the cells could also be occurring, and it is proposed that metabolic active bacteria are responsible for the synthesis of gold nanoparticles. Although the reduction pathway was not studied here, it is suggested that a rapid reduction of Au(III) to Au(I) occurs in the cell wall and is followed by a slow reduction to Au(0) [30]. This reduction pathway could compromise the S-layer [31] and a *mer*-like cluster, the latter of which confers microbial resistance to mercury by reducing the toxic metal to Hg(0) with the reductase enzyme, MerA [32]. This enzyme could also work on gold species due to the similarities between the metals [30].

Figure 6. Scheme indicating the main processes that may occur in the encapsulated cells after exposure to Au(III): adsorption through the S-layer, absorption and accumulation inside the bacterial cell and inside the alginate matrix. A possible gold nanoparticles biosynthesis was observed.

For future studies, a protocol for the extraction and characterization of the gold nanoparticles should be standardized. Also, the gold reduction pathway should be identified.

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